Effect of linoleic acid supplementation on triglyceride content and gene expression in milk fat synthesis in two- and three-dimensional cultured bovine mammary epithelial cells

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ABSTRACT
In this study, the effect of 0, 40, and 80 μmol/L of LA on triglyceride (TG) content was evaluated, as well as the mRNA expression of genes involved in milk fat synthesis in bovine mammary epithelial cells (BMECs) which were cultured in two-dimensional (2D) and three-dimensional (3D) models. TG content was measured with a TG determination kit, and the expression of genes was measured with real-time quantitative polymerase chain reactions (RT-qPCR). Adding 80 μmol/L of LA significantly increased intracellular TG content in both 2D and 3D cultured BMECs (p < .05). The mRNA expression of diacylglycerol acyltransferase 2 (DGAT2) and peroxisome proliferator-activated receptor-γ (PPARG) increased with the addition of 80 and 40 μmol/L of LA, respectively (p < .05), whereas the expression of PPARG was downregulated in the 2D model (p < .05). Adding LA significantly reduced the expression of sterol regulatory element-binding transcription factor 1 (SREBF1), acetyl-CoA carboxylase (ACC), fatty acid synthase (FASN), stearoyl-CoA desaturase (SCD), and fatty acid-binding protein 3 (FABP3) compared to the control group (p < .05). Altogether, TG content in BMECs and expression of genes involved in milk fat synthesis were significantly higher with addition of LA under 3D BMECs culture model than those of 2D (p < .05), and 40 and 80 μmol/L of LA inhibited the de novo synthesis of fatty acids and promoted the expression of DGAT2 and accumulation of TG. The mRNA expression of PPARG in 3D model was increased by addition of 40 μmol/L LA, whereas it was downregulated in 2D model.

Introduction
Essential lipids in dairy milk, including oleic acid, linoleic acid (LA) and its isomers, conjugated LA (CLA), and α-LA (ALA), have been shown not only to promote human health and protect against cancer but also to act as energy substrates for cell membrane synthesis and precursors for intracellular signalling involving transport molecules (Bauman et al. 2006; Petzold et al. 2013). As a polyunsaturated long-chain n-6 fatty acid, LA ranks among the most abundant fatty acids during bovine lactation and it is actively absorbed by bovine mammary epithelial cells (BMECs). The content of LA and isomers can be easily increased in milk fat by supplementation with oilseeds or plant oils, which are rich sources of LA in cattle diets (Lock and Bauman 2004; Dhiman et al. 2005). Adding LA to the incubation medium of bovine mammary epithelial cells (BMECs) had also significantly increased cytosolic triglyceride content (Scott and Cook, 1975; Yonezawa, et al. 2008). Moreover, essential fatty acids can bind to peroxisome proliferator-activated receptor-γ (PPARG) and regulate the expression of genes involved in cellular proliferation and death, the activation of transcription factors, nutrient metabolism, and immune response (Reyes et al. 2004; Yonezawa et al. 2008).

The development and maintenance of histological features, including polarised morphology, growth-arrested acini-like spheroids with hollow lumens, and attachment to an underlying basement membrane, are critical for epithelial cell function. However, given the lack of any glandular structure, monolayer-cultured BMECs in two-dimensional (2D) culture models can neither exactly simulate the physiological process of epithelial cells in vivo nor provide the optimal medium for understanding the regulatory mechanism of precursors on the proliferation, differentiation, and

KEYWORDS
Linoleic acid; bovine mammary epithelial cells; triglyceride; milk fat genes; three-dimensional culture model

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INTRODUZIONE
I lipidi essenziali nei latte, tra cui l’acido oleico, l’acido linoleico (LA) e i suoi isomeri, la CLA, e l’α-LA (ALA), hanno dimostrato non solo di promuovere la salute umana e di proteggere contro il cancro ma anche di agire come substrati di energia per la sintesi della membrana cellulare e come precursore per la segnalazione intracellulare coinvolgendo le molecole di trasporto (Bauman et al. 2006; Petzold et al. 2013). Come acido grasso polinsaturato a catena lunga n-6, LA si ritiene uno dei più abbondanti acidi grassi durante la lactazione bovina e viene attivamente assorbito dalle cellule epiteliali mammarie bovine (BMEC). Il contenuto di LA e degli isomeri può essere facilmente aumentato nel grasso del latte mediante la supplementazione con oleolei o oli vegetali, che sono ricchi di LA nelle alimentazioni bovine (Lock e Bauman 2004; Dhiman e collaboratori 2005). Aggiungere LA nel medio di incubazione di BMEC ha anche significativamente incrementato il trigliceride citosolico (Scott e Cook, 1975; Yonezawa e collaboratori 2008). Inoltre, gli acidi grassi essenziali possono legarsi al recettore perossisomico attivato dall’agonista di reazione ν (PPARG) e regolare l’espressione dei gene coinvolti nella proliferazione cellulare e nella morte, l’attivazione dei fattori di trascrizione, la metabolismo nutrizionale, e la risposta immunitaria (Reyes e collaboratori 2004; Yonezawa e collaboratori 2008).

Il sviluppo e l’organizzazione di struttura histologica, inclusi il morfologico polarizzato, la arresto della proliferazione acini o sferoidi con lumini vuoti, e l’adescamento ad una membrana basale, sono criticali per la funzione cellulare epiteliale. Tuttavia, data la mancanza di qualsiasi struttura glandolare, le BMEC in modello di cultura bidimensionale (2D) non possono simulare perfettamente il processo fisiologico di cellulai epiteliali in vivo né fornire lo standard ottimale per capire il meccanismo di regolazione dei precursori sulla proliferazione, differenziazione, e
were applied to study the effect of the models and LA concentrations on milk fat synthesis in BMECs. BMECs were collected from three cows, and each treatment was repeated three times in each cow. BMECs were seeded in 6-well plate, to which Matrigel (Corning, NY, 356234) was added and refed differentiation medium daily for 3 d. The three concentrations of LA were added for 48 h in lactogenic medium. The same dosage of LA was supplemented in 2D cultured BMECs to compare TG content and the expression of milk fat genes between 2D and 3D cultured BMECs.

Cell culture in the two-dimensional model

Mammary glands of healthy, multiparous mid-lactating Holstein cows of sound body condition were used to collect BMECs. Several pieces of mammary gland tissues of approximately 1 cm³ were digested by 500 U/mL collagenase II (Sigma-Aldrich, St. Louis, MO, MB2665) for 1 h at 37°C and 5% CO₂. The digesta were diluted with phosphate buffered solution (PBS; HyClone Laboratories, South Logan, UT, SH30256.018) containing penicillin–streptomycin (HyClone, SV30010) and filtered through an 80-nylon mesh. The filtered liquid was transferred into centrifuge tube and centrifuged at 150 g for 8 min. The cell pellets were cultured in 75-cm² flasks (Corning, 430720) at a concentration of 8.0 × 10⁵ cells per flask and resuspended in the proliferation medium containing Dulbecco’s modified Eagle’s medium–F12 (DMEM–F12, HyClone, SH30023.018) supplemented with 10% foetal bovine serum (FBS, HyClone, SH30084.03), 1 ng/mL of hydrocortisone (Sigma–Aldrich, H0888), 2 mmol/L of glutamine (Sigma–Aldrich, G3126), 10 ng/mL of epidermal growth factor (Gibco, Grand Island, NY, E3477), 5 μg/mL of insulin–transferrin–selenium (ITS, Sigma–Aldrich, I3146) and 1% penicillin–streptomycin (5000 units of penicillin and 5000 mg of streptomycin per mL) in conditions of 37°C and 5% CO₂. BMECs were purified from fibroblasts according to their respective sensitivities to trypsin (Gibco, 25200-056), verified by immunofluorescent staining with cytokeratin 18 until attaining 70–80% confluence, and subcultured in a lactogenic medium containing DMEM–F12 supplemented with bovine serum albumin (1 g/L) and prolactin (5 μg/mL), 1 ng/mL of hydrocortisone, 2 mmol/L of glutamine, 5 μg/mL of ITS, and 1% penicillin–streptomycin. LA was diluted in anhydrous ethanol and later in DMEM–F12 media to prepare a working solution. BMECs were randomly allocated to treatment groups with 0, 40, and 80 μmol/L of LA. BMECs in the control group were incubated with lactogenic media with a same amount of ethanol used to dilute LA in the other treatments. Each treatment was performed for 48 h in the culture system of milk components in BMECs. By contrast, three-dimensional (3D) cultured BMECs can be inoculated in reconstituted basement membrane to result in the formation of polarised, growth-arrested acini-like spheroids with hollow lumens that simulate several important characteristics of glandular architecture in vivo.

Matrigel, a kind of extracellular matrix widely used in 3D models and derived from the extracellular matrix of sarcoma in Engelbreth–Holm–Swarm mice, is primarily composed of laminin, fibre adhesion proteins, collagen, proteoglycan, and various associative growth factors (Debnath et al. 2003). Matrigel relates closely to the formation of cell morphology and provides mechanical power for the movement of cells in order to rapidly form acinar-like structures (Kozłowski et al. 2001). Nutrients, growth factors, and hormones can circulate in the pores of Matrigel to increase the use of cells (Wang 2013; Tana 2014). During the past decade, 3D culture model has received increased attention as powerful cell-based systems used to investigate the function and biological activities of genes and signal pathways in a biologically relevant context and high-throughput manner (Schmeichel and Bissell 2003; Debnath and Brugge 2005). Sasser et al. (2007) suggested that 3D breast tumour environments showed effects possibly concealed by normal 2D cell cultures. Experimental design and treatments

In this study, a 2 × 3 factorial experimental design was used. Two types of cell culture models (i.e., 2D and 3D) and three concentrations of LA (0, 40, and 80 μmol/L), following previous research (Li et al. 2016), were applied to study the effect of the models and

Materials and methods

The study was approved by the Inner Mongolia Agricultural University Animal Care and Use Committee and the Ministry of Agriculture of China for the care and use of laboratory animals.
medium in conditions of 37°C and 5% CO₂ and in triplicate.

**Cell culture in the three-dimensional model**

Epithelial cells were inoculated in Matrigel, which was thawed at 4°C for 12 h, and divided into 1.0 mL aliquots, then stored at −20°C. Matrigel remains liquid on ice but solidifies rapidly at room temperatures and should be kept on ice during experiments following sterile techniques. A precooled pipette was used to mix the Matrigel to realise homogeneity. The culture plates were rinsed 3 times by using 2 mL of precooled PBS. To each well of the culture plates was added 1 mL Matrigel spread evenly with a precooled pipette. The plates were placed in a cell culture incubator for at least 30 min in order to allow the Matrigel to solidify. Cells were seeded at a concentration of 3.0 × 10⁶ cells per well, which contained DMEM–F12 media supplemented with 5% FBS, 2% Matrigel, 1 ng/mL of hydrocortisone, 2 mM of glutamine, 5 μg/mL of ITS, and penicillin–streptomycin. Each plate was placed in a cell culture incubator in conditions of 5% CO₂ and 37°C. Cells were refed differentiation medium daily for 3 d. Various concentrations of LA (0, 40, and 80 μmol/L) were added to the culture medium, and LA treatments in the cell lysate was tested using a triglyceride test kit (Jiancheng, Nanjing, China, A045-4) and normalised for protein in each well. Protein concentrations were determined using a bicinchoninic protein kit (Jiancheng, A110-2), and TG level was expressed as mmol/mg of protein. Each experiment was performed in triplicate and repeated three times.

**RNA preparation and quantitative real-time polymerase chain reactions**

After stimulations with LA in the 2D culture model, cells were washed 3 times with cold PBS and collected in TRIsol reagent (Takara, Japan, 9180). 3D cultured cells growing in Matrigel were recovered by using cell recovery solution (Corning, 354253), which depolymerises the Matrigel on ice. Total RNA was isolated from the cells by using the TRIsol extraction method according to the manufacturer’s instructions. The quantity and purity of RNA samples were measured by using Nano Drop spectroscopy (Thermo Scientific, Waltham, MA) with the ratio of absorbance at 260 nm and 280 nm. RNA integrity was confirmed via 2% agarose gel electrophoresis. cDNA was generated by using a PrimeScript RT reagent kit with a gDNA eraser (Takara, RR047A). Real-time polymerase chain reactions (PCR) reaction, using the SYBR Premix Ex TaqII (Tli RNaseH Plus, Takara, RR820A) according to the manufacturer’s instructions, was performed on an Illumina real-time PCR machine in two steps: a cycle of initial denaturing at 95°C for 30 s, followed by 40 cycles involving denaturation at 95°C for 5 s and annealing at 60°C for 30 s. A subsequent dissociation stage produced a melting curve to verify the specificity of the amplified products. Glyceraldehyde phosphate dehydrogenase was used as the internal control. Primer sequences and parameters are presented in Table 1. Quantitative

| Table 1. Primer sequences and parameters. |
|-----------------------------------------|
| Genes       | Primer sequences | GeneBank accession no. |
|-------------|------------------|------------------------|
| GAPDH       | F: 5'-GTGTGATGGCGTGAAC-3' R: 5'-CACCTCTGGTGACATGATG-3' | XM-001034034.1 |
| ACC         | F: 5'-GTGTGATGGCGTGAAC-3' R: 5'-CACCTCTGGTGACATGATG-3' | NM_172422 |
| FASN        | F: 5'-GTGTGATGGCGTGAAC-3' R: 5'-CACCTCTGGTGACATGATG-3' | NM_001012699 |
| DGAT2       | F: 5'-CATGACACCTCATGACC-3' R: 5'-CTTCATGACACCTCATGACC-3' | NM_205793 |
| SCD         | F: 5'-AAAGTGACACCTCATGACC-3' R: 5'-CTTCATGACACCTCATGACC-3' | AY241993 |
| FABP3       | F: 5'-AAAGTGACACCTCATGACC-3' R: 5'-CTTCATGACACCTCATGACC-3' | NM_180124 |
| PPARG       | F: 5'-AAAGTGACACCTCATGACC-3' R: 5'-CTTCATGACACCTCATGACC-3' | NM_00113302 |
| SREBF1      | F: 5'-AAAGTGACACCTCATGACC-3' R: 5'-CTTCATGACACCTCATGACC-3' | NM_00113302 |

ACC: acetyl-CoA carboxylase; DGAT2: diacylglycerol acyltransferase 2; FASN: fatty acid synthase; FABP3: fatty acid-binding protein 3; GAPDH: glyceraldehyde-3-phosphate dehydrogenase; SCD: stearoyl-CoA desaturase; SREBF1: sterol regulatory element-binding transcription factor.
real-time PCR data were calculated using the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen 2001). Each experimental group was replicated in triplicate to ensure consistency.

**Statistical analysis**

Data were analysed using the mixed procedure of Statistical analysis system (2002) with a $2 \times 3$ factorial arrangement. The mixed model included the fixed effects of the culture model, LA concentration, the interactions between LA doses and cell culture type, and the random effects of replicates of each batch. Effects of the fixed factors were declared significant at $p < .05$.

**Results**

**Identification of bovine mammary epithelial cells and total RNA**

BMECs were verified by immunofluorescent staining with cytokeratin 18. In the images produced, red indicates the BMECs nucleus, whereas cytokeratin 18 appears in green. Fluorescence-activated cell-sorting analysis for cytokeratin expression in the cells showed that BMECs were nearly entirely positive (Figure 1(A)) and that the negative control of fibroblasts was negative (Figure 1(B)). Results revealed that the cultured cells were the epithelial cells of the cow mammary gland, which were used to perform a follow-up test. BMECs showed cobblestone morphology in the 2D culture model (Figure 2), which was typical of mammary epithelial cells. BMECs cultured on the basement membrane Matrigel formed 3D alveoli-like structures or mammospheres (Figure 3). The maturation of those acinar structures took roughly 5 d, during which cells proliferated to form small cell masses, and the

![Figure 1. Immunofluorescence of cytokeratin 18 (100×).](image1)

![Figure 2. Morphology of two-dimensional cultured bovine mammary epithelial cells (100×).](image2)

![Figure 3. Morphology of three-dimensional cultured bovine mammary epithelial cells (100×).](image3)
about 3000 ng/mL. RNA molecules were intact and clearly distinguishable at 28, 18, and 5.8 s (Figure 4).

**Accumulation of triglyceride**

The effect of LA supplementation on TG content in 2D and 3D cultured BMECs is presented in Table 2. Compared with the control group, groups with LA added significantly increased TG content in a concentration-dependent manner in the 3D cultured BMECs. LA in the amount of 80 μmol/L caused a significant increase in TG content compared to the 40 μmol/L LA group and control group, and TG content in the 40 μmol/L LA group was also significantly greater than that of the control group (p < .05). In the 2D cultured model, TG content in the 80 μmol/L LA group was significantly greater than that of the control group and 40 μmol/L LA group (p < .05), whereas no significant difference was observed in TG content between the 40 μmol/L LA group and the control group (p < .05).

The effect of LA treatment and culture model on TG synthesis appears in Table 3. A positive interaction effect emerged between the model and LA on TG content (p < .001). TG content of the cell culture medium in 3D cultured BMECs was significantly greater than that of 2D culture model (p < .001). LA in an amount of 80 μmol/L significantly increased TG content compared to the 40 μmol/L LA group and control group (p < .001). The combination of 3D model and 80 μmol/L LA in medium resulted in higher TG content.

**mRNA expression of genes involved in milk fat synthesis**

The effect of LA supplementation on the mRNA expression of genes involved in milk fat synthesis appears in Tables 4 and 5. The mRNA expression of acetyl-CoA carboxylase (ACC), fatty acid synthase (FASN), stearoyl-CoA desaturase (SCD), and sterol regulatory element-binding transcription factor 1 (SREBF1) were significantly lower in the 40 μmol/L LA and 80 μmol/L LA groups than in the control group, in both 2D and 3D cultured BMECs (p < .001). By contrast, the expression of diacylglycerol acyltransferase 2 (DGAT2) was significantly greater in the 80 μmol/L LA group than in the control group and 40 μmol/L LA group (p < .05). The mRNA expression of fatty acid-binding protein 3 (FABP3) was significantly lower in the 40 μmol/L LA and 80 μmol/L LA groups than that of the control group (p < .05). However, the mRNA expression of peroxisome proliferator-activated receptor-c; SCD: stearoyl-CoA desaturase; SREBF1: sterol regulatory element-binding transcription factor 1.

| Items | ACC | FASN | DGAT2 | SCD | FABP3 | SREBF1 | PPARG |
|-------|-----|------|-------|-----|-------|--------|-------|
| 0     | 1.00 ± 0.06<sup>a</sup> | 1.00 ± 0.02<sup>a</sup> | 1.00 ± 0.02<sup>a</sup> | 1.00 ± 0.02<sup>a</sup> | 1.00 ± 0.02<sup>a</sup> | 1.00 ± 0.02<sup>a</sup> | 1.00 ± 0.02<sup>a</sup> |
| 40    | 0.51 ± 0.06<sup>b</sup> | 0.51 ± 0.02<sup>b</sup> | 1.30 ± 0.12<sup>b</sup> | 0.53 ± 0.03<sup>b</sup> | 0.89 ± 0.08<sup>b</sup> | 0.69 ± 0.02<sup>b</sup> | 2.55 ± 0.69<sup>b</sup> |
| 80    | 0.38 ± 0.10<sup>c</sup> | 0.28 ± 0.04<sup>c</sup> | 2.41 ± 0.77<sup>c</sup> | 0.28 ± 0.03<sup>c</sup> | 0.56 ± 0.03<sup>c</sup> | 0.42 ± 0.13<sup>c</sup> | 0.56 ± 0.14<sup>c</sup> |

**Table 3.** Effect of linoleic acid treatment on the mRNA expression involved in milk fat synthesis in three-dimensional cultured bovine mammary epithelial cells.

| Items | ACC | FASN | DGAT2 | SCD | FABP3 | SREBF1 | PPARG |
|-------|-----|------|-------|-----|-------|--------|-------|
| 0     | 1.00 ± 0.05<sup>a</sup> | 1.00 ± 0.02<sup>a</sup> | 1.00 ± 0.02<sup>a</sup> | 1.00 ± 0.02<sup>a</sup> | 1.00 ± 0.02<sup>a</sup> | 1.00 ± 0.02<sup>a</sup> | 1.00 ± 0.02<sup>a</sup> |
| 40    | 0.51 ± 0.05<sup>b</sup> | 0.51 ± 0.02<sup>b</sup> | 1.30 ± 0.12<sup>b</sup> | 0.53 ± 0.03<sup>b</sup> | 0.89 ± 0.08<sup>b</sup> | 0.69 ± 0.02<sup>b</sup> | 2.55 ± 0.69<sup>b</sup> |
| 80    | 0.38 ± 0.10<sup>c</sup> | 0.28 ± 0.04<sup>c</sup> | 2.41 ± 0.77<sup>c</sup> | 0.28 ± 0.03<sup>c</sup> | 0.56 ± 0.03<sup>c</sup> | 0.42 ± 0.13<sup>c</sup> | 0.56 ± 0.14<sup>c</sup> |

**Table 4.** Effect of linoleic acid treatment on the mRNA expression involved in milk fat synthesis in two-dimensional cultured bovine mammary epithelial cells.

| Model | Linoleic acid concentration, μmol/L |
|-------|----------------------------------|
| 3D    | 0.31 ± 0.01<sup>a</sup> | 0.42 ± 0.03<sup>b</sup> | 0.58 ± 0.05<sup>c</sup> |
| 2D    | 0.26 ± 0.03<sup>b</sup> | 0.34 ± 0.03<sup>b</sup> | 0.49 ± 0.06<sup>c</sup> |

*Means within a line with different letters mean significant difference between LA treatments (p < .05), while with same superscript means no significant difference (p > .05).
The effect of LA treatment and culture model on triglyceride (TG) synthesis and mRNA expression of genes involved in milk fat synthesis.

### Table 5. Effect of linoleic acid treatment and culture model on triglyceride (TG) synthesis and mRNA expression of genes involved in milk fat synthesis.

| Model  | Linoleic acid concentration, μmol/L | TG content, mmol/mg protein | ACC | FASN | DGAT2 |
|--------|-----------------------------------|-----------------------------|-----|------|-------|
| 2D     | 0                                 | 0.008<sup>a</sup>          | 1.00<sup>c</sup> | 1.00<sup>c</sup> | 1.00<sup>c</sup> |
|        | 40                                | 0.01<sup>b</sup>           | 0.51<sup>a</sup> | 0.10<sup>c</sup> | 1.30<sup>b</sup> |
|        | 80                                | 0.04<sup>b</sup>           | 0.68<sup>a</sup> | 0.56<sup>c</sup> | 2.41<sup>b</sup> |
| 3D     | 0                                 | 0.02<sup>c</sup>           | 15.70<sup>a</sup> | 11.11<sup>a</sup> | 5.29<sup>b</sup> |
|        | 40                                | 0.04<sup>b</sup>           | 7.55<sup>a</sup> | 5.53<sup>c</sup> | 4.95<sup>b</sup> |
|        | 80                                | 0.08<sup>a</sup>           | 3.07<sup>d</sup> | 3.48<sup>c</sup> | 9.03<sup>a</sup> |
| SEM    |                                   | 0.001                       | 1.10  | 2.48  | 3.60  |

**Table 6. Effect of linoleic acid treatment and culture model on triglyceride (TG) synthesis and mRNA expression of genes involved in milk fat synthesis.**

| Model  | Linoleic acid concentration, mmol/L | SCD | PPARG | SREBF1 | FABP3 |
|--------|------------------------------------|-----|-------|--------|-------|
| 2D     | 0                                  | 1.00<sup>c</sup>           | 1.00<sup>c</sup> | 1.00<sup>c</sup> | 1.00<sup>c</sup> |
|        | 40                                 | 0.21<sup>c</sup>           | 0.67<sup>a</sup> | 0.49<sup>c</sup> | 0.89<sup>b</sup> |
|        | 80                                 | 0.28<sup>c</sup>           | 0.44<sup>c</sup> | 0.05<sup>c</sup> | 0.52<sup>a</sup> |
| 3D     | 0                                  | 16.45<sup>a</sup>          | 10.53<sup>a</sup> | 18.41<sup>a</sup> | 21.14<sup>a</sup> |
|        | 40                                 | 7.85<sup>a</sup>           | 16.64<sup>a</sup> | 6.33<sup>c</sup> | 18.04<sup>b</sup> |
|        | 80                                 | 5.72<sup>a</sup>           | 6.18<sup>c</sup> | 5.09<sup>c</sup> | 7.42<sup>a</sup> |
| SEM    |                                   | 1.70  | 2.18  | 5.80  | 0.64  |

**Table 6. Effect of linoleic acid treatment and culture model on triglyceride (TG) synthesis and mRNA expression of genes involved in milk fat synthesis.**

| Model  | Linoleic acid concentration, μmol/L | SCD | PPARG | SREBF1 | FABP3 |
|--------|-----------------------------------|-----|-------|--------|-------|
| 2D     | 0                                 | 1.00<sup>c</sup>           | 1.00<sup>c</sup> | 1.00<sup>c</sup> | 1.00<sup>c</sup> |
|        | 40                                | 0.01<sup>b</sup>           | 0.51<sup>a</sup> | 0.10<sup>c</sup> | 1.30<sup>b</sup> |
|        | 80                                | 0.04<sup>b</sup>           | 0.68<sup>a</sup> | 0.56<sup>c</sup> | 2.41<sup>b</sup> |
| 3D     | 0                                 | 0.02<sup>c</sup>           | 15.70<sup>a</sup> | 11.11<sup>a</sup> | 5.29<sup>b</sup> |
|        | 40                                | 0.04<sup>b</sup>           | 7.55<sup>a</sup> | 5.53<sup>c</sup> | 4.95<sup>b</sup> |
|        | 80                                | 0.08<sup>a</sup>           | 3.07<sup>d</sup> | 3.48<sup>c</sup> | 9.03<sup>a</sup> |
| SEM    |                                   | 0.001                       | 1.10  | 2.48  | 3.60  |

**Table 6. Effect of linoleic acid treatment and culture model on triglyceride (TG) synthesis and mRNA expression of genes involved in milk fat synthesis.**

| Model  | Linoleic acid concentration, mmol/L | SCD | PPARG | SREBF1 | FABP3 |
|--------|------------------------------------|-----|-------|--------|-------|
| 2D     | 0                                  | 1.00<sup>c</sup>           | 1.00<sup>c</sup> | 1.00<sup>c</sup> | 1.00<sup>c</sup> |
|        | 40                                 | 0.21<sup>c</sup>           | 0.67<sup>a</sup> | 0.49<sup>c</sup> | 0.89<sup>b</sup> |
|        | 80                                 | 0.28<sup>c</sup>           | 0.44<sup>c</sup> | 0.05<sup>c</sup> | 0.52<sup>a</sup> |
| 3D     | 0                                  | 16.45<sup>a</sup>          | 10.53<sup>a</sup> | 18.41<sup>a</sup> | 21.14<sup>a</sup> |
|        | 40                                 | 7.85<sup>a</sup>           | 16.64<sup>a</sup> | 6.33<sup>c</sup> | 18.04<sup>b</sup> |
|        | 80                                 | 5.72<sup>a</sup>           | 6.18<sup>c</sup> | 5.09<sup>c</sup> | 7.42<sup>a</sup> |
| SEM    |                                   | 1.70  | 2.18  | 5.80  | 0.64  |

**Table 6. Effect of linoleic acid treatment and culture model on triglyceride (TG) synthesis and mRNA expression of genes involved in milk fat synthesis.**

**ACC:** acetyl-CoA carboxylase; **FASN:** fatty acid synthase; **DGAT2:** diacylglycerol acyltransferase 2; **SEM:** standard error of mean.

*p* < .05 means within a line with different letters mean significant difference between model or LA treatments (p < .05), while with same superscript means no significant difference (p > .05).

**FABP3:** fatty acid-binding protein 3; **PPARG:** peroxisome proliferator-activated receptor-γ; **SEM:** standard error of mean.

*p* < .05 and *p* < .01 means within a line with different letters mean significant difference between model or LA treatments (p < .05), while with same superscript means no significant difference (p > .05).

The effect of LA treatment and culture model on the mRNA expression of genes involved in milk fat synthesis appears in Tables 3 and 6. The interaction of the model and LA significantly affected the mRNA expression of genes associated with milk fat synthesis (p < .001). The mRNA expression of genes in the 3D cultured BMECs was significantly greater than that in 2D cultured BMECs (p < .001). The mRNA expressions of **ACC, FASN, SCD, and SREBP1** significantly deceased in the 40 and 80 μmol/L LA groups compared with control group (p < .001), and the mRNA expressions of **ACC, FASN, SCD, SREBF1, FABP3, and PPARG** in the 80 μmol/L LA group were significantly lower than in the 40 μmol/L LA group and control group (p < .001). No significant difference appeared in the mRNA expression of **DGAT2** and **FABP3** between the 40 μmol/L LA group and control group. The mRNA expressions of **DGAT2** and **PPARG** were significantly increased in the 80 and 40 μmol/L LA groups, respectively (p < .001). The combination of 3D model and 80 μmol/L LA resulted in lowest **ACC, FASN, SCD, FABP3, SREBF1** expression. The high **DGAT2** expression was observed in the combination of 3D model and 80 μmol/L LA. **PPARG** expression was highest as supplement of 40 μmol/L LA in 3D model.
Discussion

During the past decade, 3D cell culture models have received increasing attention given their ability to simulate in vivo growth conditions of cells. Previous studies have shown that breast epithelial cells grown in 3D culture systems had different morphological features (Moh et al. 2005) and protein expression (Anders et al. 2003; Lee et al. 2007) compared with 2D cultured cells. When BMECs were grown in a 2D model, they lost the ability to differentiate, even in the presence of prolactogenic hormones, and cells showed cobblestone and monolayer morphology (Kozlowski et al. 2001). However, mammary epithelial cells cultured in 3D models formed acini-like spheroids with hollow lumens. Primary mouse mammary epithelial cells cultured in 3D models formed acini-like spheroids structures when cultured in basement membrane gels as well as in human mammary epithelial cells (Schmeichel and Bissell 2003; Sasser et al. 2007).

In this study, BMECs showed cobblestone morphology in a 2D culture model, whereas alveoli-like structures or mammospheres formed in 3D cultured BMECs. The aninar-like, tubular structures exposed the active site of the cellular surface receptorism, which can function more effectively with the surrounding environment and provide conditions for nutrient regulation. BMECs formed aninar-like, tubular branch structures, which could explain the significantly greater expression of genes involved in the milk protein synthesis of 3D cultured BMECs than those in 2D cultured BMECs. Previous studies have shown that β-casein in 3D cultured BMECs could be detected by western blot, but not in 2D cultured BMECs (Wang 2013). Given the same nutrient and hormone stimulation, expressions of genes involved in milk fat synthesis in 3D cultured BMECs were significantly greater than those of 2D cultured BMECs (Tana 2014), and those findings are consistent with the results of this study.

Milk fat is composed primarily of TG derived either from plasma lipids or by de novo synthesis from milk fat precursors. Therefore, TG content reflects fat synthesis in BMECs. Short-chain fatty acids significantly increased intracellular TG in BMECs cultured in a 3D model (Tana 2014), and previous studies have suggested that the accumulation of TG in BMECs was accelerated with the addition of exogenous long-chain fatty acids. Cui and Wang (2012) have indicated that supplementation with oleic acid or LA ranging from 0 to 100 μmol/L could dramatically increase the TG content in the media of BMECs. Cells cultured with linoleate at 50 to 400 M notably increased TG accumulations (Scott and Cook 1975; Yonezawa et al. 2008). The results of the present study thus concur with earlier ones. In this study, LA stimulated the accumulation of TG in a concentration-dependent manner, ranging from 0 to 80 μmol/L.

LA has also been shown to upregulate the mRNA expression of DGAT2 in BMECs. DGAT2 is pivotal in regulating milk fat synthesis, by acting as a crucial signal pathway in TG synthesis. By contrast, the lack of DGAT2 has reduced TG synthesis efficiency (Bionaz and Loor 2008b). Kadegowda et al. (2009) indicated that long-chain fatty acids promoted the synthesis of intracellular TG and upregulated the expression of the lipogenic genes AGPAT6, DGAT1, DGAT2, and GPAM (Yonezawa et al. 2008; Kadegowda et al. 2009). It is possible that the greater expression of DGAT2 induced by LA gradually increases intracellular TG accumulation.

The phenotypic control is mediated not only by the surrounding microenvironment but also by intracellular signalling pathways. PPARG acts as a lipid sensor for genes involved in lipid homeostasis in mammary glands containing SREBF1, FASN, SCD1, and ACC (Feige et al. 2006; Kang et al. 2007; Rakshandehroo et al. 2010) and genes related to the transport of fatty acids such as lipoprotein lipase and clusters of differentiation (Rakshandehroo et al. 2010). PPARG is thus a pivotal control point of bovine milk fat metabolism. Several long-chain fatty acids, particularly polyunsaturated ones, are natural ligands of PPARG in ruminants and nonruminants, along with putative target genes, are upregulated in dairy cattle (Bionaz and Loor 2008a, 2008b). In this study, mRNA expression of PPARG was upregulated with the addition of 40 μmol/L LA in 3D cultured BMECs; however, in 2D cultured BMECs, LA treatment significantly decreased the expression of PPARG compared with the control group, possibly due to the formation of a cell polarisation structure in the 3D cell culture that benefits the assimilation and use of LA and provides more favourable condition for LA’s binding to PPARG.

As a central signalling pathway, SREBF1 regulates fatty acid synthesis in the bovine and mouse mammary glands (Rincon et al. 2012). Ma and Corl (2012) have found that the mRNA expression of FASN, ACC, SCD1, and FABP3 was downregulated with the addition of 100 nM of SREBF1 siRNA in BMECs, thereby indicating that the expression of FASN, ACC, SCD1, and FABP3 mRNA were regulated by SREBF1. Previous studies have shown that polyunsaturated fatty acids can inhibit the mRNA expression of SREBP1 (Kadegowda et al. 2009; Cui and Wang 2012) and affect the de novo synthesis of short- and medium-chain fatty acids (Anderson et al. 2007), as well as mRNA expression (Bauman et al. 2008).
in BMECs. ACC and FASN are critical enzymes in fatty acids synthesis; ACC is the rate-limiting enzyme that catalyses the conversion of ACC to malonyl CoA, whereas FASN is a key lipogenic enzyme that catalyses the terminal steps in the de novo biogenesis of fatty acids (Bionaz and Loor 2008b). Previous studies have shown that long-chain fatty acids can inhibit the synthesis of short-chain fatty acids (Warntjes et al. 2008). Cui and Wang (2012) found that the expression of ACC and FASN decreased with the addition of unsaturated fatty acids. In this study, LA in doses of 40 and 80 μmol/L downregulated the mRNA expression of SREBF1, FABP3, FASN, and ACC, and those results were consistent with previous research. The inhibitory effect of de novo fatty acid synthesis by LA may stem from long-chain Acyl-CoA’s competing with newly synthesised medium-chain Acyl-CoA for the sn-2 and sn-3 positions of the TG backbone.

Compared to 2D cultured cells, BMECs cultured in 3D culture systems had different morphological features (Moh et al. 2005) and protein expression (Anders et al. 2003; Lee et al. 2007). LA regulated milk fat synthesis, as well as milk protein synthesis (Li et al. 2016). But there were little studies concerned on the effect of the interaction between culture model and LA on the milk fat synthesis. Our current research indicated that 40 μmol/L LA decreased the relative expression of PPARG at 2D culture model, while increased at 3D model, indicating there was a significant interaction between LA and culture model on milk fat synthesis. The DGAT2 abundance was increased by 80 μmol/L LA, and 3D culture model facilitated the up-regulation of LA on DGAT2 abundance. Our study demonstrated that 3D culture model accelerated up-regulation of suitable concentration of LA on the synthesis of TG. It suggested that 3D culture model in combination with LA could active the DGAT2 and PPARG to regulate milk fat synthesis. These results indicated that the interaction of culture model and LA regulated cellular energy status, and the proper combination of model and LA depressed ACC, FASN, SCD, SREBF1 and FABP3, while increased DGAT2 and PPARG. It suggested that 3D culture model had a positive effect on LA up-regulating milk fat synthesis. In concluded, the effect of interaction between culture model and LA on milk fat synthesis was related to DGAT2 and PPARG pathway, but we did not block or silence these pathways to detect the mechanism. Therefore, the exact mechanism needed to be discussed.

Conclusion

Altogether, the TG content and the mRNA expression of genes involved in milk fat synthesis in 3D cultured BMECs were significantly greater than those of 2D cultured cells. Moreover, the accumulation of TG was stimulated in a dose-dependent manner following the incremental addition of LA in BMECs. LA in amounts of 40 and 80 μmol/L inhibited the de novo synthesis of fatty acids and promoted the expression of DGAT2 and TG content. Lastly, the mRNA expression of PPARG increased with the addition of 40 μmol/L LA in the 3D model, yet was downregulated in the 2D model.

Disclosure statement

No potential conflict of interest was reported by the authors.

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References

Anders M, Hansen R, Ding RX, Rauen KA, Bissell MJ, Korn WM. 2003. Disruption of 3D tissue integrity facilitates adenovirus infection by deregulating the coxsackievirus and adenovirus receptor. Proc Natl Acad Sci USA. 100:1943–1948.

Anand P, Fu A, Teoh SH, Luo KQ. 2015. Application of a fluorescence resonance energy transfer (FRET)-based biosensor for detection of drug-induced apoptosis in a 3D breast tumor model. Biotechnol Bioeng. 112:1673–1682.

Aranda V, Haire T, Nolan ME, Calarco JP, Rosenberg AZ, Fawcett JP. 2006. Par6-aPKC uncouples ErbB2 induced disruption of polarized epithelial organization from proliferation control. Nat Cell Biol. 8:1235–1245.

Anderson SM, Rudolph MC, McManaman JL. 2007. Key stages in mammary gland development. Secretory activation in the mammary gland: it is not just about milk protein synthesis. Breast Cancer Res. 9:204.

Bauman DE, Mather IH, Wall RJ, Lock AL. 2006. Major advances associated with the biosynthesis of milk. J Dairy Res. 89:1235–1243.

Bauman DE, Perfield JW, Harvatine KJ, Baumgar LH. 2008. Regulation of fat synthesis by conjugated linoleic acid: lactation and the ruminant model. J Nutr. 138:403–409.

Bionaz M, Loor JJ. 2008a. ACSL1, AGPAT6, FABP3, LPIN1, and SLC27A6 are the most abundant isoforms in bovine mammary tissue and their expression is affected by stage of lactation. J Nutr. 138:1019–1024.

Bionaz M, Loor JJ. 2008b. Gene networks driving bovine milk fat synthesis during the lactation cycle. BMC Genomics. 9:366.

Cui RL, Wang JQ. 2012. Effects of 18-carbon fatty acids on cell proliferation and triacylglycerol accumulation in bovine mammary epithelial cells in vitro. CJAVS. 43:1064–1070.

Debnath J, Muthuswamy SK, Brugge JS. 2003. Morphogenesis and oncogenesis of MCF-10A mammary epithelial acini grown in three-dimensional basement membrane cultures. Methods. 30:256–268.
Debnath J, Brugge JS. 2005. Modelling glandular epithelial cancers in three-dimensional cultures. Nat Rev Cancer. 5:675–688.

Dhiman TR, Nam SH, Ure AL. 2005. Factors affecting conjugated linoleic acid content in milk and meat. Crit Rev Food Sci Nutr. 45:463–482.

Feige JN, Gelman L, Michalik L, Desvergne B, Wahli W. 2006. From molecular action to physiological outputs: peroxisome proliferator-activated receptors are nuclear receptors at the crossroads of key cellular functions. Prog Lipid Res. 45:120–159.

Kozlowski M, Wilczak J, Motty T, Gajewska M. 2001. Role of extracellular matrix and prolactin in functional differentiation of bovine BME-UV1 mammary epithelial cells. Pol J Vet Sci. 14:3433–3442.

Kang K, Hatano B, Lee CH. 2007. PPAR delta agonists and metabolic diseases. Curr Atheroscler Rep. 9:72–77.

Kadegowda AK, Bionaz M, Thering B, Piperova LS, Erdman RA, Loor JJ. 2009. Identification of internal control genes for quantitative polymerase chain reaction in mammary tissue of lactating cows receiving lipid supplements. J Dairy Res. 92:2007–2019.

Lee GY, Kenny PA, Lee EH. 2007. Three-dimensional culture models of normal and malignant breast epithelial cells. Nat Methods. 4:359–365.

Liva KJ, Schmittgen TD. 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2(−ΔΔCt) method. Methods. 25:402–408.

Lock AL, Bauman DE. 2004. Modifying milk fat composition of dairy cows to enhance fatty acids beneficial to human health. Lipids. 39:1197–1206.

Moh MC, Zhang C, Luo C, Lee LH, Shen S. 2005. Structural and functional analyses of a novel Ig-like cell adhesion molecule, hepaCAM, in the human breast carcinoma MCF7 cells. J Biol Chem. 280:27366–27374.

Ma L, Corl BA. 2012. Transcriptional regulation of lipid synthesis in bovine mammary epithelial cells by sterol regulatory element binding protein-1. J Dairy Res. 95:3743–3755.

Minue R, Bissell MJ. 2013. Three-dimensional cultures of mouse mammary epithelial cells. Methods Mol Biol. 945:221–250.

Li DB, Li HL, Xing YY. 2016. Effect of linoleic acid on the expression of genes associated with milk fat and milk protein synthesis of bovine mammary epithelial cells. Chin J Cell Biol. 3:257–264.

Petzold M, Meyer U, Kersten S, Spilke J, Kramer R, Jahreis G, Dänicke S. 2013. Effects of conjugated linoleic acids and dietary concentrate proportion on performance, milk composition, milk yield and metabolic parameters of periparturient dairy cows. Arch Anim Nutr. 67:185–201.

Reyes N, Reyes I, Tiwari R, Geliebter J. 2004. Effect of linoleic acid on proliferation and gene expression in the breast cancer cell line T47D. Cancer Lett. 209:25–35.

Rakshandehroo M, Knoch B, Müller M, Kersten S. 2010. Peroxisome proliferator-activated receptor alpha target genes. Cell Mol Life Sci. 61:393–416.

Rincon G, Islas-Trejo A, Castillo AR, Bauman DE, German BJ, Medrano JF. 2012. Polymorphisms in genes in the SREBP1 signalling pathway and SCD are associated with milk fatty acid composition in Holstein cattle. J Dairy Res. 79:66–75.

Scott TW, Cook LJ. 1975. Digestion and metabolism in the ruminant. In: McDonald IW, Warner ACI. Armidale, N.S.W., Australia: University of New England Publishing Unit, p. 510.

Schmeichel KL, Bissell MJ. 2003. Modeling tissue-specific signaling and organ function in three dimensions. J Cell Sci. 116:2377–2388.

Sasser AK, Mundy BL, Smith KM, Studebaker AW, Axel AE, Haidet AM. 2007. Human bone marrow stromal cells enhance breast cancer cell growth rates in a cell line-dependent manner when evaluated in 3D tumor environments. Cancer Lett. 254:255–264.

Tana N. 2014. Effect of sodium acetate and sodium p-hydroxybutyrate on milk fat synthesis in the two-dimensional and three-dimensional cultured bovine mammary epithelial cells. M.Ag. Thesis, Inner Mongolia agricultural university.

Wartnjes JL, Robinson PH, Galo E. 2008. Effects off feeding supplemental palmitic acid (C16:0) on performance and milk fatty acid profile of lactating dairy cows under summer heat[J]. Animal Feed Sci Technol. 140:241–257.

Wang XM. 2013. Effect of Two-Dimensional and Three-Dimensional Culture on the Gene Expression of Lactation Metabolism of Bovine Mammary Epithelial Cells. D.Ag. Thesis, Inner Mongolia agricultural university.

Yonezawa T, Haga S, Kobayashi Y, Katoh K, Obara Y. 2008. Unsaturated fatty acids promote proliferation via ERK1/2 and Akt pathway in bovine mammary epithelial cells. Biochem Biophys Res Commun. 367:729–735.