Shiraz University

IJVR

ISSN: 1728-1997 (Print)
ISSN: 2252-0589 (Online)

Vol. 22 No.1 Ser. No. 74 2021

IRANIAN JOURNAL OF VETERINARY RESEARCH

Original Article

In vitro culture and evaluation of bovine mammary epithelial cells from Ukraine dairy cows

Xu, P.[1, 2, 3*]; Fotina, H.[2]; Fotina, T.[2] and Wang, S.[1]

[1]Department of Veterinary Expertise, College of Animal Science and Veterinary Medicine, Henan Institute of Science and Technology, 453003 Xinxiang, China; [2]Department of Veterinary Expertise, Microbiology, Zoohygiene and Safety and Quality of Animals' Products, Faculty of Veterinary Medicine, Sumy National Agrarian University, 40021 Sumy, Ukraine; [3]Department of Biotechnology, School of Life Science and Basic Medicine, Xinxiang University, 453003 Xinxiang, China

[*]***Correspondence:*** *P. Xu, Department of Veterinary Expertise, Microbiology, Zoohygiene and Safety and Quality of Animals' Products, Faculty of Veterinary Medicine, Sumy National Agrarian University, 40021 Sumy, Ukraine. E-mail: 121558423@qq.com*

10.22099/ijvr.2020.37714.5508

(Received 14 Jul 2020; revised version 7 Nov 2020; accepted 22 Nov 2020)

Abstract

Background: Mammary epithelial cells (MECs) have been widely-used over the years as models to understand the physiological function of mammary disease. **Aims:** This study aimed to establish a culture system and elucidate the unique characteristics of bovine mammary epithelial cells (BMECs) from the milk of Ukraine Holstein dairy cows in order to develop a general *in vitro* model. **Methods:** The milk from a three-year-old lactating dairy cow was used as a source of the epithelial cell, characteristics of BMECs were examined using real time cell assay (RTCA), immunocytochemistry (ICC), reverse transcription-polymerase chain reaction (RT-PCR), and Western blot (WB). **Results:** The results showed that BMECs can be recovered from milk, grown in culture, and exhibit the characteristic cobblestone morphology of epithelial cells. **Conclusion:** The established BMECs retained MEC characteristics and secreted β-caseins even when grew on plastic substratum. Thus, the established cell line had normal morphology, growth characteristics, as well as secretory characteristics, and it could be considered as a model system and useful tool for understanding the biology of dairy cow mammary glands.

Key words: Bovine mammary epithelial cell, β-casein, Cobblestone morphology, Mammary disease, Ukraine Holstein dairy cow

Introduction

Ukraine is rich in natural resources and is the third largest grain exporter in the world. Based on its latitude, Ukraine is located in the world-class golden ranch belt and has the reputation of being a "European granary". However, due to the food culture in Ukraine, the demand for dairy products and meat is higher than for grains. Although Ukraine has a wide range of dairy products, output of high-quality dairy products is small. The mechanisms involved in milk protein expression and resistance to udder pathogens that cause contagious agalactia or secretion of abnormal milk have gained increasing attention because of the commercial value of milk. Bovine mammary epithelial cells (BMECs) function to synthesize and secrete milk are the target cells for making breast bioreactors (Capuco and Choudhary, 2020; Fu *et al*., 2020).

Bovine mammary epithelial cell models should mimic mammary gland function in order to evaluate the physiological, biochemical, and immunological function of these glands (Zhao *et al*., 2010; Lorraine and Sordillo, 2018). Several mammary epithelial cell (MEC) lines have previously been established, including human (Gaffney *et al*., 1976; Joshi *et al*., 2017), mouse (Danielson *et al*., 1984; Kittrell *et al*., 1992), bovine (German and Barash, 2002; Rose *et al*., 2002; Hu *et al*., 2009; Jedrzejczak and Szatkowska, 2014), pig (KuMuRA *et al*., 2001; Sun *et al*., 2006; Zheng and He, 2010), buffalo (Anand *et al*., 2012; Kaushik *et al*., 2013; Xu *et al*., 2019), sheep (Duchler *et al*., 1998; Ilan *et al*., 1998), and goat (Ogorevc *et al*., 2009; Ke *et al*., 2012; Tong *et al*., 2012; Zhang *et al*., 2020). In addition, methods for obtaining primary epithelial cells from human and bovine milk (Ceriani *et al*., 1979; Buehring, 1990; Taylor-Papadimitriou *et al*., 1992) as well as bovine mammary tissue (Gibson *et al*., 1991; Du *et al*., 2007; Hu *et al*., 2009) have been described. The first successful *in vitro* growth of BMECs in cell culture was done by Ebner *et al*. in 1961. Although the study of BMECs began many years ago, issues have remained that need to be addressed. It is not economical to slaughter a cow for the sole purpose of obtaining a mammary gland. Mammary gland tissue can be obtained from healthy cows via biopsy at any phase of reproductive life; however, the procedure is time-consuming and inconvenient.

The Ukraine Holstein is the main breed of dairy cow in Ukraine, but studies of Ukraine Holstein MECs have not yet been reported. With the rapid development of the

dairy industry in Ukraine, mechanisms and/or factors that might affect milk synthesis and quality have garnered much attention. A primary objective of the current study was to isolate and establish an *in vitro* BMEC culture from milk and to thoroughly characterize the BMECs via morphology, immunocytochemistry (ICC), and reverse transcription-polymerase chain reaction (RT-PCR) analysis. A culture system in which BMECs differentiate was developed to investigate the potential of BMECs for use as a model to study MEC function in this breed of cattle.

Materials and Methods

Ethics statement

All experimental procedures were approved by the Animal Care and Use Committee of the Sumy National Agricultural University, Sumy, Ukraine, and the Henan Institute of Science and Technology, Xinxiang, China, and performed in accordance with the animal welfare and ethics guidelines.

Materials for cell culture

The basal growth medium was 90% Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12 (DMEM/F12, Gibco, USA, New York, cat.12400-024) and 10% fetal bovine serum (FBS, Biological Industries, Israel, Kibbutz Beit-Haemek, cat.04-011-1A/B), supplemented with 10 ng/ml bovine epithelial growth factor (EGF, Sigma, USA, Louis, MO, cat.E4127). The storage medium consisted of 90% FBS and 10% dimethyl sulfoxide (DMSO, Sigma, USA, Louis, MO, cat.D2650). Cells were digested with 0.25% trypsin-ethylene diamine tetraacetic acid (EDTA) solution (HyClone, USA, Logan, Utah, cat.SH30042.01).

Cell preparation, culture, and frozen

Milk was obtained from several Ukrainian dairy cows in middle lactation (150-250 days after parturition). The nipples were scrubbed with sterile water, and the milk was squeezed by hand. The first 50 ml of milk was discarded, then approximately 150 ml was collected in a sterile centrifuge tube. The tube was sealed over a fire, brought back to the laboratory, stored at 37°C, and treated within 2 h. The milk was centrifuged at 1000 g for 20 min and the upper layer of milk was removed as much as possible. The bottom turbid liquid was washed several times with phosphate buffered saline (PBS) (1:1) solution containing antibiotic-antimycotic (Gibco, USA, New York, cat.15240-062) until the solution was pellucid and did not contain milk. The bottom pellet was mixed with fresh medium and transferred to an empty plastic cell culture dish (Corning, USA, New York, cat.430639). The culture dishes were incubated at 37°C under 5% CO_2. The basal medium was replaced with a fresh medium every 48 h until the cells were distributed across the bottom of the dish. The cells were subcultured and frozen when they were distributed across 80% of the bottom.

Growth characteristics

The real time cell assay (RTCA) was used to monitor the growth of the third-generation mammary gland epithelial cells. Totals of 4×10^4, 2×10^4, 1×10^4, 5000, 2500, 1250, and 625 cells per well were seeded on E-Plate, 16 plates, 150 µL medium per well. Real time detection of cells was performed to plot cell growth curves while optimizing optimal plate density.

Immunocytochemistry

The expression of cytokeratin 18 (Abcam, UK, Cambridge, cat.10830-1-AP) was examined by seeding 1×10^4 cells/well in 12-well flat-bottom culture plates. Cytostructural protein expression was examined on day 3 after seeding cells that were cultured in induction media. Before staining, the cells were washed with PBS solution and fixed with ice-cold methanol. Cells were incubated in PBS containing 0.2% Triton X-100 at 37°C for 5 min, then rinsed with PBS. Nonspecific reactivity was blocked with 5% bovine serum albumin (BSA) for 30 min at room temperature. First, anti-cytokeratin 18, antisera, was diluted 1/100 in PBS, added to the cells, and incubated for 1 h at room temperature. The cells were then washed three times for 5 min each time with phosphate buffered saline-Tween20 (PBST). Secondary antibody, fluorescein isothiocyanate (FITC)-conjugated monoclonal anti-mouse IgG (Sigma, USA, Louis, MO, cat.F4143), was diluted 1/50 in PBS, added to the cells, and incubated in the dark for 0.5 h. Cells were then washed three times for 5 min each time with PBS. DAPI (4,6-diamino-2-phenyl indole) was used as a nuclear counterstain. Finally, the slides were washed three times and visualized with a microscopy characterization facility (Zeiss, LSM7800, Germany, Oberkochen).

RT-PCR

Total RNA from mammary tissue, BMECs cultured with the induction media, and BMECs, and mammary alveolar cell-T (MAC-T) cells cultured with basal media were isolated using a PureLink™ RNA Mini kit (ThermoFisher, USA, Waltham MA, cat.12183018A). The expressions of *CSN2*, *BTN1A1*, and *GAPDH* were determined by RT-PCR. The integrity and concentration of the RNA was verified via analysis of 5 µL of each sample on a 1% agarose gel using an ultraviolet spectrophotometer (Beijing Junyi-Dongfang Electrophoresis Equipment Co., Ltd China, Beijing, JY04S-3E). The RT system (SuperScript®) was purchased from Invitrogen. *CSN2*, *BTN1A1*, and *GAPDH* primers were designed with Primer 5.0 (Table 1) and synthesized by Shanghai Sangon Biological Engineering Technology and Services Co. Ltd., China.

Western-blot (WB)

Total protein was isolated from BMECs, MAC-T, and mammary tissues using radio immunoprecipitation assay (RIPA) (Servicebio, China, Wuhan, cat.G2002). The proteins were run through a 12% polyacrylamide gel under reducing conditions, and transferred onto polyvinylidene fluoride (PVDF) (0.45 µm, GE

Table 1: Primer sequences for RT-PCR on identification of BMECs

Gene	Primers forward/reverse	Product length (bp)	Melting temperature (°C)
CSN2	5´AGGAACAGCAGCAAACAG3´ 5´TTTCCAGTCGCAGTCAAT3´	579	55
BTN1A1	5´TGTGTTGCTGCTGATAGAGTGT3´ 5´CCTCCAAGTTCCTTTATGGGAT3´	305	53
GAPDH	5´GGCAAGTTCAACGGCACA3´ 5´ACCACATACTCAGCACCAGCA3´	128	56

RT-PCR: Reverse transcription-polymerase chain reaction, and BMECs: Bovine mammary epithelial cells

Healthcare Life Sciences, Pittsburgh, PA, USA). After blocking in Tris-HCl buffered saline (TBS) (20 mM Tris and 137 mM NaCl) containing 5% BSA, the membrane was incubated overnight at 4°C with rabbit anti-bovine casein antibody (Jingmei, Jiangsu, China, cat.F030106). The membrane was washed three times for 5 min each with Tris-HCl buffered saline-Tween20 (TBST), and incubated with horseradish peroxidase (HRP) conjugated mouse anti-rabbit IgG (Beyotime, Shanghai, China, cat.TR-1003) for 1 h at 37°C.

Results

Establishment of BMECs

The whole growth process is different when culturing epithelial cells from tissue. The different culture stages are shown in Fig. 1, not including tissue culture. Single adherent cells appear on the culture dish after culturing for 3-4 days (Fig. 1A). Bovine mammary epithelial cells formed islands when cultured at low density after 5-7 days (Fig. 1B). Primary epithelial cells were elongated after culturing for 8-10 days (Fig. 1C). Mammary epithelial cells developed into different shapes, including oval, typical cobblestone, and irregular polygon. Most of the isolated cells that extended from the milk had an irregular polygon shape (Figs. 1D and E). The cells obtained after freezing and thawing maintained normal morphology and growth characteristics (Fig. 1F). In the selection of frozen storage medium, the frozen solution containing 90% FBS and 10% DMSO was more conducive to the survival of cryopreserved BMECs than DMEM/F12: FBS: DMSO= 5:4:1. For subsequent research, FBS containing 10% DMSO was used for preservation of frozen cells.

Growth characteristics of BMECs

Real time cell assay provides a remarkable method for real time monitoring of cell viability. The result showed that the best seeding density for the proliferation of BMECs was 1×10^4 cells (Fig. 2). The BMECs culture slowly grew within the first 3 days and cells entered the stable phase in the best seeding density.

Identification of cytokeratin 18 protein expression

Although the established cells appeared to have epithelial cell morphology, protein expression of cytokeratin 18, which is specific for epithelial cells, was examined. Cells exhibited strong positive staining for cytokeratin 18, indicating that the cultured cells possessed the properties of epithelial cells (Figs. 3A-C).

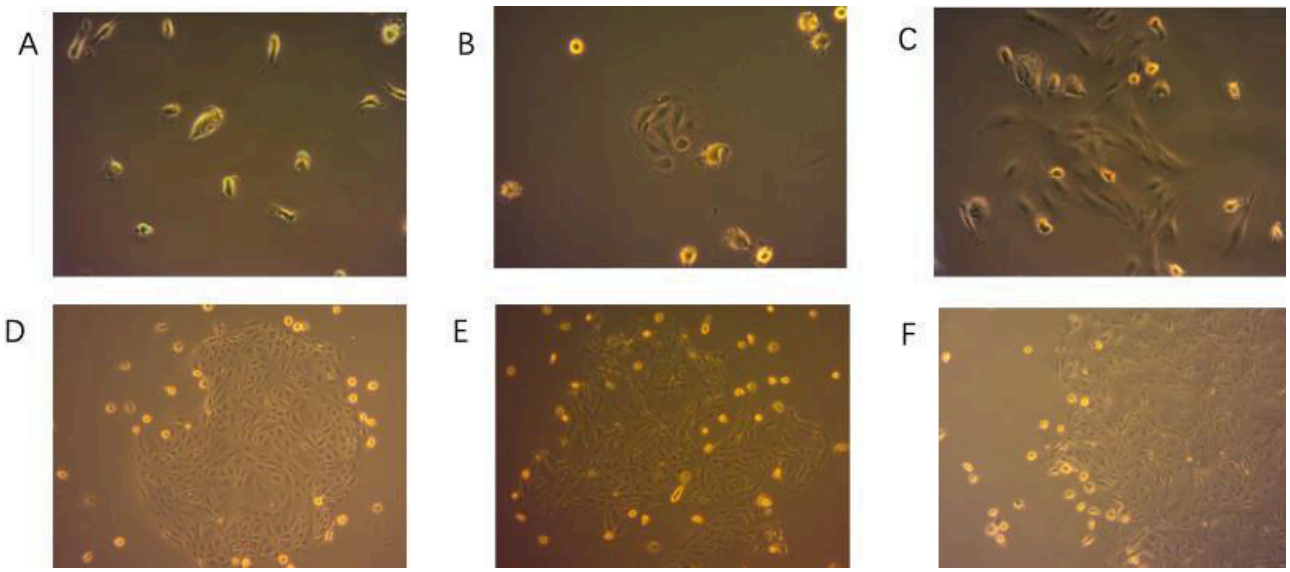

Fig. 1: Morphology of bovine mammary epithelial cells (BMECs). **A**: Cells from milk pellets on the 3rd day (×100), **B**: BMECs form islands when grown at low density between 5 and 7 days (×100), **C**: BMECs cultured between 8 and 10 days (×100), **D**: BMECs had typical cobblestone morphology between 13 and 14 days (×100), **E**: BMECs with irregular polygon shape (×100), and **F**: Morphology of resuscitated cells (×100)

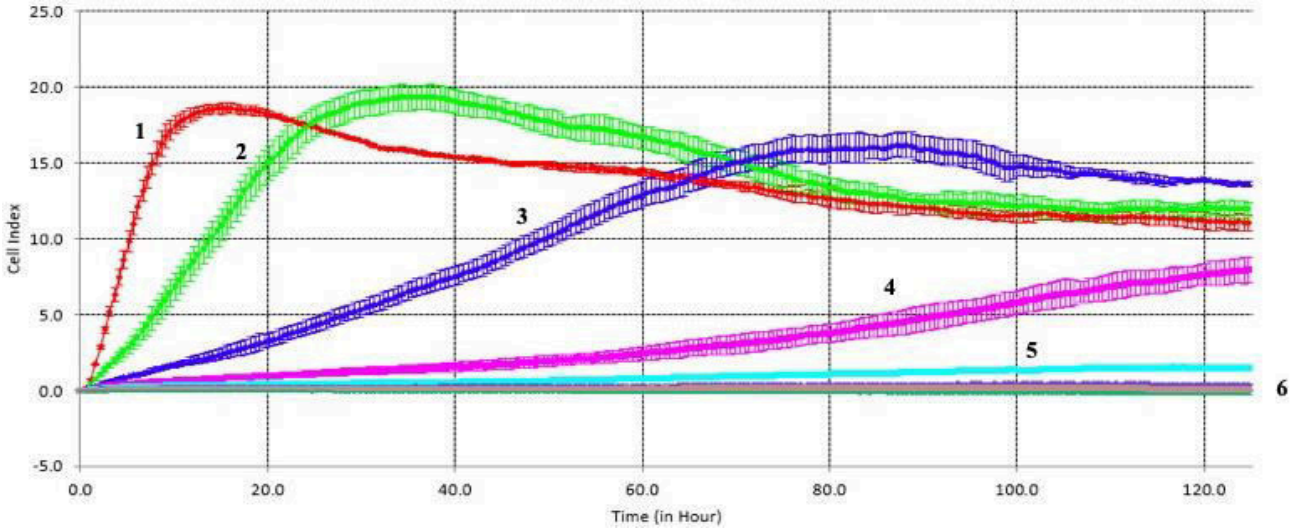

Fig. 2: Growth curves of bovine mammary epithelial cells (BMECs) with different seeding densities. Cells were plated in an E-plate at seeding densities of 4×10^4 cells (**1**), 2×10^4 cells (**2**), 1×10^4 cells (**3**), 5000 cells (**4**), 2500 cells (**5**), and 1250 cells (**6**)

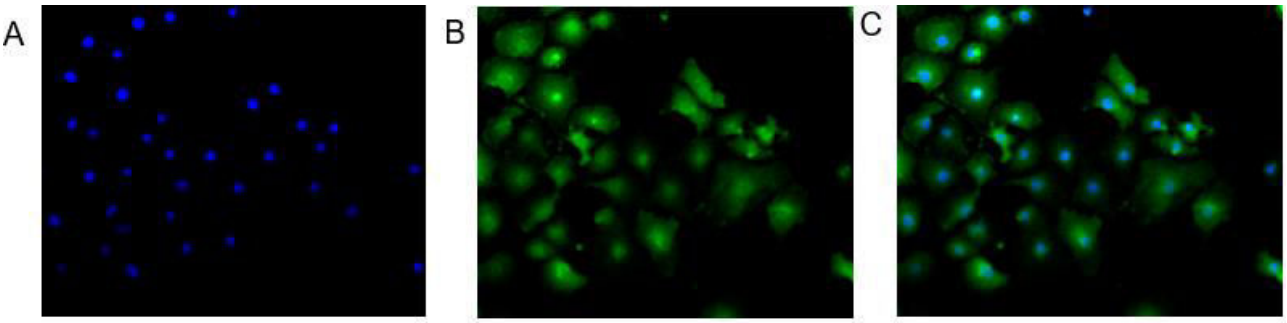

Fig. 3: Immunofluorescence identification of the isolated bovine mammary epithelial cells (BMECs) (×100). **A**: 4,6-diamino-2-phenyl indole (DAPI) staining of nucleus, **B**: 4,6-diamino-2-phenyl indole (FITC) staining of cytoplasm, and **C**: DAPI and FITC staining of BMECs

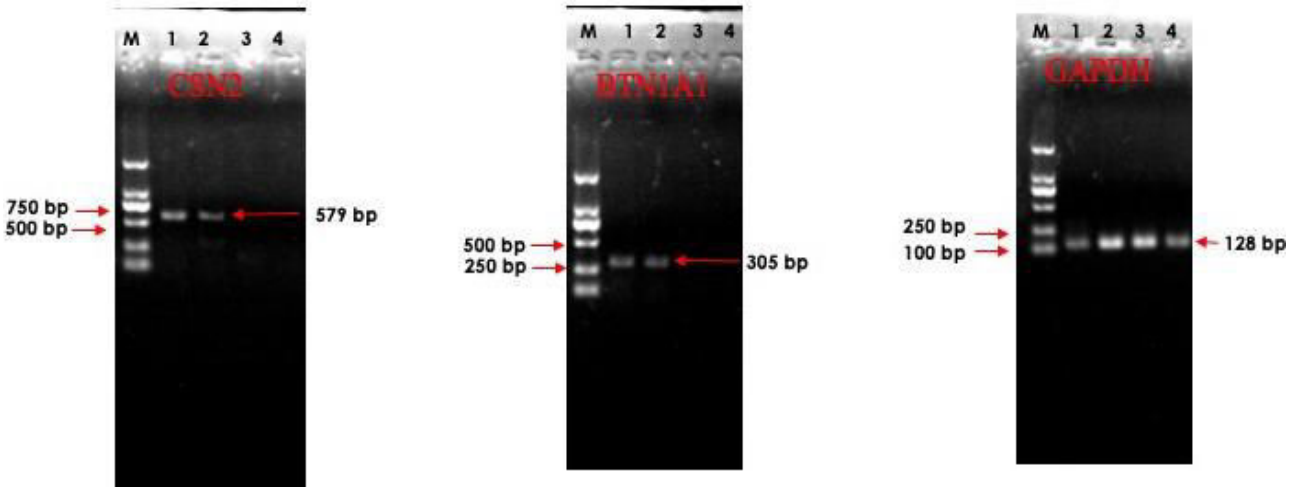

Fig. 4: Reverse transcription-polymerase chain reaction (RT-PCR) analysis for *CSN2* and *BTN1A1* in bovine mammary epithelial cells (BMECs). *CSN2* is the β-casein gene, *BTN1A1* is the butyrophilin subfamily 1 member *A1* gene, and *GAPDH* was used as reference gene. M: DL2000 marker. Lane 1: Mammary tissues (the positive control), Lane 2: BMECs cultured in induction medium (100 ng/ml insulin like growth factor (IGF) + 10 ng/ml epithelial growth factor (EGF)), Lane 3: BMECs cultured in basal medium, and 4: Bovine mammary alveolar cell-T (MAC-T) cultured in basal medium

RT-PCR and WB analysis of BMECs

It is very important that MECs express milk proteins and can mimic the *in vivo* system. Total RNA was isolated from mammary tissue, MECs cultured in

induction media, MECs cultured in basal media, and MAC-T cells. Reverse transcription-polymerase chain reaction was used to determine the mRNA expression (Fig. 4) and WB was used to determine the milk proteins expression (Fig. 5). The results confirmed the ability of the isolated cells to synthesize milk proteins.

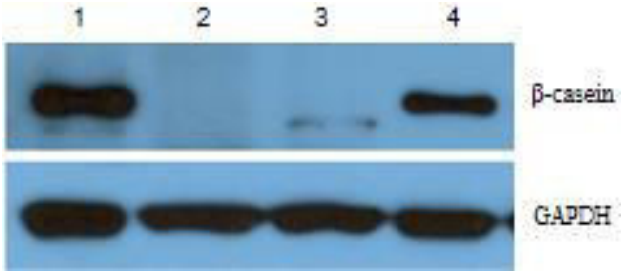

Fig. 5: Western blot analysis of β-casein in bovine mammary epithelial cells (BMECs). 1: Mammary tissues (the positive control), 2: Bovine mammary alveolar cell-T (MAC-T) cultured in basal medium, 3: BMECs cultured in basal medium, and 4: BMECs cultured in induction medium (100 ng/ml insulin like growth factor (IGF) + 10 ng/ml epithelial growth factor (EGF))

Discussion

Like other epithelial cells, MECs exfoliate and are shed into secretions. The somatic cells described in dairy manuals as part of typical milk sediment are actually leukocytes and exfoliated epithelial cells (Kitchen, 1981). Isolating and cultivating BMECs from milk by manual milking is not only simple to do but also low cost, harmless to cows, and can be carried out in most laboratories. In this study, the milk of healthy, high-producing Ukrainian Holstein dairy cows was mixed with antibody-containing PBS at a ratio of 1:1. After centrifugation, the supernatant was discarded and the pellet was cultured from which BMECs were successfully separated. Compared with the supernatant, the pellet contained a larger number of cells and fewer impurities, such as milk fat. Previous studies have reported that BMECs can be obtained by centrifugation (Buehring, 1990; Cui *et al*., 2006; Cui *et al*., 2013; Duan *et al*., 2017), however, few studies reported that using antibiotics in BMECs culture could effectively reduce the pollution. Data from the present study suggest that BMECs can be obtained from milk after culturing for 10-12 days, which is earlier than with the explant culture method. Studies have shown that BMECs were elongated from the breast tissue in 8-10 days when using the tissue adherence method, though further purification with fibroblasts is required for another 25-30 days (Rose *et al*., 2002; Hu *et al*., 2009). Therefore, although milk can be easily contaminated when using the milk separation method (Buehring, 1990; Zhong *et al*., 2006) due to factors such as recessive mastitis in cows or centrifugation defect to remove milk fat, it has the advantage of being fast. The main disadvantage of using milk as a source of BMECs is the relatively small number of mammary cells obtained compared with explant cultures. However, in this study, the breast epithelial cells in milk were generally from the breast tissue and the cell cycles were relatively long, so the isolated primary cells grew very slowly. The 3-8 generation cells grew faster than the 1-2 generation cells, however, the cells then appeared to grow slowly again, and after forming islands in isolation, they no longer covered the whole culture dish. The cause of this phenomenon needs further investigation.

The cells cultured overnight in DMEM/F12 medium containing 10% FBS and 10% EGF were distributed across 80% of the bottom. In previous studies, when only 10% FBS were used (Shamay and Gertler, 1986; McGrath, 1987; German and Barash, 2002), the cells needed to be cultured over 48 h, when they were distributed across 80% of the bottom. However, the use of EGF in this study significantly increased the cell doubling time.

Dairy cow MECs are a type of adherent culture cell that is not easily digested from a culture dish. Compared with other cells, BMECs took longer to digest (usually 6-7 min). In order to achieve complete digestion during passaging, the density of the cultured cells and cell digestion time needed to be controlled. According to the RTAC analysis, the optimal seeding density was 1×10^4 cells/0.32 cm^2. At the optimal seeding density, the logarithmic growth period of the cells was longer and various experimental treatments could be performed. However, when the cell density was high, the cells entered quickly to a stationary phase without sufficient time for other treatments. When the cell density was low, there was no stable phase.

Keratins are unique to epithelial cells of mammalian species. Cytokeratin 18 is one of the most common members of the intermediate filament gene family (Franke *et al*., 1979; Schmid *et al*., 1983). It is expressed in single layer epithelial tissues of the body and is specific for epithelial cells (Schmid *et al*., 1983; Bartek *et al*., 1985; Taylor-Papadimitriou *et al*., 1989; Pantschenko *et al*., 2000; Su *et al*., 2013). The purified BMECs showed positive staining to cytokeratin 18, thereby provided direct evidence of their epithelial nature.

Milk protein secretion is an important mammary-specific feature (Pipe *et al*., 2011). Generally, insulin, hydrocortisone, and prolactin are used in culture media to induce milk protein expression (Puissant *et al*., 1990; Strange *et al*., 1991; Akers, 2006). Casein secretion is the hallmark of BMECs (Ahn *et al*., 1995; Aoki, 2006). In the present study, variations in marker gene expression between cells cultured in basal and induction media were observed, indicating that the difference in the composition of culture media may influence functional gene expression. The expression of the *CSN2*, and *BTN1A1* genes was determined using RT-PCR. This finding suggests that BMECs exhibited normal secretory function.

This study established a method for the isolation and culture of cow MECs from the milk of Ukrainian Holstein dairy cows. By selecting the late-stage milk of

high-yielding cows without mastitis, primary cultured breast epithelial cells could be isolated. The isolated breast milk epithelial cells of Ukrainian Holstein dairy cows expressed milk protein and milk fat synthesis-related genes, which laid a foundation for further study of the lactation mechanism of dairy cows and improving the quality of dairy products.

Acknowledgements

This research was financially supported by the National Natural Science Foundation of Henan (grant No. 212300410220, 182300410086) and Key Scientific Research Projects of Higher Education in Henan Province (No. 20A180025).

References

Ahn, JK; Aoki, N; Adachi, T; Mizuno, Y; Nakamura, R and Matsuda, T (1995). Isolation and culture of bovine mammary epithelial cells and establishment of gene transfection conditions in the cells. Biosci. Biotech. Bioch., 59: 59-64.

Akers, R (2006). Major advances associated with hormone and growth factor regulation of mammary growth and lactation in dairy cows. J. Dairy Sci., 89: 1222-1234.

Anand, V; Dogra, N; Singh, S; Kumar, SN; Jena, MK; Malakar, D; Dang, AK; Mishra, BP; Mukhopadhyay, TK; Kaushik, JK and Mohanty, AK (2012). Establishment and characterization of a buffalo (*Bubalus bubalis*) mammary epithelial cell line. PloS One. 7: e40469.

Aoki, N (2006). Regulation and functional relevance of milk fat globules and their components in the mammary gland. Biosci. Biotech. Bioch., 70: 2019-2027.

Bartek, J; Durban, E; Hallowes, R and Taylor-papadimitriou, J (1985). A subclass of luminal epithelial cells in the human mammary gland, defined by antibodies to cytokeratins. J. Cell Sci., 75: 17-33.

Buehring, GC (1990). Culture of mammary epithelial cells from bovine milk. J. Dairy Sci., 73: 956-963.

Capuco, AV and Choudhary, RK (2020). Determinants of milk production: Understanding population dynamics in the bovine mammary epithelium. J. Dairy Sci., 103: 2928-2940.

Ceriani, RL; Taylor-Papadimitriou, J; Peterson, JA and Brown, P (1979). Characterization of cells cultured from early lactation milks. *In vitro* Cell Dev. Biol. Anim., 15: 356-362.

Cui, LL; Bu, PD; Wang, JQ and Luo, SP (2006). *In vitro* culture of cow mammary epithelial cells. Xinjiang Agri. Sci., 43: 394-398.

Cui, XJ; Wang, T; Liu, BC; Tao, L and Wang, X (2013). Isolation and identification of mammary epithelial cells from milk. Biotech. Bull., 3: 107-113.

Danielson, KG; Oborn, CJ; Durban, EM; Butel, JS and Medina, D (1984). Epithelial mouse mammary cell line exhibiting normal morphogenesis *in vivo* and functional differentiation *in vitro*. Proc. Natl. Acad. Sci. USA. 81: 3756-3760.

Du, J; Di, HS and Wang, GL (2007). Establishment of a bovine epithelial mammary cell line and ultrastructural changes when exposed to heat stress. Chin. J. Biotechnol., 23: 471-476.

Duan, AQ; Pang, CY; Zhu, P; Deng, TX; Lu, XR; Ma, XY; Liang, SS and Liang, XW (2017). Isolation, culture and identification of buffalo mammary epithelial cells from milk. China Anim. Hus. Vet. Med., 44: 3243-3249.

Duchler, M; Schmoll, F; Pfneisl, F; Brem, G and Schellander, K (1998). OMEG II: a new ovine mammary epithelial cell line. Biol. Cell. 90: 199-205.

Ebner, KE; Hoover, CR; Hageman, EC and Larson, BL (1961). Cultivation and properties of bovine mammary cell cultures. Exp. Cell Res., 23: 373-385.

Franke, WW; Appelhans, B; Schmid, E; Freudenstein, C; Osborn, M and Weker, K (1979). Identification and characterization of epithelial cells in mammalian tissues by immunofluorescence microscopy using antibodies to prekeratin. Differentiation. 15: 7-25.

Fu, NY; Nolan, E; Lindeman, GJ and Visvader, JE (2020). Stem cells and differentiation hierarchy in mammary gland development. Physiol. Rev., 100: 489-523.

Gaffney, EV; Polanowski, FP; Blackburn, SE; Lambiase, JT and Burke, RE (1976). Cultures of normal human mammary cells. Cell Differ. 5: 69-81.

German, T and Barash, I (2002). Characterization of an epithelial cell line from bovine mammary gland. *In vitro* Cell Dev. Biol. Anim., 38: 282-292.

Gibson, CA; Vega, JR; Baumrucker, CR; Oakley, CS and Welsch, CW (1991). Establishment and characterization of bovine mammary epithelial cell lines. *In vitro* Cell Dev. Biol. Anim., 27A: 585-594.

Hu, H; Wang, JQ; Bu, DP; Wei, HY; Zhou, LY; Li, FD and Loor, JJ (2009). *In vitro* culture and characterization of a mammary epithelial cell line from Chinese Holstein dairy cow. PLoS One. 4: e7636.

Ilan, N; Barash, I; Gootwine, E and Shani, M (1998). Establishment and initial characterization of the ovine mammary epithelial cell line NISH. *In vitro* Cell Dev. Biol. Anim., 34: 326-332.

Jedrzejczak, M and Szatkowska, I (2014). Bovine mammary epithelial cell cultures for the study of mammary gland functions. *In vitro* Cell Dev. Biol. Anim., 50: 389-398.

Joshi, PS; Modur, V; Cheng, J; Robinson, K and Rao, K (2017). Characterization of immortalized human mammary epithelial cell line HMEC 2.6. Tumor Biol., 2017: 1-12.

Kaushik, R; Singh, KP; Kumari, A; Singh, MK; Manik, RS; Palta, P; Singla, SK and Chauhan, MS (2013). Isolation, characterization, and EGFP expression in the buffalo (*Bubalus bubalis*) mammary gland epithelial cell line. *In vitro* Cell Dev. Biol. Anim., 49: 1-7.

Ke, MW; Hsu, JT; Jiang, YN; Cheng, WTK and Ju, YT (2012). Characterzation of hTERT-Immortalized caprine mammary epithelial cells. Reprod. Domest. Anim., 47: 553-561.

Kitchen, BJ (1981). Review of the progress of dairy science: bovine mastitis: milk compositional changes and related diagnostic tests. J. Dairy Res., 48: 167-188.

Kittrell, FS; Oborn, CJ and Medina, D (1992). Development of mammary preneoplasias *in vivo* from mouse mammary epithelial cell lines *in vitro*. Canner Res., 52: 1924-1932.

KuMuRA, H; Tanaka, A; Abo, Y; Yui, S; Shimazaki, K; Kobayashi, E and Sayama, K (2001). Primary culture of porcine mammary epithelial cells as a model system for evaluation of milk protein expression. Biosci. Biotech. Bioch., 65: 2098-2101.

Lorraine, M and Sordillo, MS (2018). Mammary gland immunobiology and resistance to mastitis. Vet. Clin. North Am. Food Anim. Pract., 34: 507-523.

McGrath, MF (1987). A novel system for mammary epithelial cell culture. J. Dairy Sci., 70: 1967-1980.

Ogorevc, J; Prpar, S and Dove, P (2009). *In vitro* mammary gland model: establishment and characterization of a caprine mammary epithelial cell line. Acta Agr. Slov., 94: 133-138.

Pantschenko, AG; Woodcock-Mitchell, J; Bushmich, SL and Yang, TJ (2000). Establishment and characterization of a caprine mammary epithelial cell line (CMEC). *In vitro*. 36: 26-37.

Pipe, SW; Miao, HZ; Butler, SP and Calcaterra, J (2011). Functional factor VIII made with von Willebrand factor at high levels in transgenic milk. J. Thromb. Haemostasis. 9: 2235-2242.

Puissant, C; Attal, J and Houdebine, L (1990). The hormonal control of ovine β-lactoglobulin gene in cultured ewe mammary explants. Reprod. Nutr. Dev., 30: 245-251.

Rose, MT; Aso, H; Yonekura, S; Komatsu, T; Hagino, A; Ozutsumi, K and Obara, Y (2002). *In vitro* differentiation of a cloned bovine mammary epithelial cell. J. Dairy Res., 69: 345-355.

Schmid, E; Franke, WW; Grund, C; Schiller, DL; Kolb, H and Paweletz, N (1983). An epithelial cell line with elongated myoid morphology derived from bovine mammary gland. Exp. Cell Res., 146: 309-328.

Schmid, E; Schiller, DL; Grund, C; Stadler, J and Franke, WW (1983). Tissue type-specific expression of intermediate filament proteins in a cultured epithelial cell line from bovine mammary gland. J. Cell Biol., 96: 37-50.

Shamay, A and Gertler, A (1986). A model for *in vitro* proliferation of undifferentiated bovine mammary epithelial cells. Cell Biol. Int. Rep., 10: 923-929.

Strange, R; Li, F; Friis, RR; Reichmann, E; Haenni, B and Burri, PH (1991). Mammary epithelial differentiation *in vitro*: minimum requirements for a functional response to hormonal stimulation. Cell Growth Differ. 2: 549-559.

Su, F; Liu, X; Liu, GH; Yu, Y; Wang, YS; Jin, YP; Hu, GD; Song, H and Zhang, Y (2013). Establishment and evaluation of a stable cattle type II Alveolar epithelial cell line. PLoS One. 8: e76036.

Sun, Y; Lin, C and Chou, Y (2006). Establishment and characterization of a spontaneously immortalized porcine mammary epithelial cell line. Cell Biol. Int., 30: 970-976.

Taylor-Papadimitriou, J; Shearer, M and Tilly, R (1992). Some properties of cells cultured from early-lactation human milk. J. Natl. Cancer I. 58: 1563-1571.

Taylor-Papadimitriou, J; Stampfer, M; Bartek, J; Lewis, A; Boshell, M; Lane, EB and Leigh, IM (1989). Keratin expression in human mammary epithelial cells cultured from normal and malignant tissue: relation to *in vivo* phenotypes and influence of medium. J. Cell Sci., 94: 403-413.

Tong, HL; Li, QZ; Gao, XJ and Yin, DY (2012). Establishment and characterization of a lactating dairy goat mammary gland cell line. *In vitro* Cell Dev. Biol. Anim., 48: 149-155.

Xu, W; Chen, Q; Jia, Y; Deng, J; Jiang, S; Qin, Q; Wang, X; Yang, X and Jiang, H (2019). Isolation, characterzation, and SREBP1 functional analysis of mammary epithelial cell in buffalo. J. Food Biochem., 43: e12997.

Zhang, H; Liu, T; Li, B; Zhang, K; Wang, D; Liu, Y; Ge, L; Jiang, Y and Su, F (2020). Establishment of a stable β-casein protein-secreted Laoshan dairy goat mammary epithelial cell line. Front Vet. Sci., 13: 501.

Zhao, K; Liu, HY; Zhou, MM and Liu, JX (2010). Establishment and characterization of a lactating bovine mammary epithelial cell model for the study of milk synthesis. Cell Biol. Int., 34: 717-721.

Zheng, YM; He, XY and Zhang, Y (2010). Characteristics and EGFP expression of goat mammary gland epithelial cells. Reprod. Domest. Anim., 45: e323-331.

Zhong, K; Wang, YL and Yang, GY (2006). Research progress of somatic cells in breast secretions. China Anim. Hus. Vet. Med., 12: 39-42.