Biological Trace Element Research (2019) 189:64–74
https://doi.org/10.1007/s12011-018-1468-x

Copper or Magnesium Supplementation Endows the Peptic Hydrolysate from Bovine Lactoferrin with Enhanced Activity to Human Gastric Cancer AGS Cells

Li-Ying Bo[1] · Tie-Jing Li[2] · Xin-Huai Zhao[1]

Received: 29 May 2018 / Accepted: 27 July 2018 / Published online: 1 August 2018

Abstract

A lactoferrin hydrolysate (LFH) was generated from bovine lactoferrin by pepsin, mixed with Cu^{2+} and Mn^{2+} at 0.64–1.28 and 0.28–0.56 mg/g protein, respectively; and then their in vitro effects on human gastric cancer AGS cells were assessed. With incubation times of 24 or 48 h, LFH and its Cu^{2+}/Mn^{2+} mixtures at 10–30 mg/mL in dose-dependent manner inhibited cell growth; and more, these mixtures showed higher activities than LFH alone. Cell treatments of LFH and the mixtures (25 mg/mL) for 24 h could arrest cell cycle at G0/G1-phase, damage mitochondrial membrane integrity, and induce apoptosis, while the mixtures were also more powerful than LFH to exert these three effects. Higher Cu^{2+}/Mn^{2+} supplementation level resulted in higher growth inhibition, cell cycle arrest, mitochondrial membrane potential disruption, and apoptosis induction; furthermore, Mn^{2+} was notable for its higher efficacy than Cu^{2+} to increase these four effects. Western-blot assay results revealed that four apoptosis-related proteins Bad, Bax, cytochrome c, and p53 were up-regulated, and both caspase-3 and caspase-9 also were cleaved and activated; moreover, two autophagy-related proteins LC3-II and cleaved Beclin-1 were down- and up-regulated, respectively. It is thus concluded that Cu^{2+} and especially Mn^{2+} could endow supplemented LFH with increased anti-cancer effects in AGS cells, with two proposed events as enhanced apoptosis induction (via activating apoptosis-related proteins) and autophagy inhibition (via activating autophagy-related proteins).

Keywords Lactoferrin hydrolysate · Copper · Magnesium · Gastric cancer cells · Apoptosis · Autophagy

Introduction

Gastric cancer is considered as one of the most common malignant cancers in the world, and is reported to have higher morbidity and mortality in the present Asia [1]. The main curative therapies for gastric cancer are surgery and chemotherapy; however, these approaches are very painful for cancer patients and might be slim successful if the cancer is diagnosed at a late stage [2, 3]. It is therefore necessary to find natural compounds with desired prevention on cancer development. Consequently, many studies in recent years have investigated potential anti-cancer activities of various food components to several gastric cancer cells [4].

Some compounds from plant foods have potential anti-cancer effects in various cancer cells such as BGC-823, SGC-7901, MKN-45, and MGC-803 cells [5]. Plant foods are rich in several phytochemicals including alkaloids, carotenoids, flavonoids, and other bioactive compounds. It is evident that two flavonoid members flavones and flavonols have anti-cancer effects in gastric cancer MKN-45 cells, resulting in growth inhibition, cell cycle arrest, and apoptosis [6]. Alkaloids, another type of phytochemicals, also have anti-cancer potential in human gastric cancer AGS cells through inhibiting cell growth and inducing apoptosis [7]. Among these assessed components from animal foods is lactoferrin (LF), an iron-binding glycoprotein from milk with molecular weight about 80 kDa, which has been reported with anti-cancer effects in gastric cancer cells via arresting cell cycle in sub-G1-phase [8]. Meanwhile,

✉ Tie-Jing Li
litiejing@lnu.edu.cn

✉ Xin-Huai Zhao
zhaoxh@neau.edu.cn

[1] Key Laboratory of Dairy Science, Ministry of Education, Northeast Agricultural University, Harbin 150030, People's Republic of China

[2] College of Light Industry, Liaoning University, Shenyang 110136, People's Republic of China

several proteins or their degraded products (i.e., hydrolysates) are also verified to have in vitro anti-cancer effects in gastric cancer cells. For example, the solid fraction separated from yogurt by centrifugation can inhibit the growth of primary tumor cells [9], while an isolated peptide fraction from algae protein is observed with anti-cancer activity in AGS cells, leading to a post-G1-phase arrest [10]. In China, two research groups assessed anti-cancer effects of LF and lactoferricin B (one peptide fraction isolated from tryptic LF hydrolysate) in gastric cancer SGC-7901 and AGS cells [11, 12]; their results demonstrated that both LF and lactoferricin B could promote cell apoptosis. However, to the best of our knowledge, it is still unknown that if LF hydrolysates can interact with other food components (e.g., trace metal ions), and consequently this interaction might give rise to changed effects in cancer cells. It is well-known that both proteins and protein hydrolysates can interact with some trace metals [13]; as the result, these trace metals possess higher bio-accessibility to the body [14]. It is reasonable that potential interaction between LF hydrolysates and trace metals might bring changed anti-cancer effects in cancer cells.

In natural foods, two trace elements Mn and Cu are essential to the body. Mn is a cofactor of several enzymes involved in neurotransmitter synthesis and metabolism in the brain, and is necessary for a variety of physiological processes including the metabolism of amino acids, carbohydrates, and lipids. Cu is important for the functions of many enzymes and proteins involved in energy metabolism, respiration, and DNA synthesis [15]. Cu is also a key cofactor for cytochrome oxidase, superoxide dismutase, ascorbate oxidase, and tyrosinases [16]. Both Mn^{2+} and Cu^{2+} can form complexes with many organic materials, and subsequently have been assessed for their bio-activities such as immune and anti-cancer properties [17, 18]. In a study of Zhou and coauthors, Mn complex of N-substituted di(picolyl)amine could interfere with mitochondrial function of U251 and HeLa cells, and thus exhibit enhanced growth inhibition [19]. In another study, a Cu complex containing pyridine co-ligand was observed to have enhanced anti-cancer activities in various human cancer cells [20]. LF in the stomach is digested by pepsin. If LF hydrolysate (LFH) interacts with other materials such as Mn and Cu, potential change in its activity to cancer cells is interesting, however, is not assessed yet.

In this study, LFH was intendedly mixed with copper chloride and magnesium sulfate of two levels, respectively. The resultant four mixtures were assessed for their in vitro effects including growth inhibition, cell cycle arrest, and apoptosis in AGS cells, using LFH as control. Moreover, the cells treated with these mixtures were detected for their mitochondrial membrane potential and expression changes of several proteins, to reveal possible mechanism responsible for the changed anti-cancer effects of LFH's Cu^{2+}/Mn^{2+} mixtures.

Materials and Methods

Materials

Bovine LF with respective iron and protein contents of 170 mg/kg and 979.0 g/kg was purchased from MILEI Gmbh (Leutkirch, Germany). Porcine gastric mucosa pepsin (CAS: 9001-75-6) and Dulbecco's modified Eagle's medium were purchased from Sigma-Aldrich Co. Ltd. (St. Louis, MO, USA). Cell Counting Kit-8 (CCK-8) was purchased from Dojindo Molecular Technologies, Inc. (Kyushu, Japan). Fetal bovine serum (FBS) was purchased from Wisent Inc. (Montreal, QC Canada). Annexin V-FITC Apoptosis Detection Kit, Cell Cycle Analysis Kit, BCA Protein Assay Kit, JC-1 dye, and Hoechst 33258 dye were bought from Beyotime Institute of Biotechnology (Shanghai, China). Primary anti-bodies (β-actin, caspase-3, caspase-9, cytochrome c, Bad, Bax, p53, LC3-I, LC3-II, and Beclin-1) and secondary antibody were purchased from Cell Signaling Technology, Inc. (Boston, MA, USA). Dextran T-70 and phosphate-buffered saline (PBS) were purchased from Solarbio Science and Technology Co. Ltd. (Beijing, China). 5-Fluorouracil (5-FU) was bought from Jinyao Pharmaceutical Co. Ltd. (Tianjin, China). Other chemicals used in this study were analytical grade. The used water was ultrapure water generated from Milli-Q Plus (Millipore Corporation, New York, NY, USA).

Sample Preparation and Analysis

LFH was prepared as previously described [21] with some modifications. In brief, LF was dissolved in water and added with 1 mol/L HCl to pH 2.5, which gave a final protein concentration of 50 g/L. LF solution was added with pepsin of 0.75 kU/g protein, incubated at 37 °C for 4 h, heated at 80 °C for 15 min to inactivate pepsin, and then neutralized to pH 7.0 using 1 mol/L $NaHCO_3$. After 12,000×*g* centrifugation at 4 °C for 30 min, the collected supernatant (i.e., LFH) was freeze-dried, ground into powder, and stored at − 20 °C.

LFH was mixed with copper chloride and magnesium sulfate as previously described [13] with minor modification, to achieve two Cu^{2+} and Mn^{2+} supplementation levels of 0.64–1.28 and 0.28–0.56 mg/g protein, respectively. The two levels were equivalent to 10–20 (Cu^{2+}) or 5–10 (Mn^{2+}) μmol/g protein. The mixtures were adjusted to pH 7.0 using 1 mol/L $NaHCO_3$, incubated at 22 °C for 1 h, freeze-dried, and then stored at − 20 °C before their use. In this study, Mixture I and Mixture II were designated as LFH supplemented with Cu^{2+} of 0.64 and 1.28 mg/g protein, while Mixture III and Mixture IV were designated as LFH supplemented with Mn^{2+} of 0.28 and 0.56 mg/g protein, respectively.

LF, LFH, and these mixtures were all measured for their protein contents using the Kjidahl method and a conversion factor of 6.38 [22].

Cell Line and Culture Conditions

AGS cells provided by Cell Bank of the Chinese Academy of Sciences (Shanghai, China) are recommended to be cultured in the Dulbecco's modified Eagle's medium supplemented with 10% FBS, 100 units/mL penicillin, and 100 μg/mL streptomycin. A humidified atmosphere containing 5% CO_2 and temperature of 37 °C were also required in cell culture.

Assay of Growth Inhibition

The cells were seeded in 96-well plates at a density of 2×10^4 cells/well in 100 μL medium and incubated for 24 h. After removal of the medium, the cells were treated with the medium containing LFH or Mixtures I–IV of five dose levels (10–30 mg/mL), and then incubated for 24–48 h. The cells treated with 5-FU of 200 μmol/L and medium containing 5% FBS were used as positive and negative controls. CCK-8 of 10 μL was added to each well, and the cells were incubated for 1.5 h. Optical density values at 450 nm were measured by microplate reader (Bio-Rad Laboratories, Hercules, CA, USA), and used to calculate viability. The cells treated with medium containing 5% FBS were served to have viability value of 100% (i.e., without any growth inhibition).

Assay of Cell Cycle Progression

The cells (1×10^6 cells/dish) were seeded on 100-mm cell culture dishes with 10 mL medium, incubated for 24 h, and then retreated with 10 mL/dish medium containing LFH or Mixtures I–IV (25 mg/mL) for 24 h. After that, the cells were harvested by trypsin-EDTA, washed twice with cold PBS (10 mmol/L, pH 7.3), and fixed with 70% cold ethanol by shaking once every 15 min at 4 °C overnight. The cells were washed with the cold PBS, resuspended in binding buffer (500 μL), and stained with RNase A (10 μL) and propidium iodide (PI; 25 μL) at 37 °C for 30 min in the dark. The cells without sample treatment were served as negative control. Cell cycle distributions in G0/G1-, S-, and G2/M-phases were assayed using flow cytometry (FACSCalibur, Becton Dickson, San Jose, CA, USA). Cell proportions in every phase were analyzed using the ModFit software (Verity Software House, Topsham, ME, USA).

Hoechst 33258 Staining

The cells (1×10^6 cells/well) were seeded in 6-well plates with 2 mL medium, incubated 24 h, and then retreated with the medium containing LFH or Mixtures I–IV (25 mg/mL) for 24 h. Next, the cells were stained with Hoechst 33258 dye for 5 min at room temperature (22 °C), washed twice by PBS (10 mmol/L, pH 7.3), and observed under a fluorescence microscope (Type Eclipice-Ti-S, Nikon, Japan) at × 200 magnification.

Assay of Mitochondrial Membrane Potential

Loss of mitochondrial membrane potential was determined using flow cytometry and JC-1 dye. Briefly, the cells (5×10^5 cells/well) were seeded in 6-well plates with 2 mL medium at 37 °C for 24 h, and then retreated with the medium containing LFH or Mixtures I–IV (25 mg/mL) for 24 h. After that, the cells were detached by trypsin-EDTA, stained with JC-1 dye for 20 min at 37 °C, and assessed by the flow cytometry (FACS Calibur; Becton Dickson).

Assay of Apoptosis Induction

AnnexinV-FITC and PI as fluorescent dyes were used in this assay to discriminate intact, early apoptotic, late apoptotic, and necrotic cells [23]. The cells with medium containing 5% FBS were regarded as negative control. The cells (2×10^4 cells/well) were seeded in 6-well plates with 2 mL medium, and incubated at 37 °C for 24 h. After that, the cells were retreated with the medium containing LFH or Mixtures I–IV (25 mg/mL) for 24 h, detached by trypsin-EDTA, washed twice with the PBS, resuspended in 500 μL Annexin V-FITC binding buffer, stained with 5 μL Annexin V-FITC and 10 μL PI in the dark at 20 °C for 30 min, and analyzed by the flow cytometry (FACS Calibur; Becton Dickson) to detect the proportions of intact, early apoptotic (Q4), late apoptotic (Q2), and necrotic cells.

Western-blot Assay

The cells (5×10^6 cells/dish) were seeded on 100-mm cell culture dishes with 10 mL medium at 37 °C for 24 h, and also retreated with the medium containing LFH or Mixtures I–IV (25 mg/mL) for 24 h. Subsequently, the cells were washed three times with cold PBS and lysed for 30 min on ice with 100 μL RIPA Lysis Buffer (Beyotime, Shanghai, China) containing 1 mmol/L PMSF (Beyotime, Shanghai, China). The lysates were centrifuged at 12,000×*g* for 5 min at 4 °C, and resultant supernatants were collected as total cellular protein. Protein concentrations were measured using the BCA Protein Assay Kit. Protein (50 mg) from each sample was loaded on 10−15% SDS-PAGE and transblotted to PVDF membrane. The blots were blocked with 5% fat-free powdered milk dissolved with the PBS containing 0.1% Tween-20 at 37 °C for 2 h, probed with 1:5000 dilution of the primary anti-body in blocking buffer overnight at 4 °C. The bands were incubated with an anti-rabbit secondary anti-body horseradish

peroxidase conjugate. The enhanced chemiluminescence of 200 μL was covered on PVDF membrane, and the signal on the band was detected by a Chemi Scope 6300 (Clinx Science Instrument, Shanghai, China).

Statistical Analysis

All data were reported as means or means ± standard deviations from three independent experiments and assays. Statistical analysis was performed by using the SPSS software version 16.0 (SPSS Inc., Chicago, IL, USA) using one-way analysis (ANOVA) with Duncan's multiple range tests. The statistical significance was set at a level of $P < 0.05$.

Results

Growth Inhibition of LFH and Mixtures I–IV in AGS Cells

In this study, 5-FU as one of classic chemotherapy agents displayed growth inhibition in AGS cells. The cells treated with 200 μmol/L 5-FU for 24–48 h showed growth inhibition of 44.5–56.3%. Results (Table 1) also indicate that LFH and Mixtures I–IV of 10–30 mg/mL could inhibit cell growth; and more, both prolonged treatment time and increased dose level consistently led to enhanced growth inhibition. In comparison with LFH, Mixtures I–IV at each time point showed higher inhibition, as LFH demonstrated growth inhibition of 5.4–46.5% while Mixtures I–IV displayed increased growth inhibition (6.5–85.9%). Moreover, Mixtures III–IV were roughly observed to have higher inhibition than Mixtures I–II (7.9–85.9% versus 6.5–63.6%). This fact means that Mn^{2+} was more efficient than Cu^{2+} to confer LFH with higher inhibition. Mixture I displayed lower growth inhibition than Mixture II (6.5–55.2% versus 7.0–63.6%). Similar phenomenon was also found in the case of Mixture III and Mixture IV (7.9–67.2% versus 11.2–85.9%). This indicates that higher Cu^{2+}/Mn^{2+} supplementation levels led to higher anti-proliferation. It is concluded that it was Cu^{2+} and especially Mn^{2+} supplemented to LFH that contributed enhanced inhibition.

Mixtures I–IV at dose level other than 25 mg/mL led to too weaker or stronger growth inhibition (Table 1). Accordingly, these mixtures and LFH were assessed for other effects in the cells at dose level of 25 mg/mL. And more, treatment time was fixed at 24 h.

Cell Cycle Arresting of LFH and Mixtures I–IV in AGS Cells

To further elucidate inhibition of LFH and its Cu^{2+}/Mn^{2+} mixtures on cell growth, flow cytometry analysis was performed to detect cell cycle distribution (Fig. 1), aiming to verify whether these materials were capable of disturbing cell cycle progression. Results indicate that in comparison with control cells, the cells treated with LFH for 24 h totally had increased cell proportion of G0/G1-phase (i.e., increasing from 58.3 to 62.3%); moreover, cell treatment with Mixtures I–II and Mixtures III–IV also resulted in increased cell proportion of G0/G1-phase (66.8–69.1% and 73.4–75.8%). These data indicate three findings: (1) Mixtures I–IV had dramatic effects than LFH alone to arrest cell cycle progression at G0/G1-phase; (2) Mn^{2+} brought greater cell cycle arrest, compared to Cu^{2+}; and (3) higher metal level also yielded greater cell cycle arrest. Overall, Cu^{2+}/Mn^{2+} supplementation of LFH thus brought enhanced ability to disturb cell cycle progression in AGS cells, making contribution to growth inhibition via arresting cell cycle progression at G0/G1-phase.

Apoptosis Induction of LFH and Mixtures I–IV in AGS Cells

Morphologic features of the control and treated AGS cells were observed using Hoechst 33258 staining (Fig. 2a).

Table 1 Growth inhibition (%) of lactoferrin hydrolysate (LFH) and its Cu/Mn-supplemented products at five dose levels in AGS cells with treatment times of 24–48 h

Samples	Dose levels and treatment times									
	10 mg/mL		15 mg/mL		20 mg/mL		25 mg/mL		30 mg/mL	
	24 h	48 h	24 h	48 h	24 h	48 h	24 h	48 h	24 h	48 h
LFH	5.4 ± 1.1^{a}	6.6 ± 1.7^{a}	8.4 ± 1.9^{a}	12.5 ± 2.2^{a}	9.5 ± 2.0^{a}	14.3 ± 2.1^{a}	26.0 ± 2.7^{a}	35.5 ± 2.0^{a}	40.9 ± 2.7^{a}	46.5 ± 2.4^{a}
Mixture I	6.5 ± 1.3^{a}	7.8 ± 1.1^{a}	10.5 ± 1.8^{a}	13.8 ± 2.8^{ab}	11.5 ± 1.6^{ab}	15.4 ± 2.4^{a}	34.2 ± 3.0^{b}	43.1 ± 2.7^{b}	45.2 ± 2.6^{a}	55.2 ± 2.5^{b}
Mixture II	7.0 ± 1.4^{a}	8.4 ± 2.1^{a}	11.1 ± 1.8^{a}	15.0 ± 2.2^{ab}	14.4 ± 2.3^{abc}	16.4 ± 2.4^{ab}	41.8 ± 2.5^{c}	51.7 ± 3.1^{c}	54.9 ± 2.0^{b}	63.6 ± 2.1^{c}
Mixture III	7.9 ± 1.2^{a}	9.6 ± 1.5^{ab}	12.9 ± 1.9^{a}	15.5 ± 2.3^{ab}	16.7 ± 2.6^{abc}	17.1 ± 2.3^{ab}	43.5 ± 2.4^{c}	54.6 ± 3.2^{c}	56.2 ± 2.0^{b}	67.2 ± 2.3^{c}
Mixture IV	11.2 ± 1.8^{b}	13.4 ± 2.4^{b}	15.1 ± 2.2^{b}	18.9 ± 1.7^{b}	19.6 ± 2.8^{c}	21.5 ± 2.1^{b}	55.4 ± 2.3^{d}	63.8 ± 2.9^{d}	65.4 ± 2.5^{c}	85.9 ± 2.9^{d}

Mixtures I–II represent LFH supplemented with Cu^{2+} 0.64–1.28 mg/g protein, while Mixtures III–IV represent LFH supplemented with Mn^{2+} 0.28–0.56 mg/g protein, respectively. Means within the same column with different superscript letters differ significantly ($P < 0.05$)

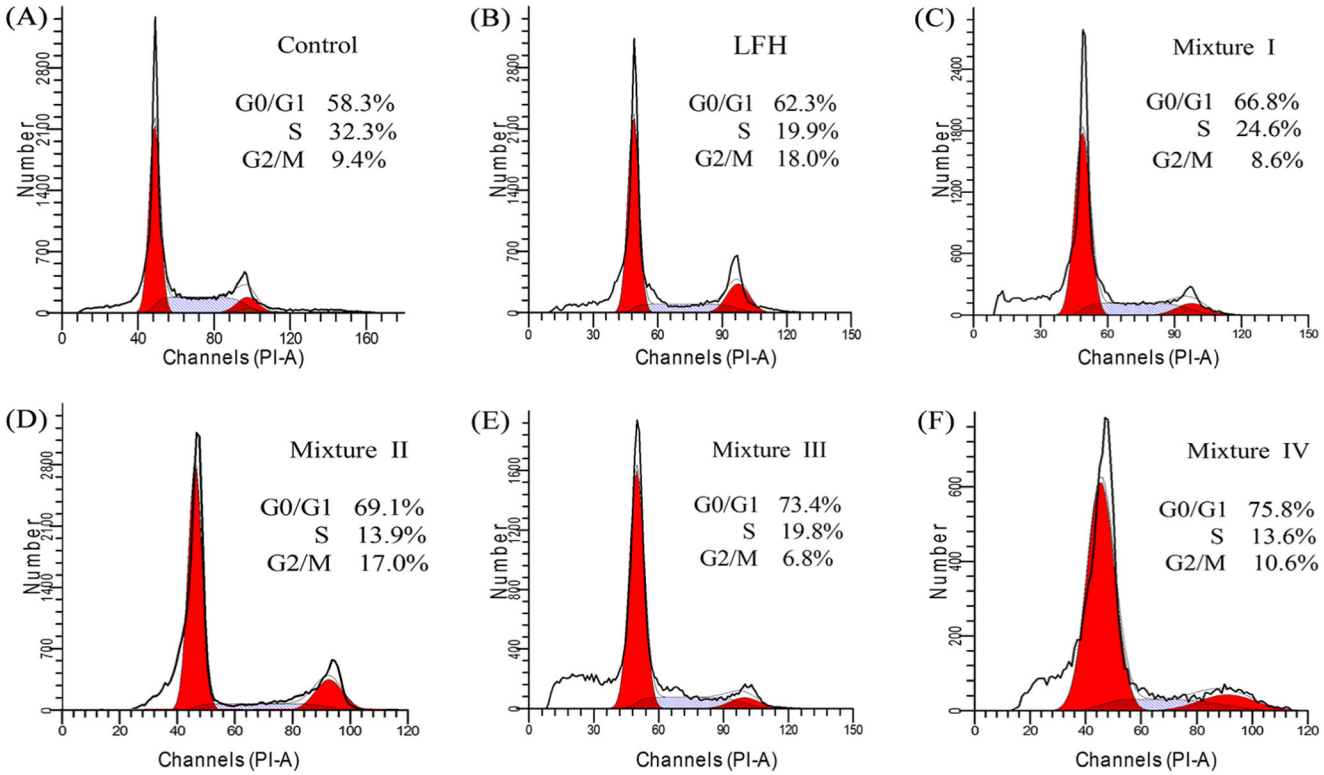

Fig. 1 Cell cycle distribution in the AGS cells treated without (**a**) or with LFH (**b**) and Mixtures I–IV (**c**–**f**) at 25 mg/mL for 24 h, respectively

Results demonstrate that the control cells without any treatment had larger cells numbers with very weaker apoptosis, as most of the cells were dimly blue (Fig. 2a). However, for the cells treated with LFH and especially its Cu^{2+}/Mn^{2+} mixtures, fewer cells were observed but some apoptotic cells with chromatin condensation and nuclear fragmentation were identified (Fig. 2b–f). Based on the morphologic changes of the cells, it is supposed that LFH and Mixtures I–IV might have apoptosis induction in AGS cells.

Apoptosis induction of LFH and its Cu^{2+}/Mn^{2+} mixtures in AGS cells were investigated by flow cytometry to identify the proportions of late apoptotic (Q2) and early apoptotic (Q4) cells. The results (Fig. 3) demonstrate that LFH and Mixtures I–IV were able to induce apoptosis. Control cells only had total apoptotic proportion (Q2 + Q4) of 9.1%, whereas the cells treated with LFH, Mixtures I–IV for 24 h showed total apoptotic proportions of 26.1%, 28.0%, 36.1%, 41.0%, and 47.2%, respectively. Based on these data, it can be seen that (1) Mixtures I–IV were more able than LFH alone to induce cell apoptosis; (2) Mn^{2+} mixtures had increased apoptosis induction, compared with Cu^{2+} mixtures; (3) the resultant apoptosis induction was governed by Cu^{2+}/Mn^{2+} concentrations in the mixtures; and (4) for these mixtures, the obtained order of apoptosis induction was completely in consistent with the measured order of cell cycle arrest. Both apoptosis induction and cell cycle arrest thus made contribution to the measured growth inhibition.

Loss of Mitochondrial Membrane Potential in Treated AGS Cells

To further verify mitochondrial dysfunction, mitochondrial membrane potentials (MMP) of AGS cells treated with LFH and Mixtures I–IV for 24 h were analyzed using JC-1 dye staining and flow cytometry. Results indicate that the cells treated with LFH had decreased MMP (cell proportion of red fluorescence 79.6%) (Fig. 4b), compared with control cells without any treatment (cell proportion of red fluorescence 91.5%) (Fig. 4a). Moreover, the cells treated with Cu^{2+} mixtures (cell proportions of red fluorescence 70.5% and 65.7%) (Fig. 4c, d) and especially Mn^{2+} mixtures (cell proportions of red fluorescence 60.3% and 54.5%) (Fig. 4e, f) led to more dramatic MMP decreases. It is evident that LFH and the mixtures had mitochondrial membrane potential disruption, which might promote the release of apoptogenic factors such as cytochrome c. The assay results also support that Mixtures I–II and especially Mixtures III–IV had higher apoptosis induction in AGS cells, compared with LFH.

Changes in Protein Expression in AGS Cells

A serial of Western-blot assay were conducted to assess expression levels of 9 proteins in the treated cells (Fig. 5), which are classified as apoptosis- and autophagy-related proteins. Results demonstrate that when the cells treated with LFH

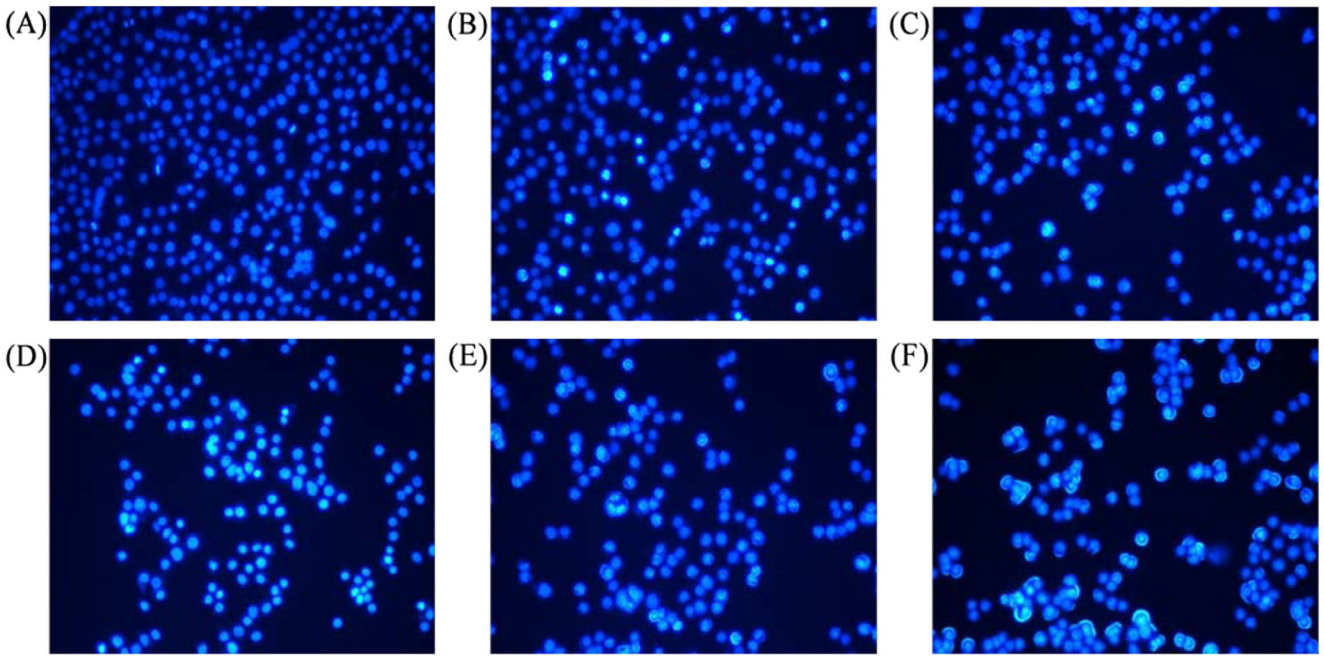

Fig. 2 Morphology of the AGS cells treated without (**a**) or with LFH (**b**) and Mixtures I–IV (**c**–**f**) at 25 mg/mL for 24 h, respectively. The images were observed under an inverted fluorescence microscope (× 200)

and Mixtures I–IV for 24 h, apoptosis-related caspase-3 and caspase-9 were cleaved and activated. The cells exposed to LFH had up-regulated Bad, Bax, cytochrome c, p53, and cleaved Beclin-1 but down-regulated LC3-II and Bcl-2. Similar results were observed following cell treatment with Mixtures III–IV, leading to slightly higher changes in

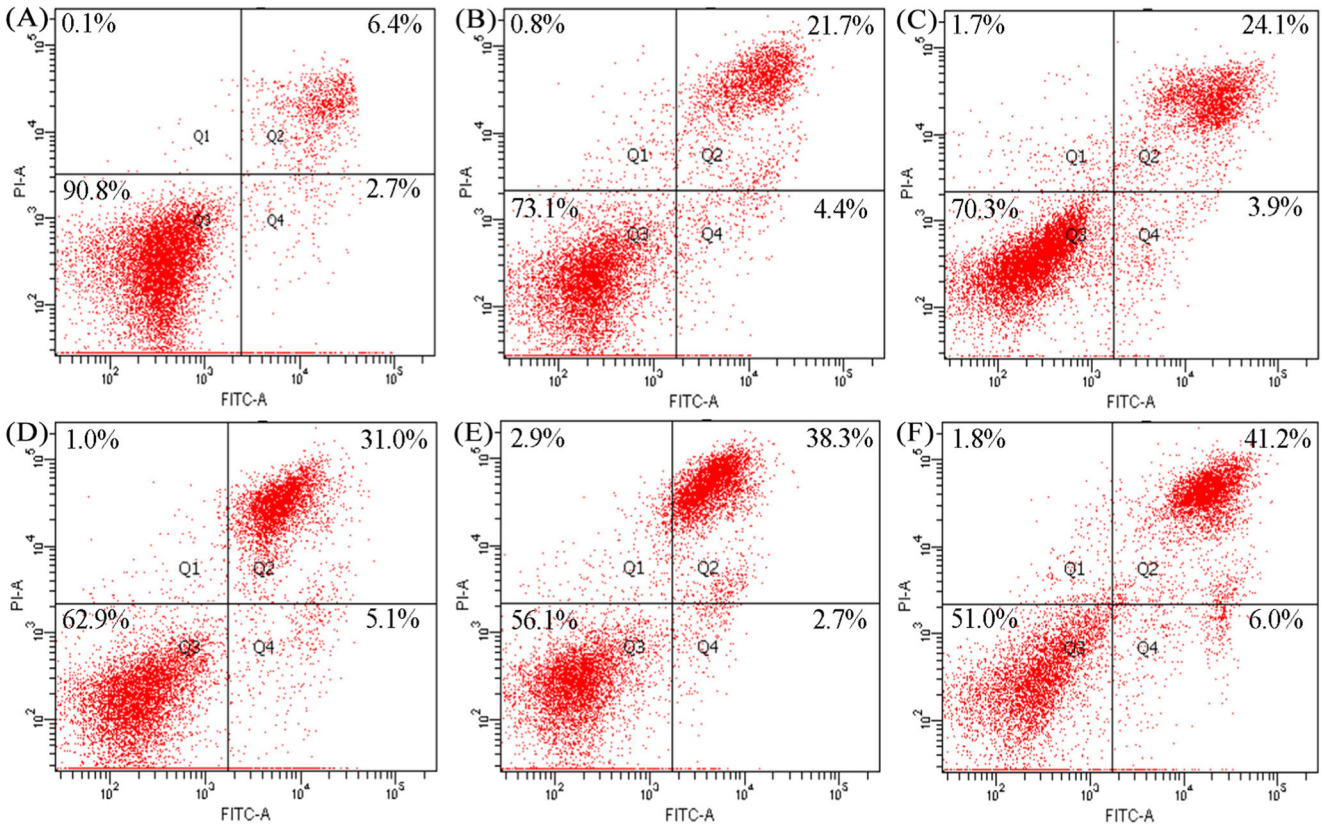

Fig. 3 Apoptosis induction of LFH and Mixtures I–IV in AGS cells. The cells were treated without (**a**) or with LFH (**b**) and Mixtures I–IV (**c**–**f**) at 25 mg/mL for 24 h, respectively

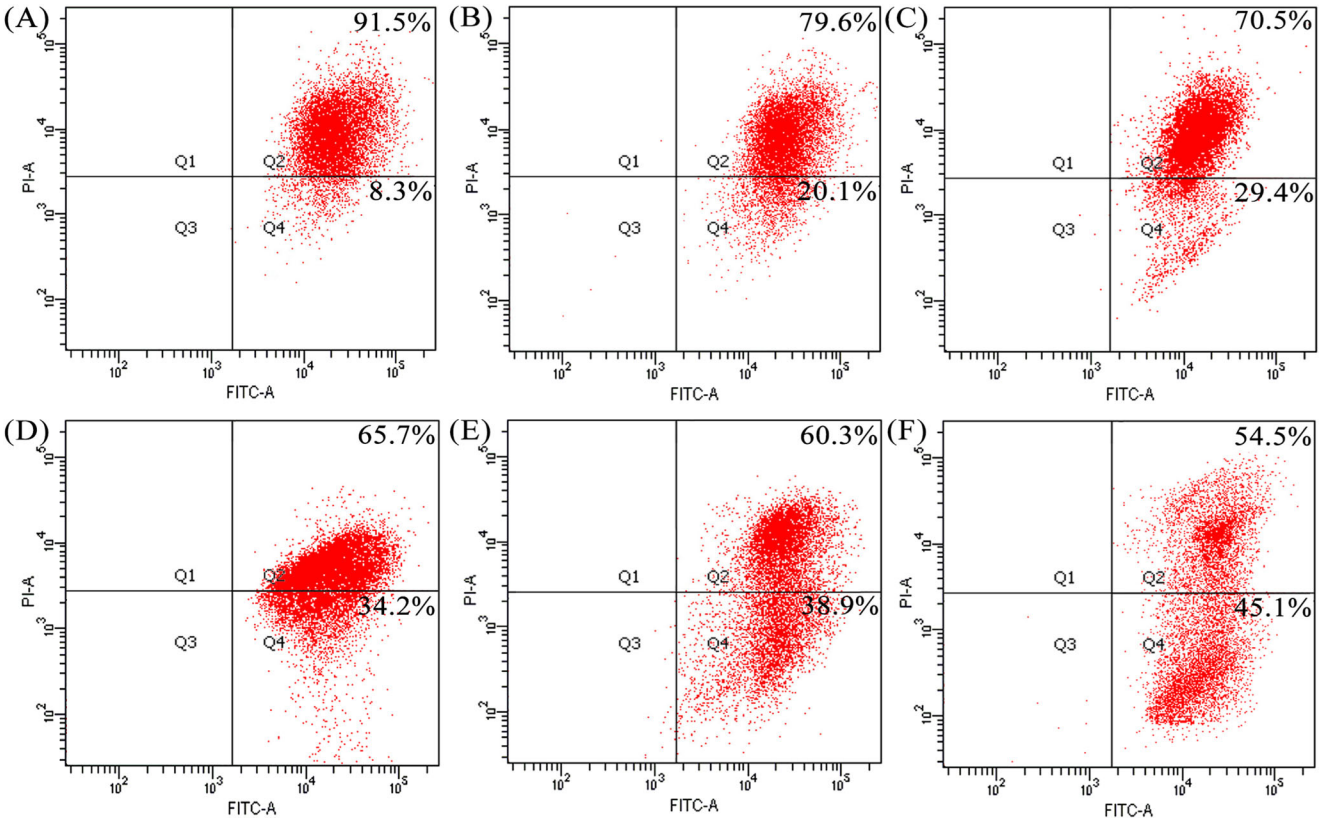

Fig. 4 Mitochondrial membrane potential of the AGS cells treated without (**a**) or with LFH (**b**) and Mixtures I–IV (**c**–**f**) at 25 mg/mL for 24 h, respectively

expression levels for these proteins. Overall, Mixture IV showed the highest activity to regulate expression levels of these proteins. The results demonstrate that Mn^{2+} supplementation was more powerful than Cu^{2+} supplementation to regulate protein expression, and higher metal concentrations in these mixtures led to greater regulation.

Based on these results, LFH and especially Mixtures I–IV are thus proposed to display anti-cancer effects in AGS cells via mediating apoptosis and autophagy, as indicated in Fig. 6. In brief, LFH and its Cu^{2+}/Mn^{2+} mixtures induced apoptosis by caspase-3, Bad, Bax, and p53 pathway, and exerted enhanced apoptosis through autophagy inhibition by LC3-II down-regulation and p53 activation. And more, a cross-talk between apoptosis and autophagy was thus verified.

Discussion

The hydrolysates from several protein resources have been evidenced to exert anti-cancer effects in various cancer cells like Hela, PC-3, DU-145, and H-1299 [24, 25]. Bovine LF is one of the native proteins with many biofunctions in the body, and also has anti-cancer activity in cancer cells but without any harmful effect on normal cells [26]. At the same time, the hydrolysates from bovine LF can display an inhibitory effect in gastric cancer cells [8, 10], inhibit liver and lung metastasis in the mice [27], and show anti-cancer activities against colon cancer cells [28]. In this study, the prepared LFH showed anti-cancer activities in AGS cells, reflected as growth inhibition, cell cycle arrest, mitochondrial membrane potential disruption, and apoptosis induction, sharing consistent conclusion to these reported works. However, when Cu^{2+} and especially Mn^{2+} were supplemented to LFH, the resultant mixtures had enhanced anti-cancer effects in AGS cells. This fact implies that some peptides in LFH might react with Cu^{2+}/Mn^{2+} to form metal-peptide complexes, and therefore possessed higher potentials to behave these assessed effects in the cells. It was found that Fe-supplemented LF could significantly inhibit the growth of HBV-infected HepG2 cells, in comparison with LF itself [29]. It is reasonable that LFH supplemented with Cu^{2+}/Mn^{2+} had enhanced anti-cancer effects in AGS cells. Furthermore, higher Cu^{2+}/Mn^{2+} supplementation level resulted in higher values for these measured activities, indicating the two metal ions indeed made contribution to anti-cancer effect. However, why Mn^{2+} had higher enhancement than Cu^{2+} on these anti-cancer activities was not clarified in this study. This phenomenon is important and should be given a special verification in future.

In general, natural compounds may exert in vitro effects on cancer cells via different pathways including growth inhibition, cell cycle arrest, apoptosis induction, and other effects. It

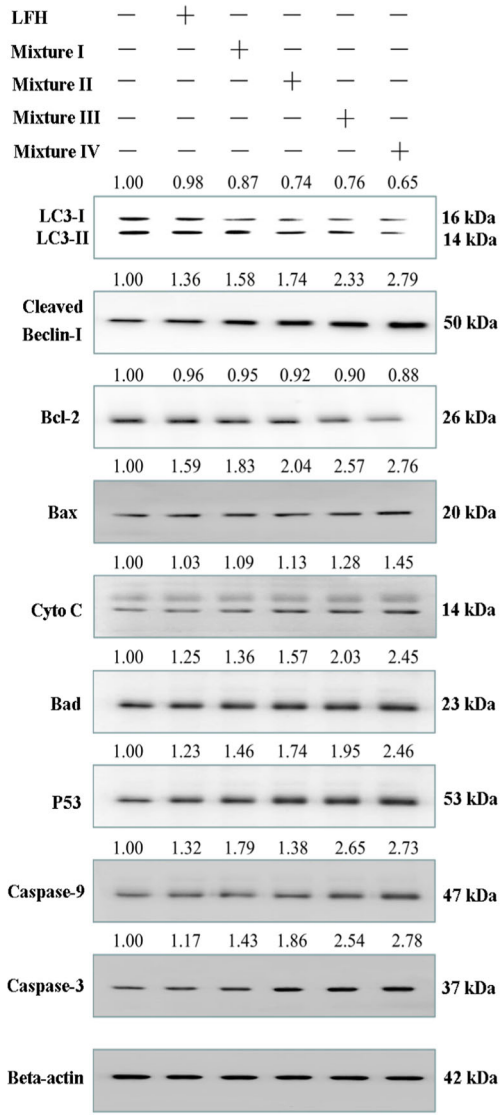

Fig. 5 Expression changes of the apoptosis- and autophagy-related proteins in AGS cells treated without or with LFH and Mixtures I–IV for 24 h. Cyto C cytochrome c. MMP mitochondrial membrane potential

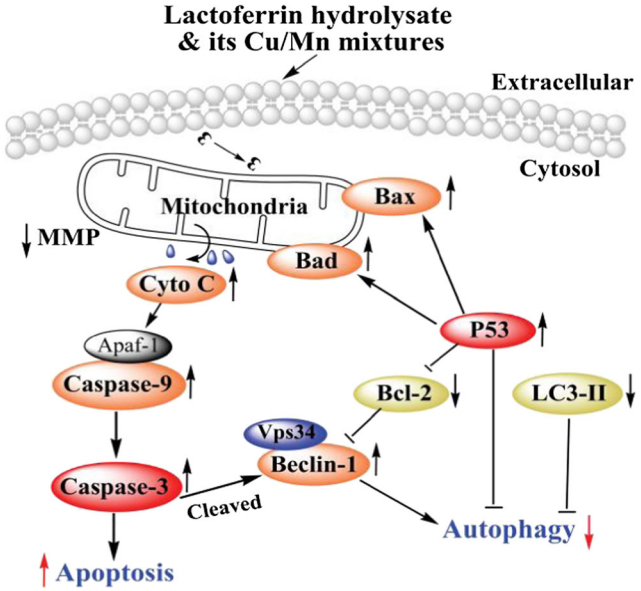

Fig. 6 Proposed molecular mechanism responsible for the anti-cancer effects of LFH and its Cu^{2+}/Mn^{2+} mixtures in AGS cells: apoptosis induction and autophagy inhibition

is known that rapid growth and development of cancer cells are achieved by continuous division of the cells, while cell cycle is a well-ordered process of cell division. Therefore, arresting cell cycle progression at some cell phases is an important approach to inhibit the growth of cancer cells [30]; for example, flavonoid compounds can arrest cell cycle progression at G0/G1-phase [6]. Protein hydrolysates can block cell cycle progression of several cancer cells. In a study conducted by Mao et al. [31], the hydrolysate from donkey milk increased cell proportion of G0/G1-phase in human lung cancer cells. Similarly, protein hydrolysates of giant grouper (*Epinephelus lanceolatus*) were found to arrest cell cycle of oral cancer cells at sub-G1 phase [32]. Importantly, apoptosis is a requisite mechanism of programmed cell death, and apoptosis induction to tumor cells is one of main strategies for cancer treatment [33]. Two previous studies reported that peptide fraction derived from algae protein and protein hydrolysates from tuna cooking juice had apoptosis induction to AGS and MCF-7 cells [10, 34]. This study also found that LFH and especially Mixtures I–IV could exert effect on AGS cells through growth inhibition, cell cycle arrest, and apoptosis induction.

Cell apoptosis can be triggered by releasing cytochrome c from mitochondria into the cytosol. Cytosolic cytochrome c can bind to Apaf-1 and then activate caspase-9, which results in caspase-3 activation and apoptosis [35]. Loss of mitochondrial membrane potential was observed for the treated AGS cells, which brought mitochondrial dysfunction and cytochrome c release (Figs. 5 and 6). The p53 has been shown to enhance its tumor-suppressing activity by apoptosis modulation [36]. Bcl-2 protein family consists of pro-apoptotic proteins (such as Bax and Bad) and anti-apoptotic proteins (such as Bcl-2 and Bcl-xl) that have opposing effects on mitochondria. Activation of pro-apoptotic Bad and Bax by p53 can increase permeability of mitochondrial membrane, leading to the release of apoptogenic factors like cytochrome c. Anti-apoptotic Bcl-2 protein which preserves mitochondrial integrity is suppressed by p53 [37]. This blocks the release of several soluble intermembrane factors like cytochrome c, which activate the executioners of cell apoptosis [38]. The results from two previous studies revealed that lactoferricin B selectively induced apoptosis in human leukemia and carcinoma cells via loss of mitochondrial membrane potential and caspases activation (such as caspase-3, caspase-6, and caspase-9) [39, 40]. Meanwhile, the peptides from rapeseed and a tuna cooking juice protein could induce MCF-7 and HepG2 cells apoptosis via up-regulated p53, Bax, and cleaved

caspase-3 as well as down-regulated Bcl-2 [34, 41]. In this study, LFH supplemented with Cu^{2+}/Mn^{2+} could up-regulate the expression of pro-apoptotic Bad, Bax, and p53 proteins but down-regulate the expression of anti-apoptotic Bcl-2 protein in AGS cells, resulting in increased release of cytochrome c from mitochondria, which subsequently triggered the activation of caspase-9 and caspase-3 as well as cell apoptosis (Fig. 6).

Autophagy is a cellular renewal mechanism, and functions in the cytoprotection of normal and cancer cells [42]. The growth of cancer cells such as pancreatic and gastric cancer cells requires autophagy [43]. Autophagy inhibition is thus proposed to have an important role on cell apoptosis. In Hela cells, autophagy inhibition is verified through caspase-3 activation, and makes positive contribution to apoptosis [44]. It also is found that cross-talk exists between autophagy and apoptosis [45]. Both Beclin-1 and p53 are classified as autophagy- and apoptosis-related proteins [46]. Beclin-1 interacts with several cofactors to modulate lipid kinase Vps-34 protein to facilitate formation of Beclin 1-Vps34-Vps15 core complexes, thereby induces autophagy [47]. However, Bcl-2 can bind to Beclin-1, thus inhibit Beclin-1-mediated autophagy [48]. Beclin-1 is a substrate for caspase-3; activated caspase-3 increases Beclin-1 cleavage, leading to inhibit autophagy in breast and other human malignancies [49]. The well-characterized tumor suppressor p53 is a critical mediator of cell death. Activation of p53 has been evidenced to inhibit autophagy [50]. And more, autophagy inhibition reflects reduction expression of LC3-II [51]. In this study, the treated AGS cells had up-regulated cleaved Beclin-1 and p53 but down-regulated LC3-II proteins (Fig. 5). LFH and its Cu^{2+}/Mn^{2+} mixtures thus had autophagy inhibition in AGS cells. At the same time, potential cross-talk between autophagy and apoptosis through caspase-3 and p53 pathway was also verified in this study. Clearly, both caspase-3 and p53 activation were essential to the assessed apoptosis induction and autophagy inhibition. It was also found in a previous study that docosahexaenoic acid induced apoptosis of SiHa cells by p53 and caspase-3 activation to inhibit autophagy [52].

Conclusion

This study declares that supplementation of peptic bovine lactoferrin hydrolysate with Cu^{2+} and especially Mn^{2+} could bring enhanced anti-cancer effects in AGS cells. In total, the supplemented hydrolysates exerted higher growth inhibition, arrested more cells at G0/G1-phase, and induced more cells into apoptotic cells. Furthermore, the supplemented hydrolysates could damage mitochondrial membrane integrity and regulate expression levels of several apoptosis- and autophagy-related proteins in AGS cells. Molecular mechanism responsible for these anti-cancer effects are proposed as enhanced apoptosis induction and autophagy inhibition. This study thus highlights anti-cancer benefits of the two trace metals to bovine lactoferrin, from a nutritive point of view.

Funding Information This work was funded by the Innovative Research Team of Higher Education of Heilongjiang Province (Project No. 2010td11). The authors thank Prof. Li-Ling Yue from Qiqihar Medical University for his kindly help in Western-blot assay, and also thank the anonymous referees for their valuable advice.

Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no competing interests.

References

1. de Martel C, Forman D, Plummer M (2013) Gastric cancer: epidemiology and risk factors. Gastroenterol Clin N Am 42:219–240. https://doi.org/10.1016/j.gtc.2013.01.003
2. Baeza MR, Giannini TO, Rivera SR, González P, González J, Vergara E, del Castillo C, Madrid J, Vinés E (2001) Adjuvant radiochemotherapy in the treatment of completely resected, locally advanced gastric cancer. Int J Radiat Oncol Biol Phys 50:645–650. https://doi.org/10.1016/S0360-3016(01)01467-5
3. Longley DB, Harkin DP, Johnston PG (2003) 5-Fluorouracil: mechanisms of action and clinical strategies. Nat Rev Cancer 3:330–338. https://doi.org/10.1038/nrc1074
4. Iqbal J, Abbasi BA, Mahmood T, Kanwal S, Ali B, Shah SA, Khalil AT (2017) Plant-derived anticancer agents: a green anticancer approach. Asian Pac J Trop Biomed 7:1129–1150. https://doi.org/10.1016/j.apjtb.2017.10.016
5. Sun Q, Lu NN, Feng L (2018) Apigetrin inhibits gastric cancer progression through inducing apoptosis and regulating ROS-modulated STAT3/JAK2 pathway. Biochem Biophys Res Commun 498:164–170. https://doi.org/10.1016/j.bbrc.2018.02.009
6. Yoshimizu N, Otani Y, Saikawa Y, Kubota T, Yoshida M, Furukawa T, Kumai K, Kameyama K, Fujii M, Yano M, Sato T, Ito A, Kitajima M (2004) Anti-tumour effects of nobiletin, a citrus flavonoid, on gastric cancer include: antiproliferative effects, induction of apoptosis and cell cycle deregulation. Aliment Pharmacol Ther 20:95–101. https://doi.org/10.1111/j.1365-2036.2004.02082.x
7. Sarkar A, Bhattacharjee S, Mandal DP (2015) Induction of apoptosis by eugenol and capsaicin in human gastric cancer AGS cells-elucidating the role of p53. Asian Pac J Cancer Prev 16:6753–6759. https://doi.org/10.7314/APJCP.2015.16.15.6753
8. Amiri F, Moradian F, Rafiei A (2015) Anticancer effect of lactoferrin on gastric cancer cell line AGS. Res Mol Med 3:11–16. https://doi.org/10.7508/rmm.2015.02.002
9. Reddy GV, Friend BA, Shahani KM, Farmer RE (1983) Antitumor activity of yogurt components. J Food Prot 46:8–11. https://doi.org/10.4315/0362-028X-46.1.8
10. Sheih IC, Fang TJ, Wu TK, Lin PH (2010) Anticancer and antioxidant activities of the peptides fraction from algae protein waste. J Agric Food Chem 58:1202–1207. https://doi.org/10.1021/jf903089m
11. Xu XX, Jiang HR, Li HB, Zhang TN, Zhou Q, Liu N (2010) Apoptosis of stomach cancer cell SGC-7901 and regulation of Akt signaling way induced by bovine lactoferrin. J Dairy Sci 93:2344–2350. https://doi.org/10.3168/jds.2009-2926
12. Pan WR, Chen PW, Chen YL, Hsu HC, Lin CC, Chen WJ (2013) Bovine lactoferricin B (LFcinB25) induces apoptosis of human

gastric cancer cell line AGS by inhibition of autophagy at a late stage. J Dairy Sci 96:7511–7520. https://doi.org/10.3168/jds.2013-7285
13. Teuwissen B, Masson PL, Osinski P, Heremans JF (1969) Metal-combining properties of human lactoferrin (red milk protein). Eur J Biochem 6:579–584. https://doi.org/10.1111/j.1432-1033.1968.tb00484.x
14. Intawongse M, Dean JR (2006) Uptake of heavy metals by vegetable plants grown on contaminated soil and their bioavailability in the human gastrointestinal tract. Food Addit Contam 23:36–48. https://doi.org/10.1080/02652030500387554
15. Bogden JD, Klevay LM (2000) Clinical nutrition of the essential trace elements and minerals. Humana Press, Totowa
16. Mertz W (1981) The essential trace elements. Science 213:1332–1338. https://doi.org/10.1126/science.7022654
17. Beard JL (2001) Iron biology in immune function, muscle metabolism and neuronal functioning. J Nutr 131:568–579. https://doi.org/10.1093/jn/131.2.568S
18. Tisato F, Marzano C, Porchia M, Pellei M, Santini C (2010) Copper in diseases and treatments, and copper-based anticancer strategies. Med Res Rev 30:708–749. https://doi.org/10.1002/med.20174
19. Zhou DF, Chen QY, Qi Y, Fu HJ, Li Z, Zhao KD, Gao J (2011) Anticancer activity, attenuation on the absorption of calcium in mitochondria, and catalase activity for manganese complexes of N-substituted di(picolyl)amine. Inorg Chem 50:6929–6937. https://doi.org/10.1021/ic200004y
20. Gou Y, Li J, Fan B, Xu B, Zhou M, Yang F (2017) Structure and biological properties of mixed-ligand Cu (II) Schiff base complexes as potential anticancer agents. Eur J Med Chem 134:207–217. https://doi.org/10.1016/j.ejmech.2017.04.026
21. Bellamy W, Takase M, Wakabayashi H, Kawase K, Tomita M (1992) Antibacterial spectrum of lactoferricin B, a potent bactericidal peptide derived from the N-terminal region of bovine lactoferrin. J Appl Bacteriol 73:472–479. https://doi.org/10.1111/j.1365-2672.1992.tb05007.x
22. AOAC (ed) (2005) Official methods of analysis of the Official Analytical Chemists International, 18th edn. Gaithersburg, AOAC Int
23. Vermes I, Haanen C, Steffens-Nakken H, Reutellingsperger C (1995) A novel assay for apoptosis. Flow cytometric detection of phosphatidylserine expression on early apoptotic cells using fluorescein labelled Annexin V. J Immunol Methods 184:39–51. https://doi.org/10.1016/0022-1759(95)00072-I
24. Pan X, Zhao YQ, Hu FY, Chi CF, Wang B (2016) Anticancer activity of hexapeptide from (*Raja porosa*) cartilage protein hydrolysate in Hela cells. Mar Drugs 14:153–163. https://doi.org/10.3390/md14080153
25. Chi CF, Hu FY, Wang B, Li T, Ding GF (2015) Antioxidant and anticancer peptides from the protein hydrolysate of blood clam (*Tegillarca granosa*) muscle. J Funct Foods 15:301–313. https://doi.org/10.1016/j.jff.2015.03.045
26. Chalamaiah M, Yu W, Wu J (2018) Immunomodulatory and anticancer protein hydrolysates (peptides) from food proteins: a review. Food Chem 245:205–222. https://doi.org/10.1016/j.foodchem.2017.10.087
27. Yoo YC, Watanabe S, Watanabe R, Hata K, Shimazaki K, Azuma I (1997) Bovine lactoferrin and lactoferricin inhibit tumor metastasis in mice. Jpn J Cancer Res 88:184–190. https://doi.org/10.1111/j.1349-7006.1997.tb00364.x
28. Freiburghaus C, Janicke B, Lindmark-Mansson H, Oredsson SM, Paulsson MA (2009) Lactoferricin treatment decreases the rate of cell proliferation of a human colon cancer cell line. J Dairy Sci 92:2477–2484. https://doi.org/10.3168/jds.2008-1851
29. Li ST, Zhou HB, Huang GR, Liu N (2009) Inhibition of HBV infection by bovine lactoferrin and iron-, zinc-saturated lactoferrin. Med Microbiol Immunol 198:19–25. https://doi.org/10.1007/s00430-008-0100-7
30. Du M, Liu Y, Zhang G (2017) Interaction of aflatoxin B 1 and fumonisin B 1 in HepG2 cell apoptosis. Food Biosci 20:131–140. https://doi.org/10.1016/j.fbio.2017.09.003
31. Mao XY, Gu JN, Sun Y, Xu SP, Zhang XY, Yang HY, Ren FZ (2009) Anti-proliferative and anti-tumour effect of active components in donkey milk on A549 human lung cancer cells. Int Dairy J 19:703–708. https://doi.org/10.1016/j.idairyj.2009.05.007
32. Yang JI, Tang JY, Liu YS, Wang HR, Lee SY, Yen CY, Chang HW (2016) Roe protein hydrolysates of giant grouper (*Epinephelus Lanceolatus*) inhibit cell proliferation of oral cancer cells involving apoptosis and oxidative stress. Biomed Res Int 23:1–12. https://doi.org/10.1155/2016/8305073
33. Edinger AL, Thompson CB (2004) Death by design: apoptosis, necrosis and autophagy. Curr Opin Cell Biol 16:663–669. https://doi.org/10.1016/j.ceb.2004.09.011
34. Hung CC, Yang YH, Kuo PF, Hsu KC (2014) Protein hydrolysates from tuna cooking juice inhibit cell growth and induce apoptosis of human breast cancer cell line MCF-7. J Funct Foods 11:563–570. https://doi.org/10.1016/j.jff.2014.08.015
35. Kluck RM, Bossy-Wetzel E, Green DR, Newmeyer DD (1997) The release of cytochrome c from mitochondria: a primary site for Bcl-2 regulation of apoptosis. Science 275:1132–1136. https://doi.org/10.1126/science.275.5303.1132
36. Symonds H, Krall L, Remington L, Saenz-Robles M, Lowe S, Jacks T, Van Dyke T (1994) p53-dependent apoptosis suppresses tumor growth and progression *in vivo*. Cell 78:703–711. https://doi.org/10.1016/0092-8674(94)90534-7
37. Ryan KM, Phillips AC, Vousden KH (2001) Regulation and function of the p53 tumor suppressor protein. Curr Opin Cell Biol 13:332–337. https://doi.org/10.1016/S0955-0674(00)00216-7
38. Donovan M, Cotter TG (2004) Control of mitochondrial integrity by Bcl-2 family members and caspase-independent cell death. Biochim Biophys Acta 1644:133–147. https://doi.org/10.1016/j.bbamcr.2003.08.011
39. Mader JS, Salsman J, Conrad DM, Hoskin DW (2005) Bovine lactoferricin selectively induces apoptosis in human leukemia and carcinoma cell lines. Mol Cancer Ther 4:612–624. https://doi.org/10.1158/1535-7163.MCT-04-0077
40. Furlong SJ, Mader JS, Hoskin DW (2006) Lactoferricin-induced apoptosis in estrogen-nonresponsive MDA-MB-435 breast cancer cells is enhanced by C6 ceramide or tamoxifen. Oncol Rep 15:1385–1390. https://doi.org/10.3892/or.15.5.1385
41. Wang L, Zhang J, Yuan Q, Xie H, Shi J, Ju X (2016) Separation and purification of an anti-tumor peptide from rapeseed (*Brassica campestris L.*) and the effects on cell apoptosis. Food Funct 7:2239–2248. https://doi.org/10.1039/c6fo00042h
42. Fu LL, Cheng Y, Liu B (2013) Beclin-1: Autophagic regulator and therapeutic target in cancer. Int J Biochem Cell Biol 45:921–924. https://doi.org/10.1016/j.biocel.2013.02.007
43. Yang SH, Wang XX, Contino G et al (2011) Pancreatic cancers require autophagy for tumor growth. Genes Dev 25:717–729. https://doi.org/10.1101/gad.2016111
44. Chakraborty D, Bishayee K, Ghosh S, Biswas R, Mandal SK, Khuda-Bukhsh AR (2012) [6]-Gingerol induces caspase-3 dependent apoptosis and autophagy in cancer cells: Durg-DNA interaction and expression of certain signal genes in Hela cells. Eur J Pharmacol 694:20–29. https://doi.org/10.1016/j.ejphar.2012.08.001
45. Fimia GM, Piacentini M (2010) Regulation of autophagy in mammals and its interplay with apoptosis. Cell Mol Life Sci 67:1581–1588. https://doi.org/10.1007/s00018-010-0248-z
46. Crighton D, Wilkinson S, O'Prey J, Syed N, Smith P, Harrison PR, Gasco M, Garrone O, Crook T, Ryan KM (2006) DRAM, a p53-

induced modulator of autophagy, is critical for apoptosis. Cell 126: 121–134. https://doi.org/10.1016/j.cell.2006.05.034

47. Kang R, Zeh HJ, Lotze MT, Tang D (2011) The Beclin-1 network regulates autophagy and apoptosis. Cell Death Differ 18:571–580. https://doi.org/10.1038/cdd.2010.191
48. Giansanti V, Torriglia A, Scovassi AI (2011) Conversation between apoptosis and autophagy: "Is it your turn or mine?". Apoptosis 16: 321–333. https://doi.org/10.1007/s10495-011-0589-x
49. Liang XH, Jackson S, Seaman M, Brown K, Kempkes B, Hibshoosh H, Levine B (1999) Induction of autophagy and inhibition of tumorigenesis by Beclin-1. Nature 402:672–676. https://doi.org/10.1038/45257
50. Feng ZH, Zhang HY, Levine AJ, Jin SK (2005) The coordinate regulation of the p53 and mTOR pathways in cells. Proc Natl Acad Sci U S A 102:8204–8209. https://doi.org/10.1073/pnas.0502857102
51. Patricia B, Rosa-Ana GP, Noelia C, Jean-Luc P, Philippe D, Nathanael L, Didier M, Daniel M, Sylvie S, Tamotsu Y, Gérard P, Patrice C, Guido K (2005) Inhibition of macroautophagy triggers apoptosis. Mol Cell Biol 25:1025–1040. https://doi.org/10.1128/MCB.25.3.1025-1040.2005
52. Jing KP, Song KS, Shin S et al (2011) Docosahexaenoic acid induces autophagy through p53/AMPK/mTOR signaling and promotes apoptosis in human cancer cells harboring wild-type p53. Autophagy 7:1348–1358. https://doi.org/10.4161/auto.7.11.16658