Bulgarian Journal of Veterinary Medicine, 2021, **24**, No 1, 97–107
ISSN 1311-1477; DOI: 10.15547/bjvm.2269

Original article

CULTURING OF PRIMARY BOVINE MAMMARY EPITHELIAL CELLS AND VALIDATION OF BIOTRANSFORMATION CAPACITY IN EXPERIMENTS WITH ENROFLOXACIN

E. VACHKOVA[1], N. VASILEV[2], N. GRIGOROVA[1] & A. MILANOVA[1]

[1]Department of Pharmacology, Physiology of Animals and Physiological Chemistry,
[2]Department of Obstetrics, Reproduction and Reproductive Disorders;
Faculty of Veterinary Medicine, Trakia University, Stara Zagora, Bulgaria

Summary

Vachkova, E., N. Vasilev, N. Grigorova & A. Milanova, 2021. Culturing of primary bovine mammary epithelial cells and validation of biotransformation capacity in experiments with enrofloxacin. *Bulg. J. Vet. Med.*, **24**, No 1, 97–107.

Many drugs and toxic compounds are subjected to disposition and metabolism in bovine mammary epithelial cells (bMECs). For rapid investigation of different compounds and their possible interactions, validated *in vitro* models are needed. Therefore, the first objective of described experiments was to develop the techniques for cell isolation, purification and culturing of bMECs. The second objective was the application of these cell cultures in a well-known substrate for one of the major biotransformation enzymes in epithelial cells. To this end, the metabolism of enrofloxacin (ENR) into its active metabolite ciprofloxacin (CPR), was studied. This conversion is known to be catalysed by enzymes of the cytochrome P4501A and P4503A family. The expression profile of these enzymes shows a close correlation with cellular ABC-efflux transporters. Primary bMECs were isolated from healthy udders of lactating cows (n=5 animals). mRNA levels of α-casein, β-lactoferrin and cyclophilin B were determined as markers of cell identity of purity of the cultures. Subsequently, bMECs cultures were incubated with ENR (10 μM). Concentrations of ENR and its main metabolite CPR in the medium and in the cells were determined by HPLC-FL analysis. Gene expression of CYP1A1, CYP1A2 and CYP3A4, bovine ABCG2 was detected by qRT-PCR. Results showed that ENR penetrated into bMECs and was converted to CPR. CPR was excreted in the medium suggesting participation of ABCG2 in fluoroquinolone efflux. In conclusion, the data showed that the established bMEC cultures expressed major CYP450 enzymes as well as the most relevant efflux transport ABGG2. This model should be further validated and can serve as an interesting model for further studies on site-specific drug/toxin metabolism and transport in the bovine mammary gland.

Key words: ABCB2, biotransformation, enrofloxacin, primary bovine mammary epithelial cells

INTRODUCTION

The understanding of specific functions of bovine mammary gland is essential for optimising milk production and application of proper care for dairy cows. In-

tramammary drug formulations are often applied in treatment of mastitis and the mammary epithelium is the first effector site. Moreover, the excretion of parenterally applied drugs and undesirable substances/toxins ingested with feed into dairy milk is of public health concern, particularly regarding the exposure of children consuming often relatively high amount of milk, compared to their body weight (Martinez *et al.*, 2018).

Mammary epithelial cells (MECs) form a single cell layer in the alveoli and are involved in milk production and secretion. *In vitro* models of homogenous culture from primary MECs are considered as optimal tools for investigating their multiple functions. At the same time, it is known that some features of primary cells can change with increasing the number of passages and according to the culture medium (Jedrzejczak & Szatkowska, 2014; Muroya *et al.*, 2016), and early passages of primary cells are preferred. A typical marker of the functionality of such primary cells in culture is the mRNA expression levels of caseins, as indicator of MEC differentiation. Moreover, the expression of β-lactoferrin is a suitable marker to verify the purity and functionality of bovine MECs (bMECs) under different culture conditions, culture substrates (plastic and collagen) and cell passages (Wellnitz & Kerr, 2004; Lopez, 2011; Stark *et al.*, 2013).

MECs become competent and ready to respond to lactogenic hormones in the presence of insulin and epithelial growth factor. Insulin increases prolactin-stimulated β-casein mRNA transcription (Shan *et al.*, 2008). Hence these hormones are often added as supplements to primary cell cultures of bMECs. However, some of these supplements can affect the function of ABC transporter proteins and the expression and activity of cytochrome P450 (CYP450) enzymes and for this reason, are commonly avoided in experimental protocols studying drug and toxin metabolism and transport.

Enrofloxacin, a widely used antibacterial drug in veterinary medicine, is metabolised to ciprofloxacin in dairy cows by CYP450 mediated oxidative N-dealkylation catalysed predominantly by CYP450-1A and CYP450-3A enzymes (Idowu *et al.,* 2010). BCRP, an ABC transporter encoded by ABCG2 gene, is expressed at high mRNA level in blood-milk barrier and it is responsible for secretion of many xenobiotics into the milk (Merino *et al.*, 2006). This effect has been also confirmed for the excretion of enrofloxacin across the blood-milk barrier into milk of dairy cows (Wassermann *et al.*, 2013). However, the available information on the expression and catalytic activity of biotransformation enzymes and BCRP in primary bMEC is still very limited.

Therefore, the current study aimed to detail the techniques for isolation and maintenance of homogenous primary culture from bMECs at early passage in a cell culture medium not specifically supplemented with galactogenic hormones. The functionality of these cultures was demonstrated by measuring the metabolism and transport of enrofloxacin in primary bMECs.

MATERIALS AND METHODS

Tissue samples from bovine mammary gland and isolation of primary MEC

Tissue samples from the mammary gland were obtained from five lactating Holstein Friesian cows in late lactation, which were intended to be slaughtered. Microbiological tests performed few days before tissue collection showed that the milk samples

from all four quarters were culture-negative. Samples from mammary gland tissue were obtained from each cow within 30 min after stunning and were transported to the lab within an hour in 50 mL RPMI 1640 medium supplemented with 100 µg/mL penicillin G, 100 µg/mL streptomycin at 20 °C. All subsequent steps were performed under sterile laboratory conditions. All reagents and culture medium were obtained from Sigma (Sigma-Aldrich, FOT Ltd., Sofia, Bulgaria). The tissue samples were washed twice in sterile PBS and cut in small pieces to less than 1– 5 mm^3. Tissue digestion was achieved by incubation in DMEM F12 (5 mL), supplemented with 100 µg/mL penicillin G, 100 µg/mL streptomycin and 600 µL of 0.1% solution of collagenase for 3 h at 37 °C during which the cell suspension was well shaken every 30 min. Cells were separated by centrifugation at 300×g for 10 min. Thereafter cells were washed twice, transferred into T25 flasks and maintained at 37 °C and 5% CO_2. The cells were incubated in DMEM F12 medium supplemented with 100 µg/mL penicillin G, 100 µg/mL streptomycin, 10% FBS and 1% ITS Liquid Media Supplement. The ITS reagent contains recombinant human insulin, human transferrin, and sodium selenite and was added in order to promote structural and biochemical properties of the mammary epithelial cells.

The cells were maintained under humidified conditions at 37 °C and 5% CO_2 till confluence. The culture medium was changed every third day.

Removal of fibroblasts and cell culturing

The cells were grown until reaching a confluence of 80–90% in DMEM F12 medium as described above. A protocol for removal of fibroblasts was applied (Lopez, 2011; Anand *et al.*, 2012) as followed: 0.025 % trypsin was added to the confluent monolayer and allowed to act for 2–3 minutes. Cell were washed and the removed fibroblasts were seeded in DMEM containing 10% FBS, 100 µg/mL penicillin G, 100 µg/mL streptomycin and 100 µg/mL L-glutamine in order to stabilise and to use those cultures as negative controls. The rest of the cells were treated with trypsin for additional 4–5 min. The reaction was stopped by adding RPMI containing 10% FBS and 100 µg/mL penicillin G and 100 µg/mL streptomycin. The detached cells were centrifuged at 4000 g for 5 min at 25 °C. The supernatant was removed, the pellet of cells was re-suspended in culture medium for bMECs and seeded in T25 flasks.

qRT-PCR analysis

Total RNA was extracted by using Tri Reagent (Genaxxon Bioscience GmbH, Germany). Tri Reagent was added to individual bMECs cultured and further steps of isolation were performed according to manufacturer's instructions. RNA concentrations were measured with spectrophotometer at A260/280 and stored at – 70 °C until reverse transcription. Single-stranded cDNAs were synthesised from 3 µg total RNA using the ReversedAid First Strand cDNA Synthesis Kit (Thermo Scientific, SGP Bio Dynamics Ltd., Sofia, Bulgaria) on a Quanta Biotech QB-96 (Quanta Biotech Ltd., Surrey, UK). The reaction mixture (20 µL) was incubated for 60 min at 42 ^{o}C, then the enzyme was heat inactivated at 70 ^{o}C for 5 min and the mixture was rapidly cooled to 4 ^{o}C. The synthetised cDNA was stored at –70 ^{o}C.

Specific primers for β-lactoferrin, α-casein, CYP1A1, CYP1A2, CYP3A4, ABCG2 and c-cyclophilin B as reference gene were used for qRT-PCR (Table 1).

Table 1. Specific bovine gene primers used in the study

Gene	NCBI number	Reversed 5`→ 3` Forward 5`→ 3`	Ta ^{o}C
β-lactoferrin[1]	L_08604	R: ATTTAGCCACAGCTCCCTGGAG F: GGCCTTTGCCTTGGAATGTATC	60
α-casein[2]	NM_181029	R: TCAGAGCCAATGGGATTAGG F: AATCCATGCCCAACAGAAAG	60
CYP1A1[3]	XM_588298	R: CCGGATGTGACCCTTCTCAA F: GACCTGAATCAGAGGTTCTACGTCT	60
CYP1A2[3]	XM_591450	R: CAATGGTGGTGCCATCAGAC F: ACCATGACCCGAAGCTGT	60
CYP3A4[3]	NM_174531	R: GCAGGTAGACGTAAGGATTTATGCT F: GCCAGAGCCCGAGGAGTT	60
ABCG2[4]	NM_001037478.2	R: GGATCCTTCCTTGCAGCTAA F: GGAGTCATGAAACCTGGCC	60
c-cyclophilin B[1]	NM_174152.2	R: GATGCTCTTTCCTCCAGTGC F: GGTCATCGGTCTCTTTGGAA	60

[1]Stark *et al.* (2013); [2] Zhou (2007); [3] Giantin *et al.* (2009); [4]Boutinaud *et al.* (2013).

The SYBR Green method was applied for the real-time PCR analysis (QuantiNova SYBR Green PCR Kit, Qiagen, SGP Bio Dynamics Ltd., Sofia, Bulgaria). qRT-PCR reaction was performed on Exicycler™ 96 Real-Time Quantitative Thermal Block and the data were analysed with Exicycler3 V 3.55.0 (Bioneer, Republic of Korea). Each reaction went through a PCR cycle with a denaturation step at 95 ^{o}C for 20 s, and annealing at 60 ^{o}C for 30 s. Gene expression values were normalised to c-cyclophilin B mRNA and are expressed as relative values (Livak & Schmittgen, 2001). The analysis was performed in duplicate for each original culture from an individual cow.

Model experiments with enrofloxacin

Primary bMECs were subjected to treatment at the sixth passage. They were seeded with a density of 1.2×10^5 cells/ well in 24-well plates. The cells in each well were supplemented with culture medium for bMECs and were kept at cell culture conditions as described above. The experiment was performed when >80% confluence of the monolayer was observed, which was equal to 2.9×10^5 cells in a well. At the day of the experiment the cells were washed once with PBS. Then the control wells with bMECs were incubated with DMEM F12 only, without any supplements. Enrofloxacin hydrochloride was added to DMEM F12 medium at a concentration of 10 μM, equivalent to 3.96 μg/mL. The bMECs were incubated at 37 °C in a 5% CO_2 humidified atmosphere for 0, 0.5, 1, 1.5, 2, 2.5 and 3 h. At each time interval the medium was removed and saved for further HPLC analysis of fluoroquinolone concentrations. Finally, bMECs were washed with PBS and were lysed by addition of ice cold deionised water and harvested. These samples were stored at – 70 ^{o}C. The protein content of bMECs was determined by the Bradford method according to the manufacturers' instructions (Sigma-Aldrich, FOT Ltd., Sofia, Bulgaria). The ex-

periments were done in duplicate with bMECs from each individual cow (n=5).

HPLC analysis of enrofloxacin and its metabolite ciprofloxacin

Reagents and enrofloxacin hydrochloride were of the highest purity (HPLC grade, Sigma-Aldrich, FOT LTD, Bulgaria). Concentrations of enrofloxacin and its main metabolite ciprofloxacin were analysed by high-performance liquid chromatography (HPLC system, Thermo Fisher Scientific Inc., Waltham, MA, USA) coupled with fluorescence detector (Haritova *et al.*, 2012). The standard solutions of fluoroquinolones (enrofloxacin and ciprofloxacin) were prepared in DMEM F12 and in lysate of bMECs cultured in DMEM F12. LOD for ciprofloxacin and enrofloxacin were 10 ng/mL and 3 ng/mL, respectively. LOQ values were 30 ng/mL and 10 ng/mL, respectively.

Statistical analysis

Data are presented as mean±SD. Test for normal distribution of the data was performed. Expression levels of β-lactoferrin and α-casein mRNAs were compared by t-test. P value < 0.05 was considered as significant (Statistica 10.0 for Windows, StatSoft Inc., USA).

RESULTS

The isolated primary bovine mammary cells were heterogeneous at the first passage. The culture consisted of both epithelial and fibroblast cells. After selective purification, homogenous cultures of bMECs were obtained at the third passage. Fig. 1 shows the typical cobble stone-like cell colonies forming a monolayer after 4–5 days of culturing.

The sixth passage was chosen as the most suitable for further experiments based on the microscopic appearance of the homogenous culture and expression levels of genes that serve as indicator of MEC differentiation. β-lactoferrin mRNA was found at higher levels of expression if compared to α-casein mRNA ($P<0.05$) in bMECs at the sixth passage (Fig. 2). They

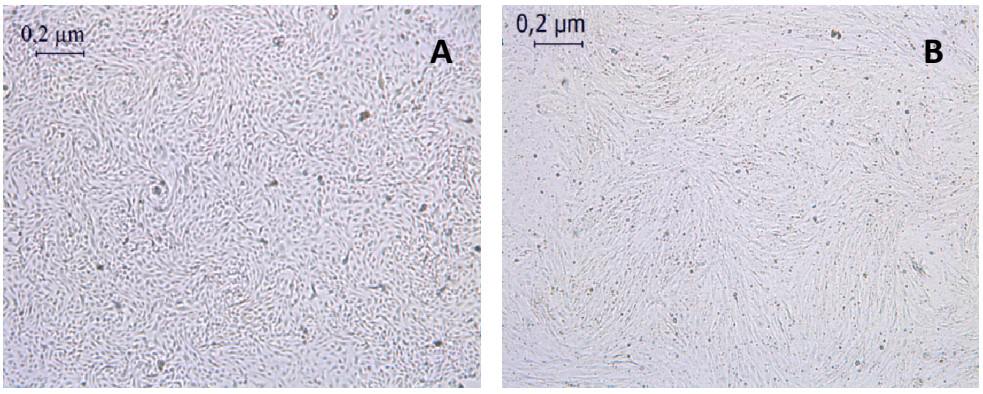

Fig. 1. A: Homogenous primary bMECs culture at third passage after removal of fibroblasts. B: Fibroblast cells culture at third passage derived from primary bMECs culture during procedure of removal of fibroblasts from heterogeneous cell suspension at early passages. Magnification 4×.

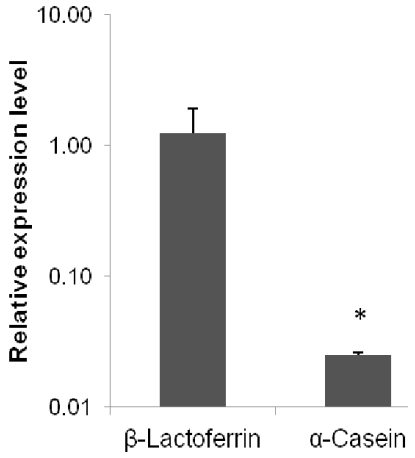

Fig. 2. Relative expression level (mean±SD) of β-lactoferrin mRNA and α-casein mRNA, in bMEC at sixth passage (n=5). c-cyclophilin B mRNA was used as reference gene. Low levels of β-lactoferrin and α-casein mRNAs were found in fibroblasts culture at second passage and were not detected at sixth passage.

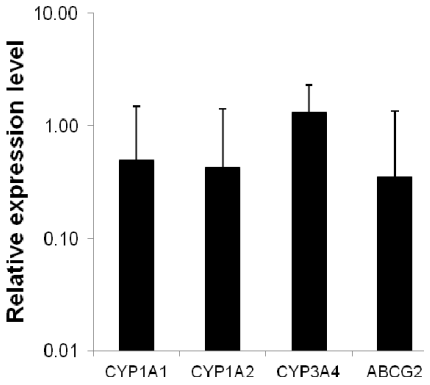

Fig. 3. Relative expression level (mean±SD) of CYP1A1 mRNA, CYP1A2 mRNA, CYP3A4 mRNA and ABCG2 mRNA in bMEC at sixth passage (n=5). c-cyclophilin B mRNA was used as reference gene.

were not found at detectable levels in fibroblast culture at the sixth passage but were detectable at the second passage. bMECs from all five cows expressed CYP1A1, CYP1A2 and CYP3A4 mRNAs and ABCG2 mRNA (Fig. 3).

Cellular concentrations of ENR in the bMECs derived from different cows showed large variations. In the group treated with ENR alone, the highest intracellular concentrations (1.33±0.96 µg/mg protein) were measured at 0.5 h of incubation. They were stable and close to 1 µg/mg protein thereafter (Fig. 4A). Ciprofloxacin, the main metabolite of ENR, was found in bMECs (0.45±0.36 - 0.34±0.11 µg/mg protein, Fig. 4A) and in the medium (0.14±0.14 µg/mL – 0.09±0.03 µg/mL) during the experiment (Fig. 4B).

DISCUSSION

The experimental protocol described here allowed the successful cultivation of primary bMECs from five different bovine mammary gland samples. After preculturing and removal of fibroblast by differential trypsinisation steps, a homogenous culture with the typical cobblestone epithelial cells was obtained. This is in line with previous studies using essentially the same method (Wellnitz & Kerr, 2004) and other studies describing the culturing of bMECs (Lopez, 2011). The current investigation confirmed also that primary bMECs from all cows (n=5) expressed highly specific genes for mammary epithelium such as β-lactoferrin and α-casein (Zhou, 2007; Stark *et al.*, 2013) at the sixth passage. However, a down regulation of caseins mRNA with increasing number of passages has been described in bMECs by Jedrzejczak & Szatkowska (2014). This may partly explain the relatively low levels of α-casein mRNA observed at sixth passage in our study. β-lactoferrin mRNA was found to be expressed at stable high levels in bMECs at different culture condition (Stark *et al.*,

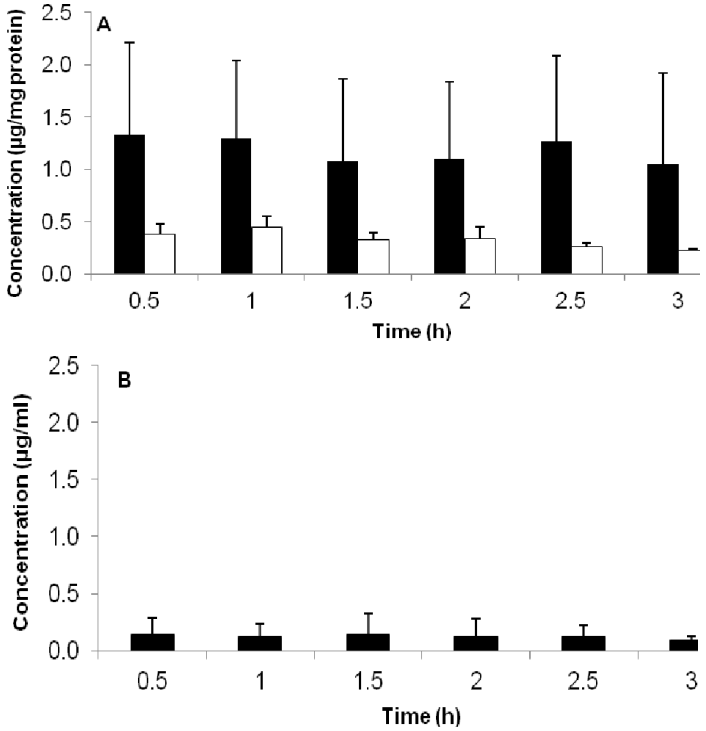

Fig. 4. A: Cellular concentration of enrofloxacin (■) and its metabolite ciprofloxacin (□) in µg/mg protein in bMECs after incubation with 10 µM enrofloxacin. B: concentration of metabolite ciprofloxacin in the medium (µg/mL, extracellular concentration) excreted from bMECs treated with enrofloxacin. The experiments were performed in cell cultures from different cows, n=5 and conducted in duplicate. Data are presented as mean±SD.

2013).

While the principle isolation and culture technique of bMECs had been described before, these experiments did not describe the expression of biotransformation enzymes or drug transporters. As the mammary gland is very often subjected to local administration of drugs and the blood-milk barrier regulates drugs excretion into the milk after injectable treatment of dairy animals, the analysis of the expression of such enzymes was the second major objective of this study. Indeed, we could show that the major CYP450 classes were expressed in our bMEC cultures, even though only ITS was used as a supplement in the cell culture medium.

Studying biotransformation and drug transport in *in vitro* models with bMECs require a re-thinking of culture conditions. Common supplements with a lactogenic potential such as corticosteroids and estrogens, which are often used in bMEC experiments, can modify the activity of metabolising enzymes (Martignoni *et al.*, 2004; Cantiello *et al.*, 2009). For example, different expression levels of ABCG2 mRNA were found in undifferentiated and differentiated (prolactin and hydrocortisone stimulated) mouse HC11 MEC line (Shan *et al.*, 2008). Although bovine MEC line BME-UV did not show signifi-

cant changes after prolactin treatment (Yagdiran, 2015), absence of ABCG2 mRNA was explained by loss of transcription factors, selection of cells during passages and culturing conditions (Monzani *et al.*, 2011; Yagdiran, 2015).

Primary bMECs at sixth passage expressed CYP1A1, CYP1A2 and ABCG2 mRNAs, genes that encode important enzymes from CYP450 family which are involved in metabolism of many drugs, including fluoroquinolones (Vaccaro *et al.*, 2003). Recently, CYP1A1 mRNA expression was described in bMECs in experiments with dioxins (Girolami *et al.*, 2018). Our results are in line with the cited studies. To confirm the functionality of the expressed CYP450 enzymes, a fluoroquinolone – enrofloxacin, was selected as a model substrate. Enrofloxacin is used in veterinary medicine among other indications in the treatment of mammary infections due to its wide tissue distribution and penetration into the intracellular space (Vallet *et al.*, 2011). The concentration of ENR used for experiments with bMECs was chosen on the basis of the levels in milk found after treatment of lactating cows with conventional doses (Kaartinen *et al.*, 1995). Similarly to investigations with white blood cells (Michot *et al.*, 2005), intracellular fluoroquinolone levels were demonstrated also in bMECs already after 30 minutes, and remained constant in bMECs during several hours of incubation. The demonstrated high intracellular concentrations of enrofloxacin are in line with the observed clinical efficacy of intramammary drug dosage forms of enrofloxacin (Viveros *et al.*, 2018). Presence of CYP450 enzymes is a prerequisite for enrofloxacin metabolism to ciprofloxacin in bMECs. ENR undergoes oxidative N-dealkylation to active metabolite ciprofloxacin in many animal species, including dairy cows (Idowu *et al.*, 2010). Experiments with primary bMECs showed that they are able to convert the parent drug to ciprofloxacin, which can be attributed to the activity of investigated enzymes.

Previous studies revealed that ENR is subjected to ATP dependent efflux into the milk of ruminants by BCRP, encoded by ABCG2 gene (Pullido *et al.*, 2006). Studies with MDCK-II cell line (Merino *et al.*, 2006) and bovine ABCG2–expressing MDCKII cells (Wassermann *et al.*, 2013; Machnke *et al.*, 2016) proved that ENR and ciprofloxacin are substrates for ABCG2. Altogether *in vitro* studies and previous pharmacokinetic investigations conducted by us or other research groups show that the protein product of ABCG2 can play significant role in efflux of fluoroquinolone drugs from MECs into the milk. Our results suggest that ciprofloxacin might be a substrate for BCRP as well and therefore this efflux transporter encoded by ABCG2 is most likely involved in its secretion into milk (Merino *et al.*, 2006).

In conclusion, primary bMECs cultured with ITS remain differentiated during six passages after their isolation and express typical lactogenic genes as demonstrated by mRNA levels of casein and lactoferrin. More importantly, these cells also expressed CYP450 1A1, 1A2, and 3A as well as ABCG2 mRNAs, and actively biotransformed and excreted enrofloxacin and its metabolite ciprofloxacin in the model study. Therefore this *in vitro* model with primary bMECs cultures can be deemed a valid model suitable for further studies with drugs and toxins relevant to bovine therapy and milk safety.

ACKNOWLEDGEMENTS

The research was financially supported by Trakia University, Stara Zagora, Bulgaria (Grant No. 04/7).

REFERENCES

Anand, V., N. Dogra, S. Singh, S. N. Kumar, M. K. Jena, D. Malakar, A. K. Dang, B. P. Mishra, T. K. Mukhopadhyay, J. K. Kaushik & A. K. Mohanty, 2012. Establishment and characterization of a Buffalo (*Bubalus bubalis*) mammary epithelial cell line. *PLoS One*, **7**, e40469.

Boutinaud, M., L. Galio, V. Lollivier, L. Finot, S. Wiart, D. Esquerré & E. Devinoy, 2013. Unilateral once daily milking locally induces differential gene expression in both mammary tissue and milk epithelial cells revealing mammary remodeling. *Physiological Genomics*, **45**, 973–985.

Cantiello, M., M. Giantin, M. Carletti, R.M. Lopparelli, F. Capolongo, F. Lasserre, E. Bollo, C. Nebbia, P.G. Martin, T. Pineau & M. Dacasto, 2009. Effects of dexamethasone, administered for growth promoting purposes, upon the hepatic cytochrome P450 3A expression in the veal calf. *Biochemical Pharmacology*, **77**, 451–463.

Giantin, M., M. Carletti, F. Capolongo, S. Pegolo, R.M. Lopparelli, F. Gusson, C. Nebbia, M. Cantiello, P. Martin, T. Pineau, & M. Dacasto, 2008. Effect of breed upon cytochromes P450 and phase II enzyme expression in cattle liver. *Drug Metabolism and Disposition*, **36**, 885–893.

Girolami, F., S. Ghadiri, E. Martignani, V. Spalenza, P. Badino & C. Nebbia, 2018. Protection against AFB1 cytotoxicity by natural antioxidants in primary cultures of bovine mammary gland. In: *Proceedings of 14th International Congress of the European Association for Veterinary Pharmacology and Toxicology*, Wroclaw, Poland, June 24–27, 2018. *Journal of Veterinary Pharmacology and Therapeutics*, **41** (Suppl. 1), 52–53.

Haritova, A., D. Petrova & S. Stanilova, 2012. A simple HPLC method for detection of fluoroquinolones in serum of avian species. *Journal of Liquid Chromatography & Related Technology*, **35**, 1130–1139.

Idowu, O. R., J. O. Peggins, R. Cullison & J. von Bredow, 2010. Comparative pharmacokinetics of enrofloxacin and ciprofloxacin in lactating dairy cows and beef steers following intravenous administration of enrofloxacin. *Research in Veterinary Science*, **89**, 230–235.

Jedrzejczak, M. & I. Szatkowska, 2014. Bovine mammary epithelial cell cultures for the study of mammary gland functions. *In Vitro Cellular & Developmental Biology – Animal*, **50**, 389–398.

Kaartinen, L., M. Salonen, L. Alli & S. Pyorala, 1995. Pharmacokinetics of enrofloxacin after single intravenous, intramuscular and subcutaneous injections in lactating cows. *Journal of Veterinary Pharmacology and Therapeutics*, **18**, 357–362.

Livak, K. J. & T. D. Schmittgen, 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-ΔΔC(T)) method. *Methods*, **25**, 402–408.

Lopez, A. C., 2011. Characterization of differentiation of bovine mammary epithelial cells. Honors Scholar Theses. 195, University of Connecticut - Storrs, USA.

Mahnke, H., M. Ballent, S. Baumann, F. Imperiale, M. von Bergen, C. Lanusse, A.L. Lifschitz, W. Honscha & S. Halwachs, 2016. The ABCG2 efflux transporter in the mammary gland mediates veterinary drug secretion across the blood-milk barrier into milk of dairy cows. *Drug Metabolism and Disposition*, **44**, 700–708.

Martignoni, M., R. de Kanter, P. Grossi, A. Mahnke, G. Saturno & M. Monshouwer, 2004. An in vivo and in vitro comparison of CYP induction in rat liver and intestine using slices and quantitative RT-PCR. *Chemico-Biological Interactions*, **151**, 1–11.

Martinez, M. N., M. H. Court, J. Fink-Gremmels & K. L. Mealey, 2018. Population

variability in animal health: Influence on dose-exposure-response relationships: Part I: Drug metabolism and transporter systems. *Journal of Veterinary Pharmacology and Therapeutics*, **41**, E57–E67.

Merino, G., A. I. Alvarez, M. M. Pulido, A. J. Molina, A. H. Schinkel & J. G. Prieto, 2006. Breast cancer resistance protein (BCRP/ABCG2) transports fluoroquinolone antibiotics and affects their oral availability, pharmacokinetics, and milk secretion. *Drug Metabolism and Disposition*, **34**, 690–695.

Michot, J. M., C. Seral, F. Van Bambeke, M. P. Mingeot-Leclercq & P. M. Tulkens, 2005. Influence of efflux transporters on the accumulation and efflux of four quinolones (ciprofloxacin, levofloxacin, garenoxacin, and moxifloxacin) in J774 macrophages. *Antimicrobial Agents and Chemotherapy*, **49**, 2429–2437.

Monzani, P. S., F. F. Bressan, L. G. Mesquita, J. R. Sangalli & F. V. Meirelles, 2011. Beta-casein gene expression by in vitro cultured bovine mammary epithelial cells derived from developing mammary glands. *Genetics and Molecular Research*, **10**, 604–614.

Muroya, S., T. Hagi, A. Kimura, H. Aso, M. Matsuzaki & M. Nomura, 2016. Lactogenic hormones alter cellular and extracellular microRNA expression in bovine mammary epithelial cell culture. *Journal of Animal Science and Biotechnology,* **7**, 8.

Pulido, M. M., A. J. Molina, G. Merino, G. Mendoza, J. G. Prieto & A. I. Alvarez, 2006. Interaction of enrofloxacin with breast cancer resistance protein (BCRP/ ABCG2): influence of flavonoids and role in milk secretion in sheep. *Journal of Veterinary Pharmacology and Therapeutics*, **29**, 279–287.

Shan, L., R. Zhang, W. Zhang, E. Lee, R. Sridhar, E. G. Snyderwine & P. C. Wang, 2008. Image-based evaluation of the molecular events underlying HC11 mammary epithelial cell differentiation. *Analytical Biochemistry*, **382**, 122–128.

Stark, A., E. Vachkova, O. Wellnitz, R. Bruckmaier & C. Baumrucker, 2013. Colostrogenesis: candidate genes for IgG1 transcytosis mechanisms in primary bovine mammary epithelial cells. *Journal of Animal Physiology and Animal Nutrition*, **97**, 1114–1124.

Vaccaro, E., M. Giorgi, V. Longo, G. Mengozzi & P.G. Gervasi, 2003. Inhibition of cytochrome p450 enzymes by enrofloxacin in the sea bass (*Dicentrarchus labrax*). *Aquatic Toxicology*, **62**, 27–33.

Vallet, C. M., B. Marquez, E. Ngabirano, S. Lemaire, M.-P. Mingeot-Leclercq, P. M. Tulkens & F. Van Bambeke, 2011. Cellular accumulation of fluoroquinolones is not predictive of their intracellular activity: Studies with gemifloxacin, moxifloxacin and ciprofloxacin in a pharmacokinetic/pharmacodynamic model of uninfected and infected macrophages. *International Journal of Antimicrobial Agents*, **38**, 249–256.

Viveros, M., R. Lopez-Ordaz, L. Gutiérrez, J.E. Miranda-Calderón & H. Sumano, 2018. Efficacy assessment of an intramammary treatment with a new recrystallized enrofloxacin vs ceftiofur and parenteral enrofloxacin in dairy cows with nonsevere clinical mastitis. *Journal of Veterinary Pharmacology and Therapeutics*, **41**, e1–e9.

Wassermann, L., S. Halwachs, D. Baumann, I. Schaefer, P. Seibel & W. Honscha, 2013. Assessment of ABCG2-mediated transport of xenobiotics across the blood-milk barrier of dairy animals using a new MDCKII in vitro model. *Archives in Toxicology*, **87**, 1671–1682.

Wellnitz, O. & D. E. Kerr, 2004. Cryopreserved bovine mammary cells to model epithelial response to infection. *Veterinary Immunology and Immunopathology*, **101**, 191–202.

Yagdiran, Y., 2015. Transport proteins in mammary epithelial cells. Thesis, Swedish University of Agricultural Sciences Uppsala, Sweden.

Zhou, Y., 2007. Effects of growth hormone and insulin-like growth factor-I on milk protein gene expression and nutrient uptake and cell proliferation in clonal bovine mammary epithelial cells. PhD thesis, Virginia Polytechnic Institute and State University, Blacksburg, Virginia, USA.

Paper received 10.07.2019; accepted for publication 27.09.2019

Correspondence:

Aneliya Milanova
Department of Pharmacology, Physiology of Animals and Physiological Chemistry,
Faculty of Veterinary Medicine, Students' Campus, Trakia University,
6015 Stara Zagora, Bulgaria
tel.: +359 42 699 696
e-mail: akmilanova@gmail.com