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Low-level Cu-fortification of bovine lactoferrin: Focus on its effect on in vitro anti-inflammatory activity in LPS-stimulated macrophages

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ABSTRACT

Bovine lactoferrin (LF) per 1 g was reacted with 0.16, 0.32, and 0.64 mg $CuCl_2$ to reach 10%, 20%, and 40% copper-saturation, respectively, aiming to assess their anti-inflammatory activities to lipopolysaccharide (LPS)-stimulated RAW264.7 macrophages. The macrophages treated with $CuCl_2$ at 0.051 μg/mL dose did not have obvious change in cell viability, lactate dehydrogenase (LDH) release, and intracellular reactive oxygen species (ROS) production. However, LF and Cu-fortified LF products (10−80 μg/mL doses) mostly showed inhibitory effects on the stimulated macrophages dose-dependently. Moreover, Cu-fortified LF products of lower Cu-fortifying levels at lower doses exerted weaker inhibition on the stimulated macrophages than LF, leading to higher cell viability but decreased LDH release. Meanwhile, LF and Cu-fortified LF products at 10 and 20 μg/mL doses showed different activities to the stimulated cells, *via* partly decreasing or increasing the production of inflammatory mediators namely prostaglandin E_2 (PGE_2), nitric oxide, tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6), IL-1β, and ROS production, depending on the used Cu-fortifying and dose levels. Compared with LF, Cu-fortified LF product (Cu-fortifying level of 0.16 mg/g LF) at 10 μg/mL dose showed enhanced inhibition on the production of PGE_2, ROS, IL-1β, and TNF-α, evidencing increased anti-inflammatory activity. However, the inhibition of Cu-fortified LF product (Cu-fortifying level of 0.32 mg/g LF) at 20 μg/mL dose on the production of these inflammatory mediators was mostly reduced. It is thus proposed that both Cu-fortifying and dose levels could affect LF's anti-inflammatory activity in LPS-stimulated macrophages, while the Cu-fortifying level of LF could govern activity change.

1. Introduction

Lactoferrin (LF), one of the minor fractions of whey proteins, belongs to the transferrin family (Takayama, 2012; Pryshchepa et al., 2022). LF is an iron-binding glycoprotein and has various bio-functions such as anti-microbial, anti-carcinogenic, anti-inflammatory, and immuno-modulatory effects (Naidu, 2000; Huma et al., 2014; Navarro et al., 2015). It is generally accepted that LF can affect the innate and adaptive immune systems, and is thus involved in both acute and chronic inflammation to regulate the immune response (Kruzel et al., 2006; Huma et al., 2014). For example, LF, as a marker of inflammatory diseases, has specific and sensitive activities to chronic inflammatory bowel diseases (Judd et al., 2011). LF can modulate cytokines or cellular activity and thus exert anti-inflammatory effect to reduce immune cell recruitment into the inflammatory sites (Legrand and Mazurier, 2010). LF also exhibits the anti-inflammatory effect on skin inflammation by decreasing the expression of tumor necrosis factor-α (TNF-α) or reducing the number of infiltrating leukocytes in human influenza virus infections (Kruzel et al., 2006; Yamauchi et al., 2006). It was verified that LF could suppress the toll-like receptor 4 (TLR-4)-related inflammatory pathway in both anoxia cell and cerebral ischemia reperfusion mouse models, while heat treatment might reduce LF activity temperature-dependently (Yang et al., 2020). With increased attention paid to the anti-inflammatory properties of LF, bovine LF, as one important protein fraction from bovine milk, is now commercially produced and used as one of the food supplements to promote human health. LF has also been

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used in clinical trials and medicine field due to its anti-inflammatory activity (Ulloa et al., 2016; Drago-Serrano et al., 2017).

The interaction between LF and other food components like essential trace elements and polyphenols could lead to activity changes (Marchetti et al., 1999; Mohan et al., 2007; Burrow et al., 2011; Chaharband et al., 2018). Several trace elements such as Zn, Cu, Fe, and Se are involved in multiple physiological processes in the body including immuno-regulation (Failla, 2003; Tsuji et al., 2016), and thus are recommended as nutritional supplements in processed foods (*e.g.* the infant formulated milk powder). Zn, Fe, and Cu also can regulate macrophage activity and have immuno-modulatory and anti-microbial effects (Stafford et al., 2013; Weiss, 2009; Tsuji et al., 2016). For instance, Zn can affect the production of inflammatory cytokine TNF-α (Foster and Samman, 2012), while Fe released by macrophages is proposed to be involved in the process of inhibiting inflammatory cytokines (Weiss, 2009). However, LF has a good affinity to Fe^{3+}, and thus can remove the free Fe^{3+} from the site of inflammation (Brock, 2012; Huma et al., 2014). Cu can decrease the secretion of interleukin-6 (IL-6) and IL-1β in cancer-induced rats (Hassan et al., 2019). When Cu is used as a nutritional supplement in the formulated infant milk powder, its suggested level is 8.4–25 μg/100 kJ (Commission of the European The Commission of the European Communities, 2006). Meanwhile, it was proposed that lower Cu levels (*i.e.* 20−30 μmol/L) did not exhibit toxicity to cells (Contreras et al., 2010; Hu et al., 2016). Chemically, the fortified Cu has the opportunity to contact or interact with bovine LF in the milk because native LF is only partly Fe-saturated. The fortified trace elements thus might increase or decrease LF activity. For example, the Se-, Mn-, and Zn-fortification of LF induced an increase in anti-oxidative, anti-bacterial, and anti-viral effects (Marchetti et al., 1999; Goldoni et al., 2000; Burrow et al., 2011). Whether the low-level Cu-fortification of LF might change the anti-inflammatory activity of LF is still less studied. A previous study of our group had verified that the Cu-fortification of a commercial bovine LF at 0.16 mg Cu per gram LF caused an enhanced immuno-modulatory effect in both splenocytes and macrophages; however, the higher Cu-fortifying level led to decreased LF activity (Zhao and Zhao, 2019). Whether the low-level Cu-fortification might change the anti-inflammatory activity of LF in lipopolysaccharide (LPS)-stimulated macrophages is an interesting issue but remains a challenge in the present.

In this study, the commercial bovine LF was thus fortified with $CuCl_2$ to reach three Cu-fortifying levels of 0.16, 0.32, and 0.64 mg Cu per gram of LF, causing 10%, 20%, and 40% Cu-saturation in LF. After then, LF and the three Cu-fortified LF products were assessed for their anti-inflammatory activities using the LPS-stimulated RAW264.7 macrophages as a cell model by measuring the LPS-induced production of inflammatory mediators like nitric oxide (NO), prostaglandin E2 (PGE2), TNF-α, IL-6, IL-1β, and intracellular reactive oxygen species (ROS). This study aimed to verify two roles of the fortified Cu in LF: (1) whether the low-level Cu-fortification of LF could cause a change in anti-inflammatory activity in this cell model, and (2) whether the Cu-fortifying level as a chemical factor might control the change of LF activity.

2. Materials and methods

2.1. Materials and reagents

The bovine LF was bought from MILEI GmbH. (Leutkirch, Germany), measured with corresponding protein and iron contents of 979.0 g and 160 mg per kilogram, and thus estimated with a Fe-saturation of about 11%. The Dulbecco's modified essential medium with high glucose (DMEM) and fetal bovine serum (FBS) used in the cell experiments were provided by HyClone Co. (Logan, UT, USA) and Wisent Inc. (Montreal, Quebec, Canada), respectively. The LPS isolated from *Escherichia coli* 055:B5 was bought from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA). The phosphate-buffered saline (PBS, pH 7.2) and $CuCl_2$ were obtained from Solarbio Science and Technology Co., Ltd. (Beijing, China) and Tianli Chemical Reagent Co., Ltd. (Tianjin, China), respectively. Other chemicals used in this study were analytical agents. Ultrapure water used in this study was generated from the Milli-Q Plus (Millipore Corporation, New York, NY, USA).

The cell counting kit-8 (CCK-8) and lactate dehydrogenase (LDH) assay kit were bought from Dojindo Laboratories (Kyushu, Japan) and Nanjing Jiancheng Biological Engineering Research Institute (Nanjing, China), respectively. The nitric oxide and reactive oxygen species assay kits were bought from Beyotime Biotechnology Co., Ltd. (Shanghai, China), while the PGE_2 ELISA kit was bought from Shanghai Enzyme-linked Biotechnology Co., Ltd. (Shanghai, China). Other ELISA kits used to measure TNF-α, IL-6, and IL-1β were provided by Wuhan Boster Biological Engineering Co., Ltd. (Wuhan, China).

2.2. Sample preparation and cell culture

Based on two well-known facts that bovine LF has a molecular weight of about 80 kDa and fully Fe-saturated LF may contain 2 mol iron per mole of LF (Huma et al., 2014), the Cu-fortification of LF was done at three fortifying levels (0.16, 0.32, and 0.64 mg per gram of LF) as previously described (Zhao and Zhao, 2019). The three levels endowed the Cu-fortified LF products with 10%, 20%, and 40% Cu-saturation, which were designed throughout this study as LF-Cu-10, LF-Cu-20, and LF-Cu-40, respectively. In detail, $CuCl_2$ was dissolved in water to generate a solution with Cu concentration of 32.0 mg/L; after that, part of this solution (0.15, 0.30, and 0.60 mL) was added into 100 mL culture medium containing 30 mg LF. The mixed medium was immediately used in cell experiments.

The RAW 264.7 macrophages were bought from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China). As the cell provider requires, the cells were cultured in the DMEM medium supplemented with 10% FBS at 37 °C in a humidified atmosphere of 5% CO_2. The cells were maintained by sub-culturing them every other day.

2.3. Assay of cell viability

The cells were seeded onto 96-well plates at cell density of 2×10^4 cells/100 μL/well and then incubated at 37 °C for 18 h. After culture removal, LF and Cu-fortified LF products (10−80 μg/mL doses) in 10% FBS-supplemented medium were added to each well for a treatment time of 30 min, followed by a co-culture with LPS (1 μg/mL) for 24 h. Meanwhile, $CuCl_2$ (dissolved in water) was used at a 0.051 μg/mL dose to treat the seeded cells similarly, while the generated Cu level was the same as that generated from LF-Cu-40 at 80 μg/mL dose.

After these cell treatments, the cells were incubated at 37 °C for 24 h. Cell culture was then removed, and 100 μL medium containing 10 μL CCK-8 solution was added into each well. The plates were incubated for another 1.5 h. Optical density at 450 nm of each well was evaluated by a microplate reader (Bio Rad Laboratories, Hercules, CA), and used to calculate cell viability (%) as previously described (Lee et al., 2011). The control cells without any treatment were served with a viability value of 100%.

2.4. Assay of LDH release

The assay of LDH activity in cell medium was used to identify the potential effect of LF, LF-Cu-10, and LF-Cu-20 (10 and 20 μg/mL doses) on the LPS-stimulated macrophages. The cells seeded onto the 96-well plates at 2×10^4 cells/100 μL/well were incubated for 18 h, incubated with these samples for 30 min, and then treated with 1 μg/mL LPS for 24 h. In brief, the culture medium was centrifuged at 2000×*g* for 20 min at 20 °C to ensure cell accumulation. The cell-free culture medium was collected and measured for LDH activity using the LDH assay kit and kit instructions. The optical density was measured using the mentioned microplate reader at 450 nm wavelength.

Similarly, the seeded cells, after an incubation of 18 h, were treated with $CuCl_2$ at the 0.051 μg/mL dose for 30 min and incubated with the DMEM medium for 24 h. The obtained culture medium was then measured for LDH activity as above. The control cells without any treatment were designed with 100% LDH release, while the LDH release of the $CuCl_2$-treated cells was expressed as % of the control cells.

2.5. Determination of intracellular ROS production

A classic DCFH/DA (2′,7′-dichlorodihydrofluorescein diacetate) method was used to determine ROS levels in the LPS-stimulated macrophages as previously described (Wu et al., 2017). The cells were cultured in 6-well plates at cell density of 1×10^6 cells/100 μL/well for 18 h, treated with LF, LF-Cu-10, and LF-Cu-20 (10 and 20 μg/mL doses) for 30 min, and then cultured with 1 μg/mL LPS for 24 h. The cells treated with the DMEM medium alone and LPS were used as corresponding negative and positive controls. Meanwhile, the seeded cells were treated with $CuCl_2$ at 0.051 μg/mL dose for 30 min and then treated with the DMEM medium for 24 h.

After the mentioned incubation, the cell cultures were centrifuged, while the collected cells were washed with PBS (0.1 mol/L, pH 7.4) and digested with trypsin. The cells were collected in the centrifuge tubes, centrifuged to discard the supernatants, and then suspended with cold PBS. The suspended cells were incubated with 2′,7′-dichlorodihydrofluorescein diacetate (DCFH-DA) solution (5 μmol/L) at 37 °C for 30 min, and washed with FBS-free medium three times. The microplate reader was employed to measure fluorescence intensity using the respective excitation and emission wavelengths of 488 and 525 nm. ROS level was thus reported as 2′,7′-dichlorofluorescein (DCH) fluorescence.

2.6. Assay of NO production

NO production was assessed as previously described (Qureshi et al., 2012). The cells were seeded onto 24-well plates (2×10^5 cells/100 μL/well), cultured for 18 h, treated with LF, LF-Cu-10, and LF-Cu-20 (10 and 20 μg/mL doses) for 30 min, and then cultured with 1 μg/mL LPS for 24 h. The cells treated with the DMEM medium and LPS only were used as negative and positive controls, respectively. After the incubation, culture supernatants were collected to measure NO concentrations using the kit, based on the Griess reagent method and the kit protocol. The optical density was also measured by the microplate reader at 540 nm.

Meanwhile, the seeded cells after a culture of 18 h were treated with $CuCl_2$ at 0.051 μg/mL dose for 30 min, and then incubated with the DMEM medium for 24 h. The obtained culture supernatant was also collected to measure NO concentrations as above.

2.7. Assays of PGE2 and cytokine production

The cells were cultured in 6-well plates at cell density of 1×10^6 cells/100 μL/well for 18 h, treated with LF, LF-Cu-10, and LF-Cu-20 (10 and 20 μg/mL doses) for 30 min, and then cultured with 1 μg/mL LPS for 24 h. The positive and negative controls were also designed as mentioned above. After the incubation, cell culture supernatants were harvested by centrifuging at 2000×*g* for 20 min, and assessed for PGE_2 concentrations using the ELISA kit and the kit protocol. Meanwhile, the concentrations of three cytokines, namely IL-6, IL-1β, and TNF-α, in the culture medium were also quantified using the respective ELISA kits according to the kit instructions. The microplate reader at 450 nm was used in these analyses to detect the values of optical density.

Also, the seeded cells were treated with $CuCl_2$ at 0.051 μg/mL dose for 30 min and then incubated with the DMEM medium for 24 h. The obtained culture supernatant was also collected to measure the IL-6 concentration as above.

2.8. Statistical analysis

All data were collected from three independent experiments or analyses and then reported as means or means ± standard deviations. Significant differences among the results from different groups were evaluated using the SPSS 16.0 software (SPSS Inc., Chicago, IL) and one-way analysis of variance (ANOVA) with Tukey's test. The difference between the results was considered significant at $P < 0.05$.

3. Results

3.1. Effect of $CuCl_2$ on cell viability, LDH release and ROS formation of macrophages

$CuCl_2$ was used in this study to generate LF-Cu-10, LF-Cu-20, and LF-Cu-40, while LF-Cu-40 had the highest Cu-fortifying level among the three samples. Cu concentration for LF-Cu-40 at the highest dose (i.e. 80 μg/mL) was calculated to be equal to a dose of 0.051 μg/mL $CuCl_2$. Thus, $CuCl_2$ at 0.051 μg/mL dose was identified for its possible effect on the macrophages. The results (Table 1) indicated that 0.051 μg/mL $CuCl_2$ did not cause a significant effect on the cells, because the cells thus treated were detected with respective cell viability and LDH relative release values of 100.3% and 98.8%. The two values were very close to those designed values of the control cells. Furthermore, $CuCl_2$ at 0.051 μg/mL dose did not promote ROS formation, because the treated cells showed a very close ROS relative level to control cells (DCF fluorescence values 9.3 *versus* 8.9). All results showed that a $CuCl_2$ dose equal to or less than 0.051 μg/mL had no toxic effect on the macrophages. In other words, the $CuCl_2$ levels used to fortify LF might have no adverse effect on the macrophages.

3.2. Effect of LF and Cu-fortified LF products on macrophage viability

CCK-8 assay was also used in this study to determine the potential toxic effect of LF, LF-Cu-10, LF-Cu-20, and LF-Cu-40 on the LPS-stimulated macrophages, using four doses (10−80 μg/mL) and cell viability as the evaluation index. The results (Table 2) showed that these assessed samples at the four doses had both growth promotion as well as growth inhibition in the cells. In most cases, these samples exerted growth inhibition because the measured viability values were usually less than 100%. LF-Cu-10 at 10 μg/mL dose had growth promotion on the cells, resulting in the highest viability value of 114.2%. However, the results from the data comparison indicated that Cu-fortification had an impact on LF's activity toward the cells. LF-Cu-10 always had lower growth inhibition than LF, because it always caused higher viability values (78.0−114.2% *versus* 71.9−94.8%). LF-Cu-20 resulted in viability values of 75.2–93.0%, and thus showed growth inhibition very close to LF. Meanwhile, LF-Cu-40 led to viability values of 72.9–89.7%, and showed higher growth inhibition on the cells than LF or LF-Cu-20. LF-Cu-40 had the highest growth inhibition on the cells at each dose and was thus not used in later assays. The two doses (40 and 80 μg/mL) were also not used in the forthcoming assays, because all samples at the two doses had greater growth inhibition on the cells. Overall, the two doses (i.e. 10 and 20 μg/mL) and two Cu-fortified LF products (i.e. LF-

Table 1
Cell viability, relative lactate dehydrogenase (LDH) release, and reactive oxygen species (ROS) production[a] of the macrophages exposed to 0.051 μg/mL $CuCl_2$ for 30 min.

Cell group	Cell Viability (% of control)	LDH release (% of control)	ROS production (DCH fluorescence)[b]
Control	100.0	100.0	8.9 ± 0.12
$CuCl_2$	100.3 ± 3.3	98.8 ± 4.0	9.3 ± 0.03

[a] Values are reported as means ± standard deviations (n = 3).
[b] DCH, 2′,7′-dichlorofluorescein.

Table 2
Cell viability[a] (percentages) of the LPS-stimulated macrophages exposed to bovine lactoferrin (LF) and the Cu-fortified LF products at four dose levels.

Sample[b]	Dose level (μg/mL)			
	10	20	40	80
LF	94.8 ± 1.2^{bD}	89.1 ± 0.7^{bcC}	80.2 ± 0.7^{aB}	71.9 ± 0.8^{aA}
LF-Cu-10	114.2 ± 1.4^{cD}	90.9 ± 0.8^{cC}	85.8 ± 1.2^{bB}	78.0 ± 1.7^{cA}
LF-Cu-20	93.0 ± 0.9^{bD}	87.7 ± 0.6^{abC}	80.5 ± 0.3^{aB}	75.2 ± 0.7^{bA}
LF-Cu-40	89.7 ± 0.9^{aD}	85.1 ± 1.9^{aC}	80.1 ± 0.3^{aB}	72.9 ± 1.0^{aA}

[a–c] The means within same column with different superscript lowercase letters differed ($P < 0.05$).
[A–D] The means within the same row with different superscript uppercase letters differed ($P < 0.05$).
[a] Values are reported as means ± standard deviations (n = 3).
[b] LF-Cu-10, LF-Cu-20, and LF-Cu-40: Cu-fortified LF products with respective 10%, 20%, and 40% Cu-saturation.

Cu-10 and LF-Cu-20) were used in the following assays.

3.3. Effect of LF and Cu-fortified LF products on LDH release in stimulated macrophages

The potential cytotoxicity of LF, LF-Cu-10, and LF-Cu-20 (10 and 20 μg/mL doses) on the stimulated macrophages was also examined using LDH release as a reflector. When the stimulated cells were served with LDH relative release value of 100%, the assessed samples mostly were able to reduce LDH release (Fig. 1). In detail, when the cells were treated with LF and LF-Cu-10, the measured LDH relative release values were reduced to 87.1−98.2% and 82.5−86.1%, respectively. LF-Cu-20 at 10 μg/mL dose led to LDH relative release value of 90.2%, but at 20 μg/mL dose might enhance LDH relative release value to 105.2%. These results demonstrated that both LF and LF-Cu-10 at the two doses had no cytotoxicity on the cells and thus could be used to assess their anti-inflammatory activities. Regarding LF-Cu-20, it at 20 μg/mL dose exerted a cytotoxic effect on the cells (*via* increasing LDH release or injuring the cells); however, it at 10 μg/mL dose might induce less cell injury than LF. Overall, the three samples and two doses could be used in later assays.

3.4. Effect of LF and Cu-fortified LF products on ROS production in stimulated macrophages

Relative ROS levels (expressed as DCF fluorescence) of the macrophages with various treatments are shown in Fig. 2. Compared with the control cells, the LPS-stimulated cells showed a significant ROS increase (DCF fluorescence values 24.3 *versus* 8.9), indicating that LPS efficiently promoted ROS production and induced cell inflammation. Additionally, ROS levels of the stimulated cells exposed to LF, LF-Cu-10, and LF-Cu-20 were 15.6–18.3, 12.1−14.1, and 15.1−17.9, respectively. The three samples thus inhibited ROS production and subsequently showed anti-inflammatory activities toward the stimulated cells. Moreover, LF gave enhanced inhibition on ROS production dose-dependently, while LF-Cu-10 and LF-Cu-20 at 20 μg/mL dose showed reduced inhibition on ROS production. LF-Cu-10 at each used dose showed higher activity than LF and LF-Cu-20, while LF-Cu-20 only at 10 μg/mL dose displayed higher activity than LF. It was suggested that a lower Cu-fortifying level and 10 μg/mL dose endowed LF-Cu-10 and LF-Cu-20 with higher activity in the cells to inhibit ROS production. The Cu-fortifying level was thus considered to be a key factor mediating the anti-inflammatory activity of LF in the LPS-stimulated macrophages.

3.5. Effect of LF and Cu-fortified LF products on NO and PGE_2 production in stimulated macrophages

NO and PGE_2 production are generally used to reflect the inflammatory response of cells. The present results confirmed that $CuCl_2$ at 0.051 μg/mL dose was unable to cause macrophage inflammation, because the treated cells showed culture NO production level of 3.5 μmol/L while the control cells without any treatment had culture NO production level of 3.8 μmol/L. LPS exposure caused macrophage inflammation efficiently, because the stimulated cells had much-enhanced culture NO production (41.2 μmol/L) (Fig. 3). LPS thus caused cellular inflammatory response by promoting NO production. Meanwhile, the stimulated cells treated with LF, LF-Cu-10, and LF-Cu-20

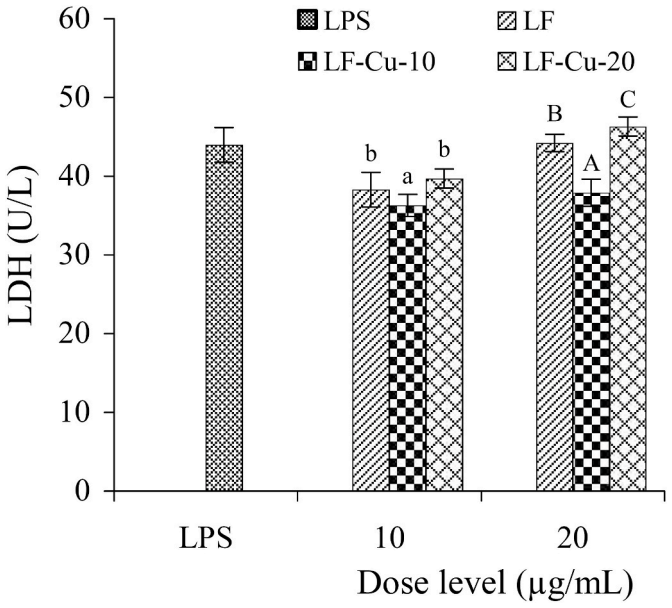

Fig. 1. Lactate dehydrogenase (LDH) activities that were measured in the cell-free cultures of the LPS-stimulated RAW264.7 macrophages exposed to LF, LF-Cu-10, and LF-Cu-20 at 10 and 20 μg/mL doses. The means collected from three independent analyses (n = 3) with different letters (a−c or A−C) differed ($P < 0.05$).

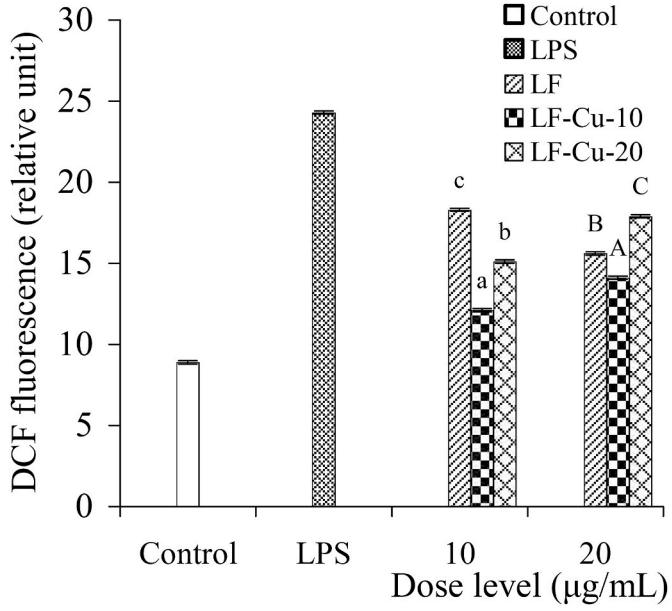

Fig. 2. ROS production levels (expressed as 2′,7′-dichlorofluorescein (DCH) fluorescence) of the LPS-stimulated RAW264.7 macrophages exposed to LF, LF-Cu-10, and LF-Cu-20 at 10 and 20 μg/mL doses. The means collected from three independent analyses (n = 3) with different letters (a−c or A−C) differed ($P < 0.05$).

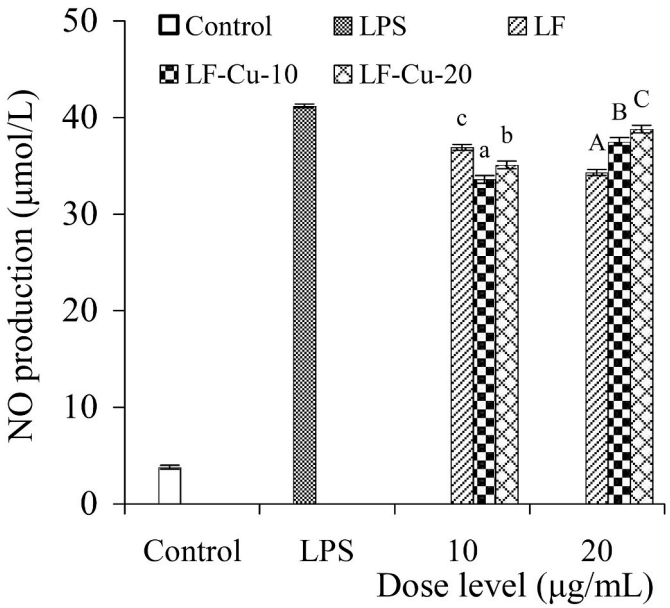

Fig. 3. Culture nitric oxide (NO) production of the LPS-stimulated RAW264.7 macrophages exposed to LF, LF-Cu-10, and LF-Cu-20 at 10 and 20 μg/mL doses. The means collected from three independent analyses (n = 3) with different letters (a–c or A–C) differed ($P < 0.05$).

had less NO production because the measured culture NO production levels were reduced to 34.3–36.9, 33.6–37.5, and 35.1–38.8 μmol/L, respectively (Fig. 3). LF, LF-Cu-10, and LF-Cu-20 could inhibit the LPS-promoted NO production, demonstrating their anti-inflammatory activities in the cells. In addition, the results (Fig. 4) also revealed that culture PGE_2 levels in the control and LPS-stimulated cells were 11.2 and 90.8 pg/mL, while those levels in the LF-, LF-Cu-10-, and LF-Cu-20-treated cells were 41.7–63.0, 21.8–34.1, and 34.5–49.4 pg/mL, respectively. The data also showed that LPS induced cellular inflammation by promoting culture PGE_2 production, but LF, LF-Cu-10, and LF-Cu-20 had anti-inflammatory activities in the stimulated cells by reducing the LPS-promoted PGE_2 production.

The results also indicated that LF dose-dependently decreased NO and PGE_2 production. Both LF-Cu-10 and LF-Cu-20 at 10 μg/mL dose showed higher anti-inflammatory ability, but they at 20 μg/mL dose gave reduced anti-inflammatory ability (*i.e.* causing less reduction in culture NO and PGE_2 production). LF-Cu-10 at each used dose showed higher ability than LF-Cu-20, while both LF-Cu-10 and LF-Cu-20 at 10 μg/mL dose had higher anti-inflammatory activity than LF. However, both LF-Cu-10 and LF-Cu-20 at 20 μg/mL dose occasionally exerted weaker activity than LF in the cells. These results thus suggested that only lower Cu-fortifying levels endowed LF with higher anti-inflammatory activity in the stimulated cells. Overall, the Cu-fortifying level should thus be considered a key factor controlling LF's anti-inflammatory activity in the stimulated macrophages.

3.6. *Effect of LF and Cu-fortified LF products on cytokine secretion in stimulated macrophages*

The results of this study showed that the control cells had culture IL-6 level of 4.8 pg/mL, while the macrophages treated with 0.051 μg/mL $CuCl_2$ for 30 min had culture IL-6 level of 5.1 pg/mL. IL-6 is one of the pro-inflammatory cytokines. This fact meant that a $CuCl_2$ dose equal to or less than 0.051 μg/mL was ineffective in causing macrophage inflammation, because cellular IL-6 production was only slightly enhanced. However, LPS could induce a significant increase in culture IL-6 level (from 4.8 to 747.5 pg/mL) (Fig. 5). Meanwhile, LF, LF-Cu-10, and LF-Cu-20 showed an ability to suppress IL-6 production in the stimulated cells (Fig. 5), by reducing culture IL-6 levels to 513.3–674.9 pg/mL. That is, the three samples had anti-inflammatory activities to the LPS-caused macrophage inflammation.

Other results (Fig. 6) also showed that the control cells had culture IL-1β and TNF-α levels of 4.8 and 34.9 pg/mL, while LPS alone caused the stimulated cells with much-increased culture IL-1β and TNF-α levels of 132.3 and 3848.1 pg/mL, respectively. Both IL-1β and TNF-α are also the reflectors of cell inflammation. The enhanced IL-1β and TNF-α production confirmed the LPS-induced cell inflammation. LF dose-dependently showed anti-inflammatory activity in the stimulated cells by decreasing respective culture IL-1β and TNF-α levels to 81.7–117.2

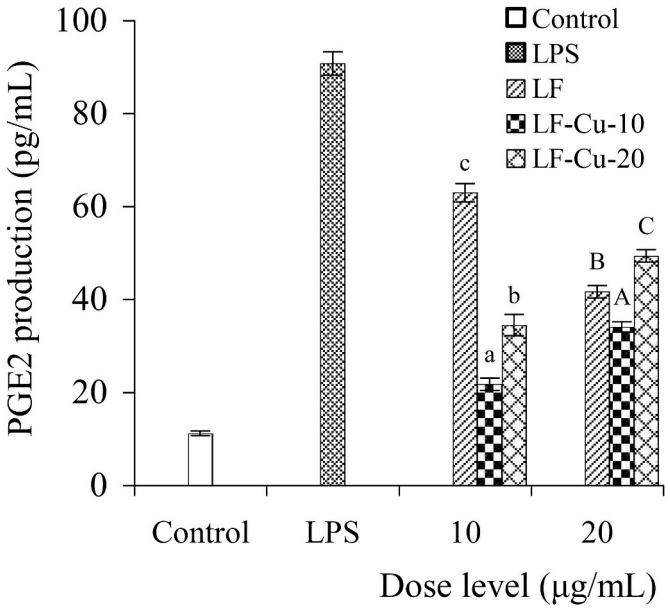

Fig. 4. Culture prostaglandin E_2 (PGE_2) levels of the LPS-stimulated RAW264.7 macrophages exposed to LF, LF-Cu-10, and LF-Cu-20 at 10 and 20 μg/mL doses. The means collected from three independent analyses (n = 3) with different letters (a–c or A–C) differed ($P < 0.05$).

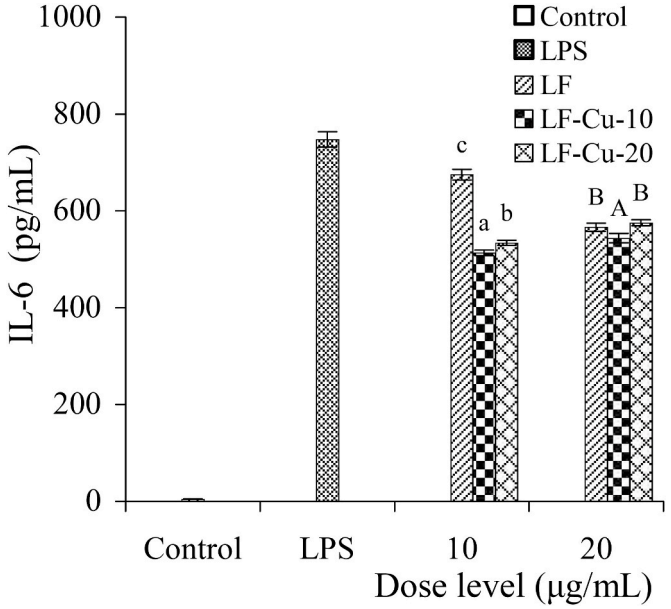

Fig. 5. Culture IL-6 levels of the LPS-stimulated RAW264.7 macrophages exposed to LF, LF-Cu-10, and LF-Cu-20 at 10 and 20 μg/mL doses. The means collected from three independent analyses (n = 3) with different letters (a–c or A–C) differed ($P < 0.05$).

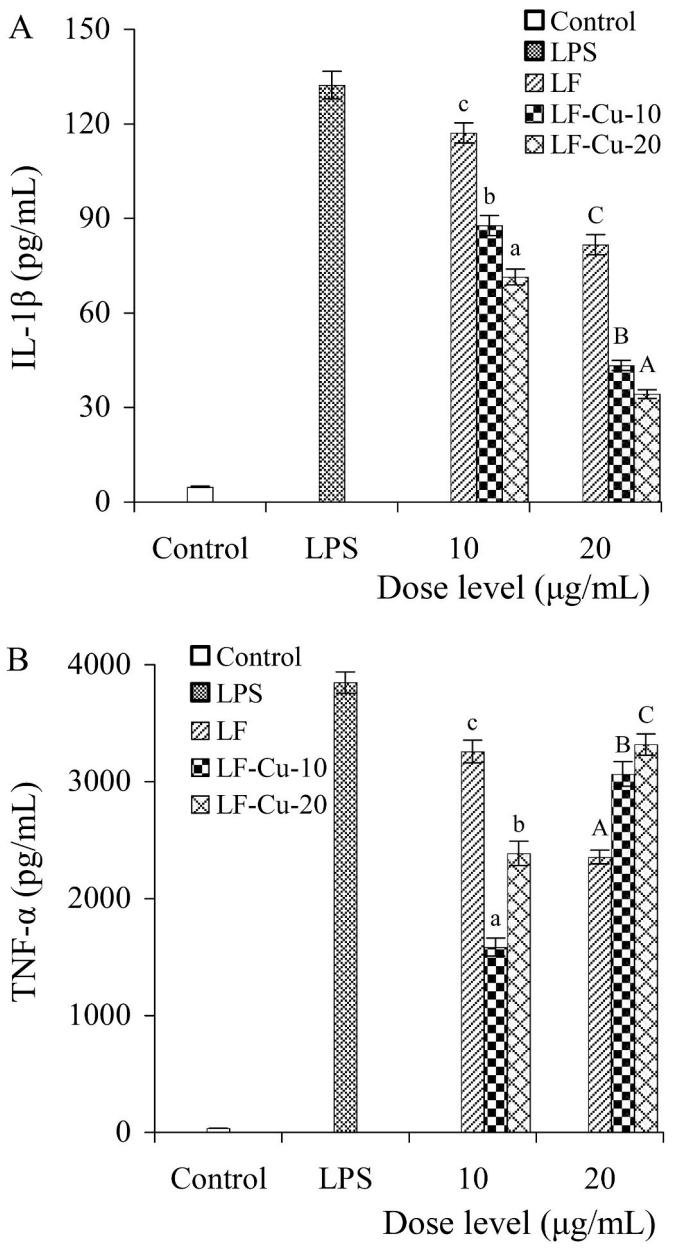

Fig. 6. Culture IL-1β (A) and TNF-α (B) levels of the LPS-stimulated RAW264.7 macrophages exposed to LF, LF-Cu-10, and LF-Cu-20 at 10 and 20 μg/mL doses. The means collected from three independent analyses (n = 3) with different letters (a–c or A–C) differed ($P < 0.05$).

and 2356.6–3257.7 pg/mL. LF-Cu-10 at 10 and 20 μg/mL doses was more active than LF by reducing IL-1β production to 43.4–87.8 pg/mL. LF-Cu-20 at the two doses was the highest active to suppress IL-1β production, causing much-reduced culture IL-1β levels of 34.3–71.4 pg/mL. Meanwhile, LF-Cu-10 at the two doses showed the highest activity in the stimulated cells, and thus decreased culture TNF-α levels to 1585.3–3065.5 pg/mL. Additionally, LF-Cu-20 at the two doses could reduce culture TNF-α production levels (2387.9–3318.2 pg/mL). These data mostly supported that the three assessed samples could combat against the LPS-caused macrophage inflammation.

To sum up, LF-Cu-10 and LF-Cu-20 at the two doses mostly showed higher capacity than LF to suppress the LPS-promoted production of IL6, IL-1β and TNF-α, and thus were regarded to have higher anti-inflammatory activities to the stimulated cells. It was thus suggested that the lower Cu-fortifying level resulted in increased anti-inflammatory activity for LF. Although LF-Cu-10 was less active than LF-Cu-20 to suppress IL-1β production, it was generally more efficient than LF-Cu-20 to suppress both IL-6 and TNF-α production. Collectively, the Cu-fortifying level was proven to be a key factor governing LF's ability to inhibit the secretion of the three pro-inflammatory cytokines in the stimulated macrophages.

4. Discussion

From a biological point of view, inflammation is a protective response to stress when the body is exposed to a variety of harmful stimuli such as infection, tissue damage, trauma, or endotoxin (*e.g.* PLS) exposure, which will give rise to inflammation (Wang et al., 2015). Consequently, macrophages as effector cells in inflammation and infection are activated to release a large number of pro-inflammatory cytokines such as IL-1β, TNF-α, IL-6, and other inflammatory mediators like NO, cyclooxygenase-2 (COX-2), and induced nitric oxide synthase (iNOS) (Shao et al., 2013). Macrophages thus play a unique role in the immune system and give rise to the innate immune response (Carralot et al., 2009). LPS is a main component of the cell walls in Gram-negative bacteria, and as a bacterial endotoxin can active macrophages or initiate the TLR4 signaling pathway; subsequently, phosphorylation of the mitogen-activated protein kinase (MAPK) and transcription factor nuclear factor-kappa B (NF-κB) is activated, while pro-inflammatory cytokines are secreted. Pro-inflammatory cytokines such as iNOS, NO, IL-1β, TNF-α, IL-6, and COX-2, and others can thus be used as evaluation indices in related research (Chae et al., 2009; Reis et al., 2011). In this study, LPS-stimulated RAW264.7 macrophages were used to evaluate and compare the anti-inflammatory activities of LF and Cu-fortified LF products. The results showed that LF and Cu-fortified LF products could attenuate the LPS-induced production of NO, PGE_2, ROS, and the three pro-inflammatory cytokines including IL-1β, TNF-α, and IL-6 (Fig. 3–6), proving their anti-inflammatory ability and especially activity changes in response to the conducted Cu-fortification. Consistent with the present results, it was found in past studies that the sweetfish-derived protein and *Rotula aquatica* Lour had anti-inflammatory ability in the LPS-stimulated RAW264.7 macrophages, *via* inhibiting the secretion of inflammatory mediators NO, PGE_2, TNF-α, and IL-6 (Sung et al., 2012; Vysakh et al., 2017).

NO and PGE_2 as two pro-inflammatory mediators are produced by iNOS and COX-2, respectively, while iNOS and COX-2 levels are significantly up-regulated in inflammatory disorders (Chun et al., 2012; Shao et al., 2013). NO usually has a beneficial biological effect on a variety of normal cells involved in immune regulation and inflammation, but at high levels may exert cytotoxicity and cause inflammatory diseases such as arteriosclerosis and septic shock (Davis et al., 2001; Pacher et al., 2007; Terao, 2009). Both NO and PGE_2 are also capable of promoting the secretion of various inflammatory cytokines (such as IL-1β and IL-6) in the T-cells, which in turn stimulate the inflammatory response (Damlar et al., 2015). NO pathway can induce ROS production (Liu et al., 2005; Jung et al., 2010). ROS also plays an important role in LPS-induced inflammatory responses and can activate the NF-κB-related signal pathway (Asehnoune et al., 2004). The activated NF-κB as a transcription factor leads to increased pro-inflammatory cytokines like TNF-α and IL-1β (Jr. Janeway and Medzhitov, 2002). Thus, a targeted substance can possess anti-inflammatory activity if it can reduce the excessive production of NO, PGE_2, and ROS in the LPS-stimulated RAW264.7 macrophages. For example, the sweet-tasting protein brazzein, casein glycomacropeptides, and surface-layer protein from *Lactobacillus acidophilus* NCFM had anti-inflammatory activities by inhibiting the production of NO, PGE_2, and ROS (Chung et al., 2018; Li et al., 2018; Wang et al., 2018). These reported results provided scientific support for our results.

The pro-inflammatory cytokines IL-1β, TNF-α, and IL-6 also play critical roles in the inflammatory response and are associated with the induction of some inflammatory mediators such as NO and ROS (Jr. Janeway and Medzhitov, 2002; Gupta et al., 2010). Both TNF-α and IL-6

are involved in some chronic inflammatory diseases (such as rheumatoid arthritis), while IL-1β plays a major role in initiating the inflammatory response caused by microbial infection (Roux et al., 2006; Kim and Moudgil, 2008; Kim et al., 2016). Thus, blocking the action of these pro-inflammatory cytokines is an effective way to treat or prevent inflammatory diseases. Bovine LF can inhibit the LPS-induced TNF-α secretion in RAW264.7 macrophages, or decrease the LPS-induced IL-6 secretion in THP-1 macrophages (Choe and Lee, 2000; Haversen et al., 2002). These results supported that the assessed samples of this study had anti-inflammatory ability by inhibiting IL-6 and IL-1β secretion in the stimulated cells.

LF can remove free Fe^{3+} from the inflammatory sites, and then bind with Fe^{3+} to prevent its usage by the pathogenic microorganisms (Brock, 2012). LF also can bind with LPS to inhibit the interaction between LPS and mCD14, and may compete with the LPS-binding proteins to bind LPS (Naidu, 2000). CD14, a glycoprotein on the surface of monocytes/macrophages, can interact with LPS to cause the release of the inflammatory mediators (such as TNF-α and IL-1) from monocytes/macrophages (Naidu, 2000). LF thus shows anti-inflammatory ability by binding free Fe^{3+} and LPS (Cutone et al., 2014). Moreover, LF can also inhibit the formation of superoxide free radicals, which play critical roles in inflammation-induced tissue damage. Thus, Cu and other trace elements had been verified for their anti-inflammatory ability; for example, Zn at a lower dose could inhibit NF-κB activation (Cortese-Krott et al., 2014), while Cu might down-regulate NF-κB to suppress inflammation and decrease the production of pro-inflammatory cytokines (Hassan et al., 2019). In Jurkat T cells, Cu^{2+} could inhibit NF-κB activation, which consequently promoted the secretion of pro-inflammatory cytokines (Kudrin, 2000). Thus, the low-level Cu-fortification in this study was a reasonable event that caused enhanced anti-inflammatory ability for LF. On the contrary, excessive Cu-fortification of LF might lead to decreased anti-inflammatory activity in the stimulated macrophages. Although Cu is very important to the immune system, it was also found that higher Cu levels led to an immuno-suppressive effect on the primary human mononuclear cells (Steinborn et al., 2017). It is thus reasonable that LF-Cu-20 had lower anti-inflammatory activity than LF-Cu-10 in the stimulated cells. Other supporting findings come from past studies involving another trace element Zn. It was evident that excessive Zn^{2+} led to cytotoxicity to both IHDPSCs and IMBMM cells, and also impacted the down-regulation of pro-inflammatory mRNA expression in the LPS-stimulated IMBMM cells (Lee et al., 2017). An in vitro study also showed that high Zn^{2+} dose caused a pro-inflammatory effect in human cells dose-dependently, while Zn^{2+} treatment combined with LPS brought about a coordinated stimulus on the production of pro-inflammatory cytokines (Foster and Samman, 2012). It is possible that high level of Cu-fortification and LPS yielded a synergistic stimulation to increase the secretion of these pro-inflammatory cytokines in the stimulated macrophages. In other words, a high level of Cu-fortification decreased the anti-inflammatory activities of the Cu-fortified LF products. Thus, the Cu-fortifying level of LF was regarded to govern the increase or decrease of anti-inflammatory activity.

5. Conclusion

Cu(II) could be employed to fortify bovine LF, while the used copper amount alone totally did not affect RAW264.7 macrophages. Compared with the unfortified LF, Cu-fortified LF products had both growth promotion and growth inhibition on the LPS-stimulated macrophages, depending on the used Cu-fortifying and dose levels. Under the lower Cu-fortifying level (0.16 mg/g LF) and lower dose (10 μg/mL), the Cu-fortified LF product showed higher anti-inflammatory activity than the unfortified LF in the stimulated macrophages; however, Cu-fortified LF product with higher Cu-fortifying level (0.32 mg/g LF) at higher dose (20 μg/mL) mostly showed weakened ability to decrease the production of inflammatory and pro-inflammatory factors. The present results therefore confirmed that Cu(II) could increase the anti-inflammatory activity of LF to the stimulated macrophages, while the applied Cu-fortifying level for LF was a key factor governing activity increase or decrease. Thus, it can be proposed that native LF may receive activity increase once it contacts with Cu(II) that is intentionally added to dairy products at a lower level.

CRediT authorship contribution statement

Qiang Zhang: Formal analysis, Methodology, Funding acquisition, Writing – original draft. **Hui-Juan Zhao:** Formal analysis, Methodology. **Liu-Yan Huang:** Formal analysis. **Chun-Li Song:** Formal analysis. **Hua-Qiang Li:** Methodology. **Xin-Huai Zhao:** Conceptualization, Supervision, Project administration, Funding acquisition, Writing – review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

No data was used for the research described in the article.

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