Effect of bovine lactoferrin and human lactoferrin on the proliferative activity of the osteoblast cell line MC3T3-E1 in vitro

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ABSTRACT

We conducted a comparative in vitro study on the proliferative effects of natural human lactoferrin (nhLF) and bovine lactoferrin (bLF) on osteoblasts. We investigated cell proliferation, cell survival, cell cycle, and mRNA and protein expression of proliferating cell nuclear antigen. Results indicated that treatment with 100 µg/mL of bLF or nhLF promoted the proliferation and sustenance of osteoblasts, and increased the length of the G₂/M and S phases compared with the untreated osteoblasts. Results of real-time quantitative PCR and Western blot showed that mRNA and protein expression of proliferating cell nuclear antigen by osteoblasts treated with bLF or nhLF were greater than those of the untreated control. At the same concentration, bLF demonstrated a greater effect on osteoblast proliferation than did nhLF. This study provides insights of significance in the utilization of bLF in healthy food formulas.

Key words: natural human lactoferrin, bovine lactoferrin, proliferation effect, osteoblast

INTRODUCTION

Lactoferrin (LF) is an 80-kDa iron-binding glycoprotein belonging to the transferrin family, and it is produced by mammal epithelial cells of exocrine glands, including human, cow, sheep, and horse (García-Montoya et al., 2012). As a pleiotropic factor, it is involved in many biological processes such as regulation of the levels of free iron in body fluids and immunoregulation, and it is known to have antitumor, antimicrobial, and antiviral activities (Naot et al., 2005). Studies have implicated bovine LF (bLF) as a growth factor that promotes mitosis of osteoblasts, stimulates proliferation and maturation of primary osteoblasts, accelerates the

differentiation of osteoblasts, and protects osteoblasts apoptosis from serum withdrawal (Cornish et al., 2004; Balasubramanian et al., 2012; García-Montoya et al., 2012).

The use of bLF in foods has been regarded as safe by the scientific community. According to the European Food Safety Agency (EFSA), the maximum allowed inclusion of bLF is 1,000 mg/L in infant formulas, 3,000 mg/100 g in chewing gums, and 133 mg/100 g in dairy products (EFSA, 2012). In the United States, the approved bLF range is 13 to 100 mg/100 mL for infant formula, 100 mg/100 g for yogurt, 400 mg/100 g for powdered milk, 200 mg/100 g for milk dessert and sugars, and 30 mg/g for sweets (FDA, 2011).

Natural human lactoferrin (nhLF) is an ideal food supplement for infants; however, little human lactoferrin is available for use in the industry; thus hindering its use compared with bLF, which is readily available in animals. Both bLF and recombinant human lactoferrin (rhLF) have been studied with respect to their effects on osteoblasts. The proliferative effect of rhLF on rat osteoblast-like cells was reported to be less than that of bLF (Grey et al., 2004). However, few studies have investigated nhLF with respect to proliferative effects on osteoblasts.

Several studies have indicated that bLF, nhLF, and rhLF cannot substitute for one another because of marked differences: nhLF and bLF consist of 691 and 689 AA, respectively, with 69% homology and similar 3-dimensional structures (Lorget et al., 2002). In vitro and in vivo investigations have demonstrated differences in their antiviral and cancer prevention mechanisms (Arnold et al., 2002; Tsuda et al., 2002). Additionally, rhLF contains more mannose residues in its polysaccharide chains than nhLF, especially when produced using a fungal express system. Consequently, rhLF has a shorter half-life than nhLF, which is thought to be related to the mannose content (van Veen et al., 2004; Kruzel et al., 2013). Other differences between rhLF and nhLF include composition of polysaccharide chains, digestive stability, and antimicrobial activity (van Veen et al., 2004; Kruzel et al., 2013). In addition,

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diverse *N*-glycan structures (fucosylated and sialylated) in bLF, nhLF, and rhLF have been reported, although rhLF *N*-glycans are more similar to those in bLF than to those in nhLF (Le Parc et al., 2017).

Our understanding of the proliferation effect of nhLF on osteoblasts is currently limited. Therefore, the objective of this study was to investigate the proliferative effects of bLF and nhLF on osteoblasts to improve the utilization of bLF in healthy food formulations.

MATERIALS AND METHODS

Reagents and Materials

The bLF used in this experiment was donated by Kanpure Ltd. Co. (Harbin, Heilongjiang, China). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (**MTT**) and nhLF were purchased from Sigma-Aldrich (St. Louis, MO). Cell counting kit-8 (**CCK-8**) was supplied by WST (Dojinbo, Japan); α -minimal essential medium (α -MEM), trypsin-EDTA, and penicillin-streptomycin were all purchased from Hyclone (Logan, UT); and fetal bovine serum was from Gibco (Grand Island, NY). Primary proliferating cell nuclear antigen (**PCNA**; rabbit monoclonal) antibody was from Abcam (Cambridge, MA), and the primary GADPH (polyclonal) antibody was prepared by Bioworld (St. Louis, MO). Horseradish peroxidase (HRP)-goat anti-rabbit IgG secondary antibody was made by KPL (Milford, MA). Solarbio (Beijing, China) provided the enhanced HRP-DAB (3,3'-diaminobenzidine) chromogenic substrate kit. The polyvinylidene fluoride (PVDF) membrane was purchased from Millipore (Danvers, MA). All-in-One qPCR Mix was purchased from GeneCopoeia (San Diego, CA).

Cell Culture and Assays

The MC3T3-E1 subclone 14 osteoblast cell line was purchased from the cell library of the Chinese Academy of Sciences (Shanghai, China). The cells were cultured in α -MEM medium with 10% fetal bovine serum and 1% penicillin-streptomycin under a humidified atmosphere consisting of 5% CO₂ and 95% air at 37°C (Steri-cycle CO₂ incubator, Thermo Fisher Scientific Inc., Waltham, MA), and the medium was changed every 2 to 3 d. For subcultures, cells at 90% confluence were passaged after detachment in 0.25% trypsin. For assays, the cell number was counted by using a hemocytometer and adjusted to a cell density of 1×10^5 cells/mL. For the proliferation and survival assays, 100 μ L of cells was seeded into 96-well plates and sextuplicates conducted at each concentration. For the cell cycle,

real-time quantitative PCR, and Western blot assays, 7 mL of cells was seeded into 90-mm plates, with triplicates conducted at each concentration. After overnight attachment, the medium with experimental compounds (1, 10, and 100 μ g/mL bLF or nhLF) was refreshed and cells incubated again. The incubation periods used in the proliferation, survival, and cell cycle assays were 24, 48, and 72 h, whereas those for PCR and Western blot assays were 48 h.

Iron Saturation of bLF and nhLF Analysis

Ten milliliters of solution of bLF or nhLF (2 mg/mL) was digested in nitric acid and the resulting residue was transferred into a 10-mL volumetric flask and made up to 10 mL with deionized water. The iron concentration was determined with the aid of an optical emission spectrometer (Optima 5300 DV, PerkinElmer, Waltham, MA). Iron saturation was calculated according to the following formula:

$$X1 = \frac{(C - C_0) \times V \times f}{m \times 1,000},$$

where *X1* is the iron mass fraction of sample (mg/g); *C* is the iron concentration of sample (mg/L); *C*₀ is the iron concentration of control (mg/L); *V* is the volume of sample (mL); *f* is the dilution ratio; and *m* is the mass of sample (g).

Then, iron saturation was calculated as follows:

$$X = \frac{X1}{1.4} \times 100\%,$$

where *X* is the iron saturation of sample (%) and 1.4 is the correction factor, which was derived as follows:

$$CF = \frac{MWI \times nFI}{RLF},$$

where *CF* is the correction factor; *MWI* is the molecular weight of iron (55.85); *nFI* is the number of molecules of ferric iron (2); *RLF* is the relative molecular mass of LF (80,000); and multiplied by 1,000 to convert grams to milligrams.

Proliferative Activity of bLF and nhLF on MC3T3-E1

Proliferative activity of bLF or nhLF on osteoblasts was measured according to the method of Hou et al. (2014). After incubation, 10 μ L of MTT (5 mg/mL)

was added to each well and further incubated in the dark for 4 h at 37°C. The solution of each well was removed and 150 μ L of dimethyl sulfoxide was added to dissolve the blue formazan crystals, which was followed by shaking at room temperature for 10 min. The absorbance was read at 490 nm using a microplate reader (BioTek-Eon, Winooski, VT).

Survival Assay of bLF- and nhLF-Treated MC3T3-E1

Cell survival was assessed using the CCK-8 assay according to the method of Ju et al. (2015). After incubation, 10 μ L of CCK-8 was added to each well and further incubated in the dark for 4 h. The absorbance was read at 450 nm using a microplate reader.

Cell Cycle of bLF- and nhLF-Treated MC3T3-E1

The cell cycle was determined by a flow cytometer (BD Bioscience, San Jose, CA) according to the method of Miao et al. (2015). Cells from each plate were harvested and transferred to new tubes, which were then centrifuged at $100 \times g$ for 5 min. The supernatant was carefully removed and 1 mL of buffer solution was added to each tube to resuspend cells overnight at 4°C. Propidium iodide solution (BD Bioscience) was added to each tube and the contents were filtered through a 50- μ m nylon mesh, after which they were analyzed using a flow cytometer.

RNA Isolation and Real-Time Quantitative PCR

Total cellular RNA was purified using a GeneMark kit (GMBiolab Co., Ltd., Taiwan), and cDNA was synthesized with a GeneCopoeia kit. Independent reaction mixtures were performed using the same cDNA for both the target gene and internal control. After an initial denaturation (95°C for 10 min), 40 amplification cycles were performed, with each cycle including denaturation at 95°C for 10 s, annealing at 57°C for 20 s, and extension at 72°C for 30 s using a 7300 Real Time PCR System (Applied Biosystems, Hercules, CA). Relative levels of gene expression were calculated by the $2^{-\Delta\Delta CT}$ method (where CT = cycle threshold). Oligonucleotide primers used for amplification are presented in Table 1.

Protein Extraction and Western Blot Analysis

Total cellular protein was extracted by using radio-immuno-precipitation assay (RIPA) buffer (Solarbio, Beijing, China), and the concentrations of lysates were detected by the bicinchoninic acid (BCA) assay (BCA Protein Assay kit; Thermo Fisher Scientific). The concentration of all lysates was adjusted to be the same according to the results of the BCA assay. Protein from each sample was loaded onto a 12% SDS-PAGE gel and semi-dry transferred (Trans-Blot SD, Bio-Rad Laboratories, Hercules, CA) onto the PVDF membranes for immune blot analysis. The PVDF membranes were blocked with 3% BSA at room temperature for 1 h, followed by the addition of primary antibodies and further incubated overnight at 4°C. The membranes were washed with $1 \times$ Tris-buffered saline (TBS)-Tween (twice) and $1 \times$ TBS (once) after which they were incubated with secondary antibody for 1 h. The membranes were washed using the above-mentioned washing sequence and 2 mL of DAB was added to each membrane. To check the equal loading of the proteins, GAPDH served as the internal control. Band area intensity was quantified using the Quantity One software from Bio-Rad Laboratories.

Statistical Analysis

Experimental data in triplicates or sextuplicates were analyzed using the Statistical Package for the Social Sciences v. 20.0 (SPSS/IBM Corp., Chicago, IL). A one-way ANOVA was used to compare mean values using a significance level of $P < 0.05$. The differences among the mean values within groups and their classification were achieved using Tukey's post hoc test.

RESULTS

Iron Saturation of bLF and nhLF

To determine whether iron saturations of bLF and nhLF reflected the nonmodified (native) state, iron concentrations of bLF and nhLF were measured using an optical emission spectrometer and their iron saturations calculated according to the formulas above. The iron mass fraction and iron saturation of bLF were

Table 1. Oligonucleotide primer sequences for target genes used in real-time quantitative PCR

Gene of interest	Primer sequence (5'-3')	Annealing temperature (°C)
PCNA	Forward: GAAGGCTTCGACACATACCGC	60
	Reverse: CAGCTGTACTCTGTTCTGGG	
GAPDH	Forward: TGAACCCTAAGCCAACC	60
	Reverse: CCACAGGATTCCATACCC	

0.2508 mg/g and 17.91%, respectively, and those of nhLF were 0.1551 mg/g and 11.08%, respectively.

Effects of bLF and nhLF on MC3T3-E1 Proliferation

The results of proliferative effects of bLF and nhLF on MC3T3-E1 are presented in Figure 1. We observed a linear increase in proliferation of MC3T3-E1 cells with increasing bLF and nhLF concentrations and incubation periods. At 72 h, 100 µg/mL bLF exhibited the greatest effect in enhancing proliferation of cells compared with the control or the other bLF treatments (1 and 10 µg/mL bLF; $P < 0.05$). At 24 h, the 1 and 10 µg/mL bLF treatments did not differ significantly from the control. No significant difference was found between the 1 µg/mL bLF treatment and the control at either 48 or 72 h.

Similar to bLF, 100 µg/mL nhLF resulted in greater cell production compared with the control or the other 2 treatments (1 and 10 µg/mL nhLF; $P < 0.05$). At 72 h, the differences in the proliferation effect of 10 and 100 µg/mL nhLF were significant ($P < 0.05$), but no difference was observed between 1 µg/mL nhLF and the control. At 24 h, no significant differences were observed between any of the nhLF treatments and the control. Furthermore, at 48 h, 100 µg/mL nhLF had a significantly greater effect than the control ($P < 0.05$).

Effect of bLF and nhLF on MC3T3-E1 Viability

The effects of bLF and nhLF on the cell viability are shown in Figure 2. The cell numbers increased after 24 h and cell mortality began at 48 h in both bLF and nhLF treatments. At 72 h, cell numbers decreased 36.65% with the 100 µg/mL bLF treatment and 42.56% with the 100 µg/mL hLF treatment. The cell numbers of bLF and hLF treatments were 45.65 and 32.73% higher than that of the control ($P < 0.05$). Regardless of the treatment timepoint, the cell numbers remained higher with increasing treatment concentrations for both bLF and nhLF. The 100 µg/mL bLF treatment resulted in a significantly higher ($P < 0.05$) survival rate than other treatments. The 1 and 10 µg/mL bLF treatments were not significantly different from each other.

Effect of bLF and nhLF on Cell Cycle of MC3T3-E1

The effects of bLF and nhLF treatments on the cell cycle are presented in Table 2. The maximum effect on cell cycle was observed after 48 h of treatment by 100 µg/mL bLF or nhLF. The G_0/G_1 phase of cells treated with 100 µg/mL bLF was 73.90%, which was significantly shorter ($P < 0.05$) than that of the control (87.03%). Meanwhile, the G_2/M and S phases were 8.09

Figure 1. Effects of different concentrations of bovine lactoferrin (bLF) and natural human lactoferrin (nhLF) on osteoblast proliferation. The cell number of control at 24 h was normalized to a value of 1. Different letters between groups across all treatments and timepoints represent significant differences ($P < 0.05$). Error bars indicate SD.

and 18.29%, which were significantly longer ($P < 0.05$) than those of the control (7.05 and 5.39%).

Similar to bLF, 100 µg/mL nhLF resulted in a better effect than the other nhLF concentrations on shortening the cell cycle: the G_0/G_1 phase decreased (76.34%), and the G_2/M and S phases increased (7.87 and 15.33%, respectively). However, bLF performed better than nhLF ($P < 0.05$).

Figure 2. Effects of different concentrations of bovine lactoferrin (bLF) and natural human lactoferrin (nhLF) on cell viability (survival). The cell number of control at 24 h was normalized to a value of 1. Different letters between groups across all treatments and timepoints represent significant differences ($P < 0.05$). Error bars indicate SD.

Figure 3. Expression of mRNA and protein of proliferating cell nuclear antigen (PCNA) treated with bovine lactoferrin (bLF) and natural human lactoferrin (nhLF). Expression levels of PCNA in the control (medium only) were normalized to a value of 1. Different letters between treatments within mRNA or protein (PCNA) expression represent significant differences ($P < 0.05$). Error bars indicate SD. (A) Fold change in mRNA and protein levels of PCNA; (B) internal control (GAPDH expression) in osteoblasts treated with nhLF, bLF and control; and (C) PCNA expression in osteoblasts treated with nhLF, bLF, and control.

Changes in mRNA and Protein Expression of PCNA

The mRNA and protein expression of PCNA are shown in Figure 3. The bLF treatment resulted in 66.00 and 20.00% increases in mRNA and protein expression of PCNA, respectively, compared with the control ($P < 0.05$). Likewise, 28.00 and 10.40% increases in mRNA and protein expression, respectively, of PCNA was noted after treatment with nhLF compared with the control ($P < 0.05$). It is important to note that increases in mRNA and protein expression of PCNA were 27.45 and 16.82% higher, respectively, with bLF than nhLF ($P < 0.05$).

DISCUSSION

In our study, the iron saturations of bLF and nhLF were about 18 and 11%, respectively. This is in line with a study that reported an iron saturation range of 15 to 20% for bLF and about 10% for nhLF (Gibbons et al., 2015). Hence, both LF in our study retained their native states. It has been demonstrated that LF at its physiological concentration promotes the proliferation and accelerates the differentiation of osteoblasts in vitro (Naot et al., 2011). Both bLF and nhLF showed time- and dose-dependent relationships on the proliferation of osteoblasts. The 1 $\mu\text{g/mL}$ bLF treatment had no significant effect on proliferation compared with the control. However, the 10 and 100 $\mu\text{g/mL}$ bLF treatments increased proliferation significantly. The same pattern was seen with the nhLF treatments. The proliferation effect of bLF and rhLF on osteoblasts was compared with the results of other studies (Cornish et al., 2004), which reported that the proliferation effect with 10 $\mu\text{g/mL}$ of bLF was 2 times higher than that with 10 $\mu\text{g/mL}$ of rhLF. Our results indicated that the proliferation effect of 10 $\mu\text{g/mL}$ bLF was 1.17 times

Table 2. Effect of different concentrations of bovine lactoferrin (bLF) and natural human lactoferrin (nhLF) on the percentage of total cell cycle time ($\pm$ SD) in each phase of the osteoblast cell line MC3T3-E1

Phase	Time (h)	Treatment ¹						
		B1	B10	B100	C	H1	H10	H100
G ₁ /G ₀	24	87.76 $\pm$ 1.94 ^{bc}	85.64 $\pm$ 0.7 ^{abc}	82.24 $\pm$ 1.98 ^a	90.19 $\pm$ 0.92 ^c	88.44 $\pm$ 2.11 ^{bc}	86.57 $\pm$ 2.81 ^{abc}	84.29 $\pm$ 2.09 ^{ab}
	48	79.39 $\pm$ 2.26 ^{ab}	75.97 $\pm$ 1.74 ^a	73.90 $\pm$ 1.51 ^a	87.03 $\pm$ 2.44 ^c	81.53 $\pm$ 2.40 ^{bc}	80.46 $\pm$ 2.43 ^b	76.34 $\pm$ 1.96 ^{ab}
	72	83.44 $\pm$ 2.11 ^{bc}	81.25 $\pm$ 2.08 ^{abc}	77.53 $\pm$ 1.73 ^a	89.05 $\pm$ 1.93 ^d	84.13 $\pm$ 2.17 ^{bcd}	85.17 $\pm$ 1.94 ^{cd}	79.30 $\pm$ 1.78 ^{ab}
G ₂ /M	24	6.73 $\pm$ 0.69 ^{ab}	7.09 $\pm$ 1.14 ^b	7.95 $\pm$ 0.98 ^b	4.72 $\pm$ 0.43 ^a	5.86 $\pm$ 0.78 ^{ab}	7.22 $\pm$ 0.74 ^b	7.54 $\pm$ 0.64 ^b
	48	7.25 $\pm$ 1.09 ^c	7.90 $\pm$ 0.75 ^{ab}	8.09 $\pm$ 0.93 ^a	7.05 $\pm$ 0.94 ^c	6.51 $\pm$ 0.57 ^c	7.20 $\pm$ 1.00 ^b	7.87 $\pm$ 0.96 ^{ab}
	72	6.53 $\pm$ 0.73 ^b	6.90 $\pm$ 0.78 ^b	7.69 $\pm$ 0.70 ^b	4.20 $\pm$ 0.79 ^a	5.79 $\pm$ 0.76 ^{ab}	6.43 $\pm$ 0.57 ^b	6.68 $\pm$ 0.67 ^b
S	24	5.27 $\pm$ 0.60 ^a	7.16 $\pm$ 0.69 ^{bc}	9.44 $\pm$ 0.62 ^d	4.85 $\pm$ 0.76 ^a	5.42 $\pm$ 0.54 ^{ab}	6.49 $\pm$ 0.53 ^{abc}	8.07 $\pm$ 0.78 ^{cd}
	48	13.44 $\pm$ 1.71 ^{bc}	15.17 $\pm$ 1.94 ^{bc}	18.29 $\pm$ 2.11 ^c	5.39 $\pm$ 0.56 ^a	12.10 $\pm$ 1.83 ^b	14.55 $\pm$ 1.94 ^{bc}	15.33 $\pm$ 1.78 ^{bc}
	72	9.70 $\pm$ 0.96 ^{ab}	11.40 $\pm$ 0.56 ^{bed}	14.53 $\pm$ 1.81 ^d	6.56 $\pm$ 0.57 ^a	10.02 $\pm$ 0.86 ^{bc}	8.22 $\pm$ 0.78 ^{ab}	13.23 $\pm$ 1.79 ^{cd}

^{a-d}Means with different letters between groups are significantly different ($P < 0.05$).

¹Treatments B1, B10, and B100 = 1, 10, and 100 $\mu\text{g/mL}$ of bLF, respectively; C represents control; H1, H10, and H100 represent 1, 10, and 100 $\mu\text{g/mL}$ of nhLF, respectively.

higher than that of 10 µg/mL nhLF ($P < 0.05$). Thus, the proliferation effect of nhLF appears to be better than that of rhLF, even though a direct comparison has not been made. The results of the study also demonstrated that the proliferation effect improved as the dose increased (to 100 µg/mL of bLF or rhLF; Cornish et al., 2004). This was in line with our findings in which proliferation occurred in a dose-dependent manner. However, the proliferation effect decreased when the concentration reached 1,000 µg/mL bLF and there was no significant difference compared with 10 µg/mL bLF (Cornish et al., 2004). The 100 µg/mL bLF treatment performed significantly better than either 10 µg/mL or 1,000 µg/mL bLF (Cornish et al., 2004; Grey et al., 2004). Overall, in the current study, bLF showed better proliferation effects on osteoblasts than nhLF at the same concentration and the same treatment time.

Cornish and coworkers (2006) demonstrated that iron saturation had no significant effect on proliferation when osteoblasts were treated with 1 µg/mL bLF; however, at 10 and 100 µg/mL bLF, iron saturation affected the proliferation of the osteoblast significantly. Their study also indicated that LF with a lower iron saturation had a greater proliferation effect than LF with a higher iron saturation, and that a higher iron saturation reduced cell proliferation (Cornish et al., 2006). In the current study, the iron saturation of bLF was 18%, which was higher than that of nhLF (11%); thus, we presume that the proliferation effect of bLF on osteoblasts should be lower than that of nhLF. However, our findings revealed otherwise. Although the reason for this phenomenon is not clear, the differences in polysaccharides chains between bLF and nhLF could be a plausible explanation. This assumption is due to the fact that polysaccharide chain composition has been reported to have an effect on digestive stability and antimicrobial activity of LF (van Veen et al., 2004; Kruzel et al., 2013). Although the reason for this phenomenon is not clear, difference in polysaccharide chains between bLF and nhLF could be a plausible explanation. The polysaccharides could affect the digestive stability and antimicrobial activity, and may affect the cell proliferation. Research has demonstrated that increases in tryptophan and α -helix content and a decrease in β -structure content were ensured by an increased iron saturation of LF. The same study indicated that the osteogenic activity of LF decreased with increasing iron saturation (Wang et al., 2013).

Besides iron saturation, other factors may affect cell proliferation. It is well documented that LF reduces apoptosis in osteoblasts and thus is an effective mechanism for cell survival because LF increases the expression of IGF-1 to exert anti-apoptosis activity. Both bLF and nhLF had higher anti-apoptosis activity than the

control. Lactoferrin also stimulated the proliferation of osteoblasts by shortening the cell cycle. In the current study, a flow cytometer was used to detect changes in the cell cycle of osteoblasts treated with bLF and nhLF. Both bLF and nhLF shortened the cell cycle in a dose-dependent manner, which was accompanied by a remarkable increase in length of the cell cycle of both the S phase and the G₂/M phase. The G₀/G₁ phase was 3.20% shorter after bLF treatment at 100 µg/mL than after nhLF at the same dose. The bLF treatments of G₂/M and S phases at that same dose were 5.44 and 19.31% longer than that of nhLF with the same dose. This suggests that bLF was more effective at shortening the cell cycle than nhLF. The S phase was the most upregulated phase by bLF treatment, and the speed of cell division was accelerated.

It is well known that LF has DNA-binding properties, acting as an enzyme to catalyze some reactions to accelerate the expression of mRNA (García-Montoya et al., 2012). One study demonstrated that LF upregulated intestinal mRNA expression in postnatal piglets and decreased expression of the *luxS* gene in *Streptococcus pneumoniae* (Kasala et al., 2015). Proliferating cell nuclear antigen plays an important role in cell proliferation and is a good indicator of cell proliferation (Zhao et al., 2013), and its induction stimulates the proliferation of vascular smooth muscle cells (Kulldorff et al., 2000). In addition, PCNA is expressed during the late G₀/G₁ phase, S phase, and early G₂/M phase (Nakajima et al., 2011). In this study, we found that bLF and nhLF upregulated the mRNA expression of PCNA and lengthened the S and G₂/M phases. Considering both these results, we speculate that LF could promote cell proliferation by simultaneously regulating PCNA expression and the cell cycle.

CONCLUSIONS

This study evaluated the effects of bLF and nhLF on cell proliferation, cell survival, cell cycle, and mRNA and protein expression of PCNA. Both bLF and nhLF significantly influenced these factors compared with the control. However, bLF had a greater proliferative effect on osteoblasts than did nhLF. Further studies should investigate the role of polysaccharide chains in the proliferation activity of bLF and nhLF and the utilization of bLF in the food industry.

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