

Bovine milk extracellular vesicles induce the proliferation and differentiation of osteoblasts and promote osteogenesis in rats

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Abstract

Bone is constantly balanced between the formation of new bone by osteoblasts and the absorption of old bone by osteoclasts. To promote bone growth and improve bone health, it is necessary to promote the proliferation and differentiation of osteoblasts. Although bovine milk is known to exert a beneficial effect on bone formation, the study on the effect of bovine milk extracellular vesicles (EVs) on osteogenesis in osteoblasts is limited. In this study, we demonstrated that bovine milk EVs promoted the proliferation of human osteogenic Saos-2 cells by increasing the expression of cell cycle-related proteins. In addition, bovine milk EVs also induced the differentiation of Saos-2 cells by increasing the expression of RUNX2 and Osterix which are key transcription factors for osteoblast differentiation. Oral administration of milk EVs did not cause toxicity in Sprague-Dawley rats. Furthermore, milk EVs promoted longitudinal bone growth and increased the bone mineral density of the tibia. Our findings suggest that milk EVs could be a safe and powerful applicant for enhancing osteogenesis.

Practical applications

Until now, calcium and vitamin D have been prescribed to promote bone formation or to prevent bone diseases such as osteoporosis. Recently, several studies to find bioactive molecules that regulate cellular functions of osteoblasts or osteoclasts are actively underway. Milk basic proteins and lactoferrin present in milk are known to promote bone formation, but they exist in small quantities and the isolation of these proteins is complicated making mass production difficult. Recently, it has been found that milk contains large quantities of EVs, and that they promote bone formation. Studies on the effect of Milk EVs on osteoblasts during osteogenesis will help in the development of biomaterials for osteogenesis.

KEY WORDS

extracellular vesicles, milk, osteoblast proliferation, osteogenesis, osteogenic differentiation

1 | INTRODUCTION

In normal bone tissue, the process of bone reabsorption and bone formation is balanced. These two consecutive coupled events are called bone remodeling (Crockett et al., 2011; Hadjidakakis & Androulakis, 2006). In bone remodeling, osteoclasts that removing mineralized bone and osteoblasts that forming and mineralizing bone matrix are involved. However, due to various causes, if the activity of the osteoblasts decrease or the activity of the osteoclasts increase, this balance is destroyed and bone diseases such as osteoporosis occur (Raisz, 2005). Therefore, in order to promote bone growth and to improve bone health, it is important to identify substances that can enhance the activity of osteoblasts.

Milk contains several effective substances that promote bone formation. Milk has nutritional value, contains calcium and vitamin D for bone mineralization, and it also provides rich sources of proteins. Milk also contains bioactive substances that can directly regulate the activity of osteoblasts or osteoclasts. Milk basic protein (MBP) present in milk has been reported to increase bone mineral density (BMD) of a healthy adult woman by inhibiting the osteoclast-mediated bone resorption (Aoe et al., 2001; Matsuoka et al., 2002; Yamamura et al., 2002). In addition, lactoferrin, which is an iron-binding glycoprotein present in milk is known to promote the proliferation and differentiation of osteoblasts and increases bone formation in a mouse model (Cornish et al., 2004; Hou et al., 2014). However, these proteins exist in trace amounts in milk and the isolation process is complicated. Recently, it has been found that milk contains a large number of extracellular vesicles (EVs), and research on the biological functions of milk EVs in osteogenesis is actively underway (Golan-Gerstl et al., 2017; Martin et al., 2018; Oliveira et al., 2016; Samuel et al., 2017; Zempleni et al., 2017).

Extracellular vesicles (EVs) are nanoparticles secreted by almost all types of cells. EVs contain proteins, lipids, and nucleic acids of originating cells and are known to transmit those bioactive molecules to target cells to change the phenotype of the cells (Simons & Raposo, 2009; Thery et al., 2009). EVs exist in various biological fluids including cerebrospinal fluid, blood, urine, saliva, and milk. Recently, research on the biological function of milk EVs has been actively conducted. EVs from breast milk are known to control the activity of immune cells (Admyre et al., 2007; Zempleni et al., 2017). EVs present in the bovine milk is known to inhibit enterocolitis in the mouse model by activating the goblet cell function (Li et al., 2019). However, there has been insufficient research on the effect of bovine milk EV on the proliferation and differentiation of osteoblasts. In this study, we demonstrated that milk EVs promote the proliferation and differentiation of human osteoblastic Saos-2 cells and pre-osteoblastic MC3T3-E1 cells in vitro, and confirmed that the milk EVs promote osteogenesis and they do not cause any toxicity in Sprague-Dawley rats.

2 | MATERIALS AND METHODS

2.1 | Culture of human osteoblastic Saos-2 cell line

Saos-2 cell line were obtained from the American Type Culture Collection (Manassas, VA, USA). The cells were cultured with DMEM/F12 media (Hyclone) supplemented with 10% (v/v) fetal bovine serum (FBS; Gibco BRL) and 100 U/ml penicillin/streptomycin (Gibco BRL). The cells were grown in a humidified 5% CO₂ incubator at 37°C. For in vitro differentiation, when the cells grew confluence, the cell culture medium was replaced with 50 µg/ml ascorbic acid, 1 mM β-glycerophosphate, and 10 nM dexamethasone in a complete cell culture medium. The differentiation medium was replaced every other day.

2.2 | Isolation of bovine milk EVs

The commercial pasteurized milk was centrifuged at 2,000g for 10 min to remove the cells and debris. The supernatant was centrifuged at 10,000g for 40 min to remove the fat globules. The supernatants were centrifuged at 35,000g for 1 hr. Then the milk-derived EVs were isolated from the supernatant by ultracentrifugation at 100,000g for 1 hr. The isolated EVs were quantified by Bradford assay (Bio-Rad) and nanoparticle tracking analysis. Milk EVs were stored at -80°C.

2.3 | Western blot

Protein extracts from milk, milk EVs or Saos-2 cells were separated via 10%-15% of SDS-PAGE and then the proteins were transferred to a 0.2 µm PVDF membrane. Blocking was performed using a 3% skimmed milk or protein-free blocking buffer (for CD9 antibody, Thermo fishers) for 1 hr, and primary antibodies against CD81 (1:1000, SC-166029, Santa Cruz), CD9 (1:1000, NB500-494, Novus Biologicals), CDK2 (1:1000, SC-6248, Santa Cruz), CDK4 (1:1000, SC-56277, Santa Cruz), Cyclin E (1:1000, SC-377100, Santa Cruz), Cyclin D1 (1:1000, SC-20044, Santa Cruz), RUNX2 (1:1000, SC-390351, Santa Cruz), Osterix (1:1000, SC-393325, Santa Cruz) and β-actin (1:1000, SC-47778, Santa Cruz) were incubated at RT for 2 hr. After washing with 0.05% TBS-T, the membrane was incubated with an HRP-conjugated goat anti-mouse IgG secondary antibody (Santa Cruz). The bands were detected by enhanced chemiluminescence (ECL; Amersham Pharmacia Biotech, England, UK).

2.4 | Nanoparticle tracking analysis (NTA)

The diameter and particle concentrations of milk EVs were determined with NTA using NanoSight LM10-HS system (Malvern Instruments

Ltd.) equipped with 688 nm laser. Nanoparticle Tracking Analysis software version 2.3 (Malvern Instruments Ltd.) was used for analysis.

2.5 | Cryo-electron microscopy

For cryo-electron microscopy, Milk EVs were absorbed to 300-mesh EM carbon grids with a hydrophilic surface and frozen using Vitrobot (Thermo Fisher Scientific) in liquid nitrogen. The grids were observed and analyzed using Talos L120C cryoTEM (Thermo Fisher Scientific), and images were recorded at 13,000 magnifications.

2.6 | Cell proliferation assay

Saos-2 cells were seeded in 96-well culture plates (5,000 cells/well) overnight. After treatment with milk EVs, cell proliferation was determined using a modified MTT assay in which the tetrazolium salt was converted to formazan by mitochondrial dehydrogenase. The absorbance at 450 nm was measured using a microplate reader (Varioskan LUX, Thermo Fisher Scientific).

2.7 | Cell-cycle analysis

The cells were harvested and fixed for 2 hr at -20°C with 70% ethanol. After washing twice with cold PBS, the cells were incubated at 4°C for an hour with RNase and DNA-intercalating dye PI (Sysmex). The cell cycle analysis of the PI-stained cells was performed by flow cytometry (Sysmex). Events were recorded for at least 10^4 cells per sample and the data were analyzed using the FCS Express 5 software (DeNovo).

2.8 | Human angiogenesis antibody array

To measure the expression of osteogenesis-related proteins in bovine milk EVs, milk EVs were diluted with 1x blocking buffer at 200 µg/ml and treated to a human angiogenesis antibody array membrane (Abcam, Cambridge, UK) following the manufacturer's protocol.

2.9 | Measurement of ALP activity and OCN concentration

For the measurement of ALP activity in Saos-2 cells, cells were harvested and washed with cold PBS. The cell pellet was resuspended with 1X assay buffer and homogenized with a sonicator. The sample was centrifuged at 12,000g for 15 min and ALP activity was measured using an Alkaline Phosphatase Assay Kit (Abcam, Cambridge, UK) following the manufacturer's instructions. For the measurement

OCN in conditioned medium, the conditioned medium was harvested after treating the Saos-2 cells with various doses of milk EVs, and the concentration of OCN was determined using an Osteocalcin Human Instant ELISA™ Kit (Thermo Fisher Scientific, Waltham, MA, USA).

2.10 | In vivo toxicity of bovine milk EVs

To evaluate the toxicity of bovine milk EVs in vivo, male and female Sprague-Dawley rats were orally administered with various doses of milk EVs (0, 0.5, 5, 50 mg/kg) once. The experiment ended on the 15th day after milk EVs administration. During the experiment, survival, body weight, and clinical signs were monitored. Blood was collected at the end of the experiment and hematological profiles and serum chemistry were analyzed. The hematological profiles were analyzed using a hematology analyzer (XN-V, Sysmex, Japan) after 1 ml of blood was put into an EDTA-containing tube. The serum chemistry was analyzed using a chemistry analyzer (7180, Hitachi, Japan) after centrifugation of blood at 3,000 RPM for 10 min.

2.11 | Measurement of the length and BMD of tibia using a micro-computed tomography

Six-week-old female Sprague-Dawley rats were obtained from Orient Bio Inc (Seongnam, Republic of Korea). After 7 days of acclimatization, the rats were randomly divided into three groups: control, growth hormone (GH), and milk EVs ($n = 5$). The control group was orally administered with the same volume of PBS as the milk EVs. The GH group was subcutaneously injected with 200 µg/kg/day of somatotropin (Sigma Aldrich, MO, USA) for 14 days. The milk EVs group was orally administered with 50 mg/kg/day of milk EVs for 14 days. At the end of the experiment, all rats were sacrificed and the tibiae were collected for micro-computed tomography (µ-CT) analysis. The length and BMD of tibia were analyzed using a Skyscan1173 (Bruker, Kontich, Belgium). Samples were scanned through a 180° rotation angle with rotation steps of 0.4° and the images were reconstructed using a Skyscan CT-analyser software. For the tibial length measurement, the distance between the head of the tibia and the most distal aspect of the medial malleolus was measured. For volumetric BMD measurement, the volume of interest for the trabecular and cortical bone was defined as the region 0.5–4.3 mm and 4.3–5.3 mm distally from the proximal growth plate, respectively.

2.12 | Statistical analysis

All data was displayed as the mean \pm standard error of the mean. Statistical significance was analyzed with the Student's t-test or one-way ANOVA. We used the Bonferroni-Dunn test to make

comparisons of three or more groups. P values $< .05$ were considered statistically significant.

3 | RESULTS

3.1 | Isolation and characterization of bovine milk EVs

To isolate the milk EVs from commercial pasteurized milk, milk was centrifuged at 2,000g for 10 min and 10,000g for 40 min to remove milk cells, debris, and milk fat globules (Figure 1a,b). The skimmed milk was further ultracentrifuged at 35,000g for 1 hr to remove caseins. This supernatant was ultracentrifuged at 100,000g for 1 hr to pellet milk EVs (Figure 1a,b). An equal volume of nine fractions was obtained from the supernatant and the pellet was resuspended with PBS and designated as fraction 10. An equal volume of all fractions was analyzed by western blot for EV marker protein CD81 to determine which fraction contains milk EVs. As expected, the CD81 signal was the highest in fraction 10 (Figure 1c). In addition, fraction 10 showed the highest particle concentrations among all fractions (Figure 1d). Cryo-electron microscopy showed that milk EVs

have spherical lipid bi-layered structure (Figure 2a) and nanoparticle tracking analysis results showed that the average diameter of milk EVs was 227.8 ± 6.7 nm with 30–600 nm size range (Figure 2b). Western blot analysis for EV marker proteins such as CD81 and CD9 in milk and milk EVs showed that milk EVs are enriched with EV marker proteins compared to milk (Figure 2c). It was known that commercial bovine milk EVs contain bioactive TGF- β , which can regulate immune cell functions (Pieters et al., 2015). We hypothesized that the milk EVs also express other growth factors or cytokines that are associated with osteoblast activation. We confirmed the expression of these proteins in milk EVs using an antibody array membrane which is sensitive and cost-effective. The antibody array showed that osteogenesis-related proteins such as bFGF, IGF-1, TGF- β , and VEGF exist in milk EVs (Figure 2d). In addition, we determined whether milk EVs contain milk proteins such as lactoferrin and β -lactoglobulin using Western blot. Interestingly, we found that lactoferrin and β -lactoglobulin are also present not only in the milk but also in the bovine milk EVs (Figure S1). This result is consistent with the previous proteomic analysis of bovine milk EVs identifying the lactoferrin and β -lactoglobulin as one of the major proteins of milk EV proteome (Benmoussa et al., 2019; Sanwlani et al., 2020; Yang et al., 2017). Extracellular soluble proteins can be attached

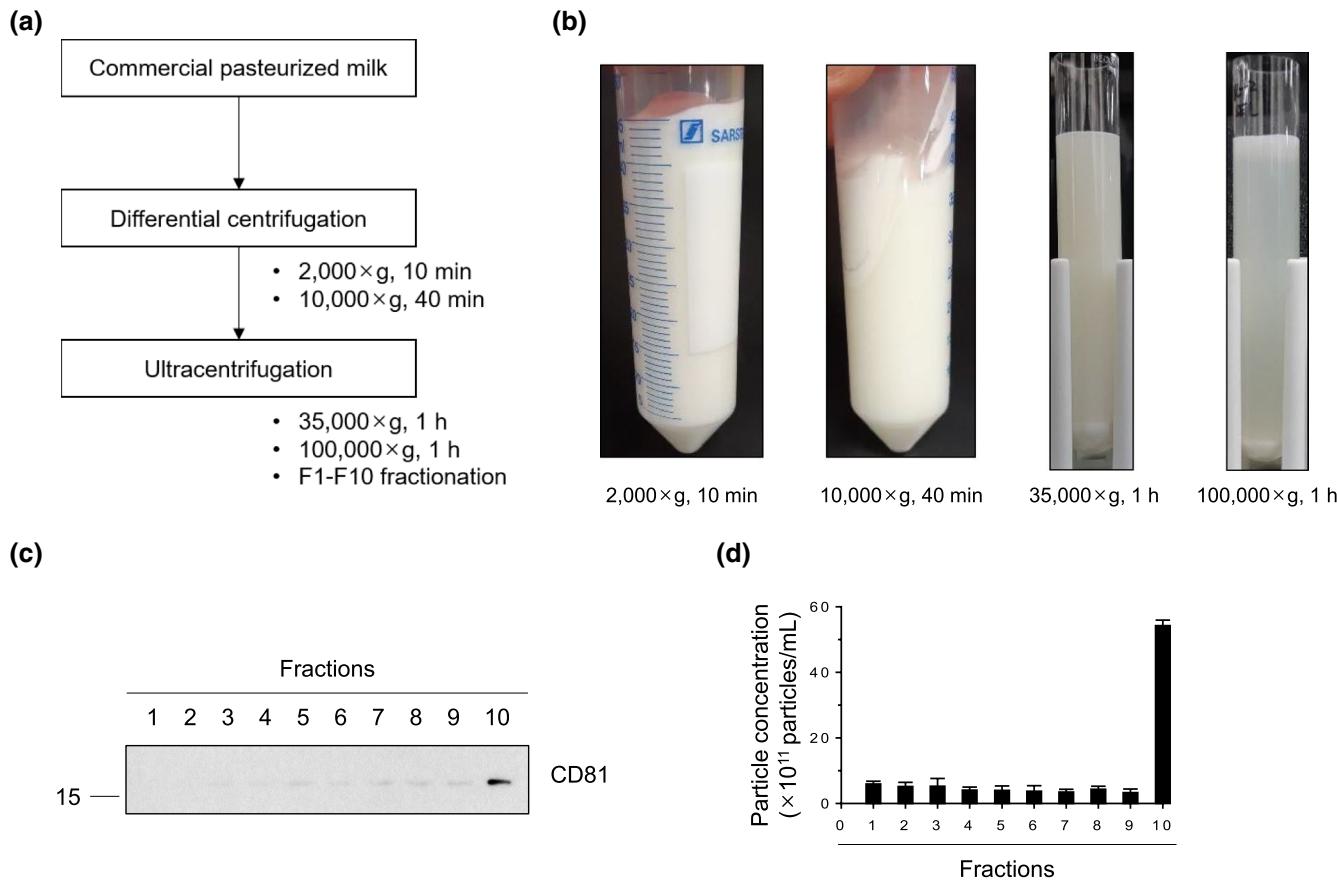


FIGURE 1 Isolation of bovine milk extracellular vesicles (milk EVs) from commercial pasteurized milk. (a) Flowchart of isolation procedure of milk EVs. (b) Images of separated bovine milk layers after differential centrifugation and ultracentrifugation. (c) Western blot analysis for CD81 in fractions obtained after ultracentrifugation at 100,000g for 1 hr. EV marker protein CD81 was enriched in fraction 10. (d) Particle concentration of each fraction measured by nanoparticle tracking analysis ($n = 3$)

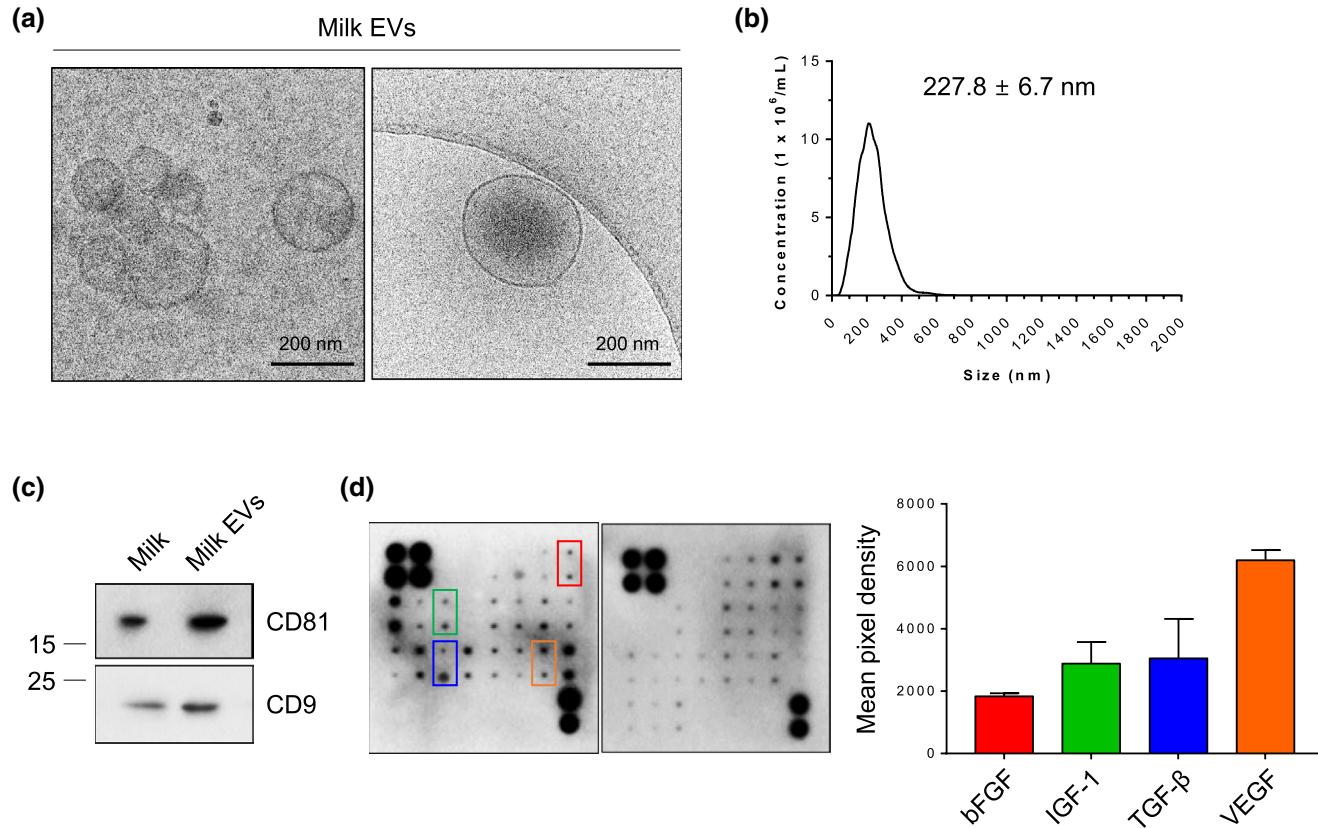


FIGURE 2 Characterization of bovine milk EVs. (a) Representative cryo-electron microscopy images of milk EVs. Milk EVs showed spherical lipid bi-layered structure (scale bar = 200 nm). (b) Size distribution of milk EVs measured by nanoparticle tracking analysis. Milk EVs had an average size of 227.8 ± 6.7 nm. (c) Western blot analysis of EV marker protein CD81 and CD9 in milk and milk EVs. (d) Immunoblot analysis of osteogenesis-related proteins in milk EVs. Mean pixel density of immunoblot is shown as a bar graph ($n = 2$). The values represent mean \pm SEM

to the surface of EVs and co-isolated with EVs (Choi et al., 2020). Therefore, the milk proteins co-isolated with EVs can also exert their effects on the proliferation and differentiation of osteoblasts. These results suggest that bovine milk EVs are nanovesicles containing osteogenesis-related proteins that can promote bone formation.

3.2 | Bovine milk EVs promote the proliferation of human osteoblastic Saos-2 cells

To determine whether milk EVs promote the proliferation of Saos-2 cells, various doses of milk EVs (100, 200, 500, and 1,000 $\mu\text{g}/\text{ml}$) were treated to Saos-2 cells for 24 hr and cell proliferation assay was performed (Figure 3a). Milk EVs dose-dependently increased the proliferation of Saos-2 cells (Figure 3a). In addition, milk EVs time-dependently increased the proliferation of Saos-2 cells (Figure 3b). Furthermore, cell cycle flow cytometry analysis also revealed that the treatment of milk EVs (1,000 $\mu\text{g}/\text{ml}$ for 72 hr) increased the percentage of cells in the S phase (Figure 3c). The expression levels of cell cycle-associated proteins such as CDK2, cyclin E, CDK4, and cyclin D1 in Saos-2 cells treated with milk EVs (1,000 $\mu\text{g}/\text{ml}$) were determined by Western blot (Figure 3d). The treatment of milk EVs significantly increased the expression of cell cycle-associated

proteins in Saos-2 cells. We also determined the effect of milk EVs on the proliferation of pre-osteoblastic MC3T3-E1 cells. The treatment of milk EVs dose-dependently increased the proliferation of MC3T3-E1 cells (Figure S2a). The milk EVs also significantly increased the cell population of the S-phase compared to the control (Figure S2b). These results indicate that bovine milk EVs significantly promote the proliferation of osteoblasts in vitro.

3.3 | Bovine milk EVs promote the differentiation of human osteoblastic Saos-2 cells

To confirm that Milk EVs promote the differentiation of osteoblasts in vitro, the expression of RUNX2 and Osterix in saos-2 cells was measured after treatment of various doses of milk EVs (0, 1, 10, 100 $\mu\text{g}/\text{ml}$). RUNX2 and Osterix are the essential transcription factors for the differentiation of osteoblasts and osteogenesis. Treatment of milk EVs significantly increased the expression of RUNX2 and Osterix (Figure 4a). To further confirmed whether milk EVs promote the differentiation of osteoblasts, the activity of alkaline phosphatase (ALP) and the expression of osteocalcin (OCN) in Saos-2 cells were measured. Treatment of milk EVs increased the ALP activity of Saos-2 cells (Figure 4b). The expression

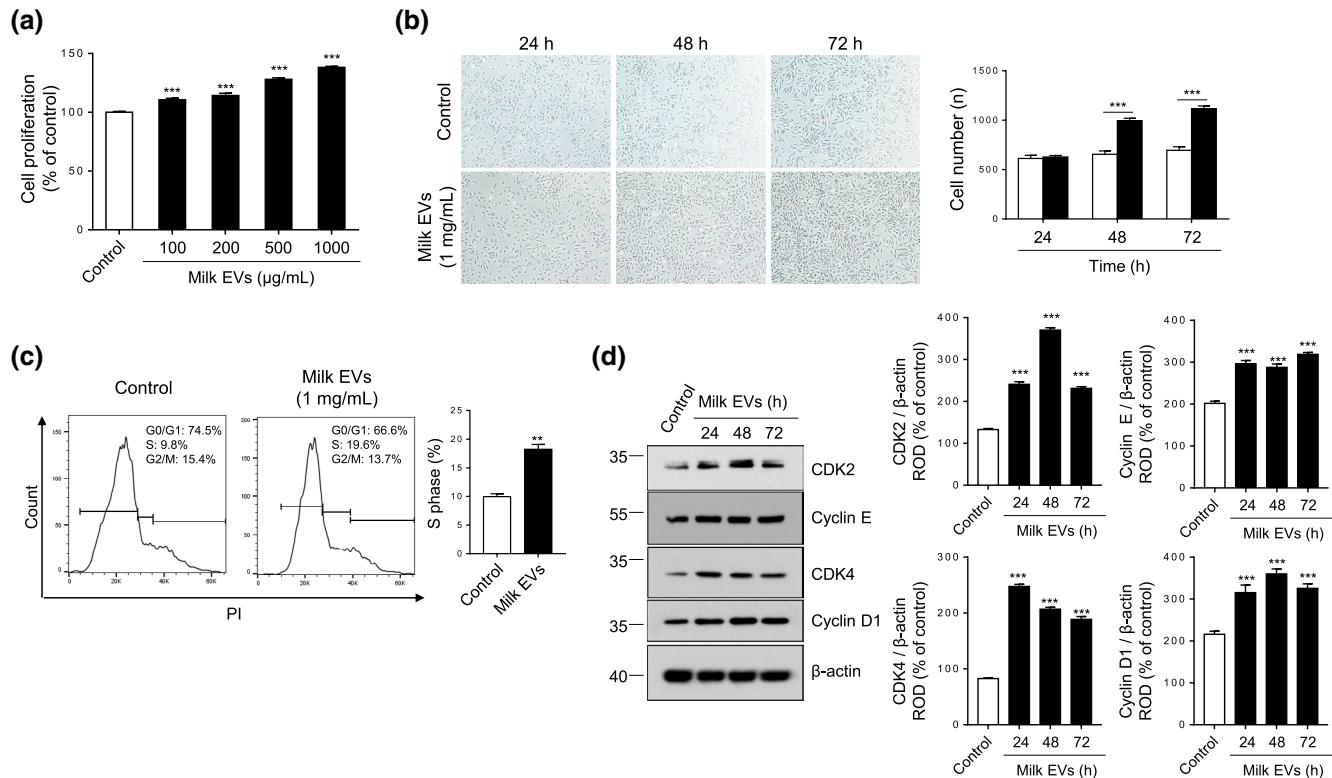


FIGURE 3 Milk EVs promote the proliferation of human osteoblastic Saos-2 cells. (a) Cell proliferation assay of Saos-2 cells treated with various doses of milk EVs (100, 200, 500, and 1,000 $\mu\text{g}/\text{ml}$) for 24 hr. The values represent mean \pm SEM. *** $p < .001$ versus control. (b) Representative images of Saos-2 cells after treatment of milk EVs (1 mg/ml). The values represent mean \pm SEM. *** $p < .001$ versus control. (c) Cell-cycle analysis of control and milk EV-treated Saos-2 cells. Milk EVs significantly increased the cell population in the S phase ($n = 5$). The values represent mean \pm SEM. ** $p < .01$ versus control. (d) Western blot analysis for CDK2, cyclin E, CDK4, and Cyclin D1 in milk EV-treated Saos-2 cells ($n = 3$). Protein levels were determined by densitometry relative to that of β -actin. The values represent mean \pm SEM. *** $p < .001$ versus control

of osteocalcin, which is involved in the extracellular matrix formation, was also dose-dependently increased by treatment of milk EVs (Figure 4c). The milk EVs also significantly increased ALP activity and OCN expression in MC3T3-E1 cells (Figure S3). These results suggest that bovine milk EVs also promote the differentiation of osteoblasts as well as proliferation.

3.4 | In vivo toxicity of bovine milk EVs

To confirm the toxicity of milk EVs *in vivo*, various doses of milk EVs (0.5, 5, 50 mg/kg) were administered once to male and female Crl:CD Sprague-Dawley rats by oral gavage. All animals survived during the experiment (Figure 5a) and had no noticeable changes in body weight (Figure 5b), clinical signs, and diet intake. To further confirmed the toxicity of milk EVs, the animals were sacrificed and blood was collected after 15 days of EV administration and a complete blood count test (Tables S1 and S2) and blood chemistry test (Tables S3 and S4) were performed. Administration of milk EVs did not result in any significant changes in hematological parameters such as red blood cell (RBC), platelet (PLT), white blood cells (WBC). In addition, milk EVs did not change the liver function-related parameters (ALT, AST, ALP), kidney function-related parameters

(BUN, creatinine) compared to untreated animals, suggesting that treatment of milk EVs did not adversely affect liver and kidney function. All other biochemical and hematological parameters remained unchanged indicating that milk EVs did not cause toxicity *in vivo*.

3.5 | Bovine milk EVs promote osteogenesis in Sprague-Dawley rats

To determine the effect of milk EVs on longitudinal bone growth and BMD, milk EVs were administered orally to Sprague-Dawley rats at 50 mg/kg/day for 14 days, and then the length and BMD of tibia were analyzed using a micro-computed tomography (μ -CT). The milk EV dose was set to 50 mg/kg/day, where no toxicity was observed in the *in vivo* toxicity test. The positive control group was subcutaneously injected with 200 $\mu\text{g}/\text{kg}/\text{day}$ of growth hormone (GH) and the negative control group was orally administered with the same volume of PBS as the milk EVs. The increase of average tibia length was observed after milk EVs administration, although there was no statistically significant difference compared to the negative control (35.64 ± 0.71 mm vs 36.24 ± 0.62 mm) (Figure 6a). It is probably because the dosage or administration

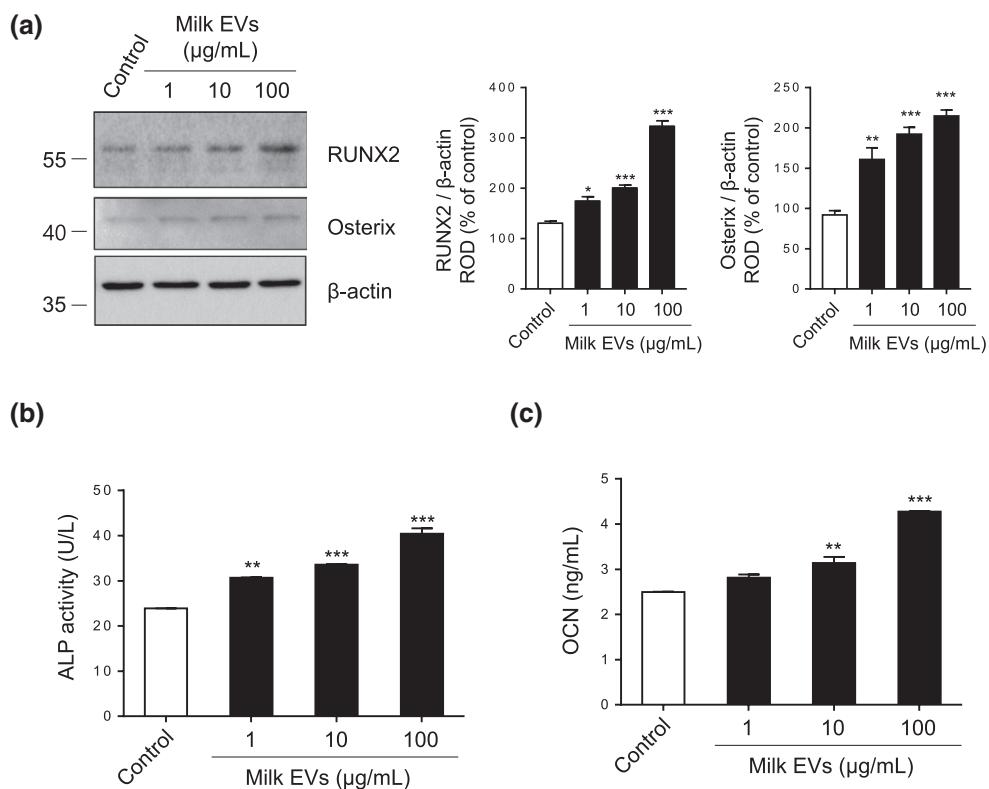


FIGURE 4 Milk EVs promote the differentiation of human osteoblastic Saos-2 cells. (a) Western blot analysis of RUNX2 and Osterix in Saos-2 cells treated with various doses of milk EVs (0, 1, 10, and 100 µg/ml) for three days ($n = 3$). Protein levels were determined by densitometry relative to that of β-actin. The values represent mean \pm SEM. * $p < .05$, *** $p < .001$ versus control. (b) Alkaline phosphatase (ALP) activity of Saos-2 cells treated with various doses of milk EVs (0, 1, 10, and 100 µg/ml) after 5 days of initiation of differentiation ($n = 3$). The values represent mean \pm SEM. ** $p < .01$, *** $p < .001$ versus control. (c) The concentration of osteocalcin (OCN) in the conditioned media of Saos-2 cells treated with various doses of milk EVs (0, 1, 10, and 100 µg/ml) after 5 days of initiation of differentiation ($n = 3$). The values represent mean \pm SEM. ** $p < .01$, *** $p < .001$ versus control

period was insufficient to observe the significant difference. In BMD measurement, milk EVs treatment significantly increased the trabecular (0.269 ± 0.031 mm vs 0.329 ± 0.062 mm) and cortical (0.746 ± 0.028 mm vs 0.766 ± 0.025 mm) volumetric BMD compared to the negative control (Figure 6b,c). These results suggest that bovine milk EVs efficiently promote osteogenesis possibly by activating the proliferation and differentiation of osteoblasts *in vivo*.

4 | DISCUSSION

Bone tissue is continuously restructured, and is maintained by the balance between the osteoclasts that absorb the old bones and the osteoblasts that form new bones. In order to promote bone growth and to treat bone-related diseases such as osteoporosis, the activity of osteoblasts should be increased. In this study, we have demonstrated that bovine milk EVs promote the proliferation of osteoblastic Saos-2 cells by increasing the expression of cell cycle-related proteins. Milk EVs also promote the differentiation of Saos-2 cells by increasing the expression of RUNX2 and Osterix. We also confirmed that milk EVs promote longitudinal bone growth and an increase in BMD of the tibia without noticeable toxicity. These results

indicate that bovine milk EVs can be used as functional materials to help bone formation or as a drug for bone-related diseases such as osteoporosis.

EVs contain proteins, lipids, and nuclear acids, such as DNA, RNA, and miRNA, which act as extracellular messengers to crosstalk between cell and cell (Nordgren et al., 2019). In particular, various cytokines and growth factors in milk EVs mediate several physiological processes including epigenetic regulation for development, inflammatory responses, immune activation, immune-modulatory activities, and pathological development (Arntz et al., 2015; Melnik et al., 2013, 2016; Nordgren et al., 2019; Qin et al., 2009). Consumption of cross-species milk beyond the early postnatal period may be involved in the development by increasing the growth hormone, IGF-1 (Nordgren et al., 2019). Milk-derived EVs modulate inflammatory response by regulating the expression of inflammatory-mediated proteins, such as IL-6, TNF-α, IL-10, IL-12/23, and arginase (Nordgren et al., 2019). Furthermore, miRNAs in bovine EVs regulate these physiological and pathological conditions (Manca et al., 2018; Wolf et al., 2015). In this study, we revealed that bovine milk EVs expressed bFGF, IGF-1, TGF-β, and VEGF. VEGF and bFGF promote osteogenesis and proliferation (Martin et al., 1997; Quarto & Longaker, 2006). In addition, IGF-1 and TGF-β are bone-derived growth factors that affect the replication and differentiation of osteoblasts (Staal

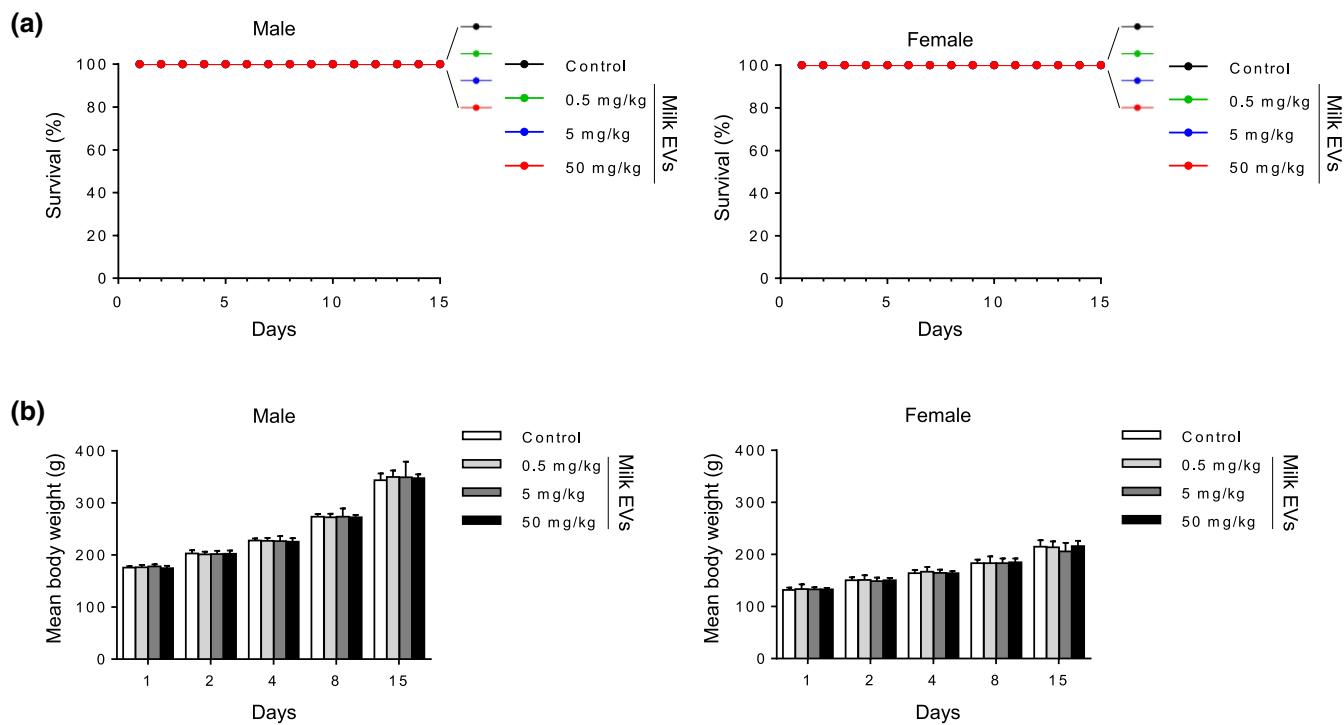


FIGURE 5 In vivo toxicity analysis of milk EVs. (a) Various doses of Milk EVs (0, 0.5, 5, 50 mg/kg) were administered to male and female Sprague-Dawley rats by oral gavage once. Survival was monitored every day ($n = 5$). (b) Mean body weight of male and female Sprague-Dawley rats after oral administration of various doses of milk EVs (0, 0.5, 5, 50 mg/kg). Body weight was monitored on day 1, 2, 4, 8, and 15. The values represent mean \pm SEM

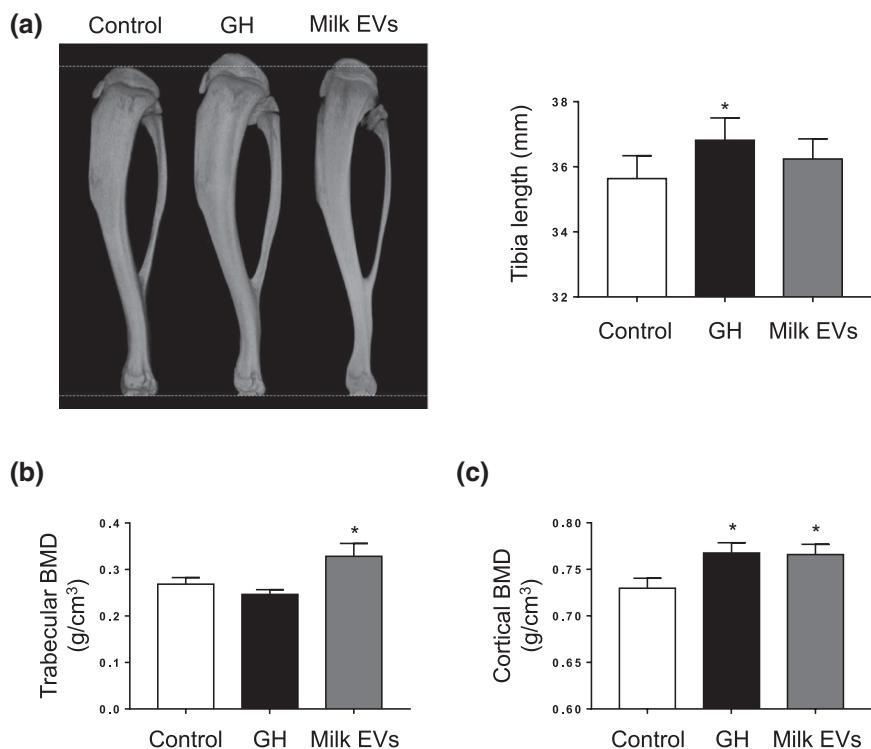


FIGURE 6 The effects of milk EVs on the longitudinal bone growth and volumetric BMD of the tibia. (a) Representative μ -CT images of the tibia of female Sprague-Dawley rats after oral administration of milk EVs (50 mg/kg/day) for 14 days. The growth hormone (GH) was used as a positive control (200 μ g/kg/day of somatotropin). The negative control group was orally administered with the same volume of PBS as the milk EVs ($n = 5$). (b, c) Trabecular (b) and cortical (c) volumetric BMD of the tibia of female Sprague-Dawley rats ($n = 5$). The values represent mean \pm SEM. * $p < .05$ versus control

et al., 1998). These findings indicate that bovine milk EVs stimulate the proliferation and osteogenesis by increasing the expression of bFGF, IGF-1, TGF- β , and VEGF.

In this study, we demonstrated that the milk EVs increase the expression of RUNX2 and Osterix. As a major transcription factor of osteoblast differentiation and skeletal morphogenesis, RUNX2 regulates the expression of various bone-related genes such as osteocalcin, osteopontin, and alkaline phosphatase. (Ducy et al., 1997; Stein et al., 2004). It is well known that RUNX2 is regulated by the BMP signaling pathway (Phimphilai et al., 2006). However, several different signaling pathways can also regulate the expression of RUNX2. For example, transforming growth factors (TGFs) (Lee et al., 1999, 2000, 2003) and fibroblast growth factors (FGFs) (Kim et al., 2003) is known to regulate the expression of RUNX2. We demonstrated that milk EVs contain the TGF- β and bFGF (Figure 2d). Therefore, milk EVs may regulate the expression of RUNX2 and induce osteoblast differentiation possibly via the bFGF or TGF- β signaling pathway.

The previous study has shown that milk-driven insulin/IGF-1 signaling accelerates cell growth and proliferation via up-regulation of the mTORC1 pathway (Melnik et al., 2013). Activation of mTORC1 promotes lipid synthesis, protein translation, and protein synthesis, resulting in increase in cell growth and proliferation (Melnik et al., 2013). In this mechanism, milk EVs containing miR-21 may trigger mTORC1 signaling through inhibition of phosphatase and tensin homolog (PTEN), sprouty 2, and programmed cell death 4 (PDCD4) (Melnik et al., 2013). Milk EVs contain diverse bioactive miRNAs that affect the proliferation and differentiation of osteoblasts. MiR-29b, found in bovine milk EVs, is known to increase the expression of RUNX2 by directly targeting HDAC4 mRNA which suppresses the expression of RUNX2. MiR-148a and miR-21 that exist in milk EVs have been shown to promote osteogenic differentiation of human bone marrow-derived stem cells (Chen & Wang, 2017; Xu et al., 2014). Bovine milk EVs have recently been reported to be osteoprotective by decreasing the osteoclast numbers in bone loss mouse models (Oliveira et al., 2020). Therefore, a complete regulatory network of milk EV-derived miRNAs has the potential to increase osteogenesis.

The most direct and accurate method for evaluating the effect of milk EVs the bone health is to measure the bone growth and BMD in an in vivo model. We demonstrated that milk EVs promote tibia bone growth and increased BMD in rats. Based on the results that milk EVs induce the differentiation of osteoblasts, it is expected that milk EVs could enhance bone fracture healing. Therefore, further studies are needed to confirm the effect of milk EVs on bone fracture healing in an in vivo model. In addition, miRNA isolation and profiling analysis for the regulation of osteogenesis is needed to investigate.

Taken together, our study revealed that bovine milk EV augments the proliferation and differentiation of osteoblasts possibly by the expression of bFGF, IGF-1, TGF- β , and VEGF in milk EVs. Moreover, administration of bovine milk EVs promoted longitudinal bone growth and an increase in BMD without any toxicity in male

and female Sprague-Dawley rats. Our findings suggest that milk EVs could be a safe and effective bioavailable material to stimulate osteogenesis.

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CONFLICTS OF INTEREST

Juhee Jeon is an employee of Stembio, Ltd. Sang Hun Lee is a founder and stockholder of Stembio, Ltd.

AUTHOR CONTRIBUTIONS

Gyeongyun Go: Investigation; Methodology; Writing-original draft; Writing-review & editing. **Juhee Jeon:** Data curation; Formal analysis; Writing-review & editing. **Gaeun Lee :** Formal analysis; Investigation; Methodology. **Jun Hee Lee :** Data curation; Formal analysis; Writing-review & editing. **Sang Hun Lee:** Conceptualization; Funding acquisition; Project administration; Resources; Supervision; Writing-review & editing.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

ETHICS STATEMENT

All animal care procedures and experiments were approved by the Institutional Animal Ethics Committee of Biotoxtech Co., Ltd and Soonchunhyang University Seoul Hospital (SCH-S2020-05). Six-week-old male and female Sprague-Dawley rat (Orient Bio Inc., Seongnam, Republic of Korea) were used for animal experiments. The animals were maintained on a 12 hr light/dark cycle at 20°C–25°C in accordance with the regulations of the Institutional Animal Ethics Committee of Biotoxtech Co., Ltd and Soonchunhyang University Seoul Hospital.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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