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Antibiotics for Mastitis Negatively Affect *in vitro* Cultures of Bovine Mammary Epithelial Stem/Progenitor Cells

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Abstract: Antibiotics are commonly used to treat bovine mastitis, but their effect on bovine mammary stem/progenitor cells (MaSC) has never been studied so far. MaSC are the driving force for the remarkable regenerative capacity of the mammary gland and factors negatively influencing normal functioning of these cells, might have a negative impact. The aim of this study was therefore to study the *in vitro* effects of three antibiotics, commonly used to treat mastitis, on proper functioning of bovine MaSC (bMaSC) by evaluating the viability, the capacity to form colonies (colony forming unit assays or CFU) and the capacity to form mammospheres. Our salient findings showed that antibiotic treatment significantly reduced cell viability and impaired the CFU capacity of bMaSC. Moreover, we observed that antibiotic treatment inhibited mammosphere formation, especially at higher concentration. In conclusion, this is the first study to show that antibiotics which used for the treatment of mastitis may negatively affect functional characteristics of bMaSC. However, more research is needed for further substantiate these findings and future studies could include another drugs that are used for treating mastitis or other udder problems in cows.

Key words: Bovine · Mastitis · Treatment

INTRODUCTION

Bovine mastitis is defined as an inflammation of the mammary gland and it has an infectious or non-infectious etiology [1-3]. In general, mastitis is a complex disease dealing with the interaction of microorganisms and the cow's anatomy and physiology, dairy husbandry and management, milking equipment and procedures and environment [4]. The disease is characterized by physical, chemical and mainly bacteriological changes in milk, as well as pathological changes in glandular tissues of the udder and affects the quality and quantity of milk [5,6]. Bovine mastitis can cause considerable economical losses due to reduced milk production, treatment cost, increased labor, milk withheld following treatment and premature culling [7].

For more than fifty years, antibiotics have been used to treat infectious mastitis caused by bacteria [8], although it has become clear that some antibiotics can greatly influence proper functioning of immune cells [9]. For example, *in vitro* studies where antibiotics were added to culture medium, at a concentration which was equivalent to doses detected in milk immediately after an intramammary injection, showed significant alterations in the morphology of bovine mammary gland-isolated polymorphonuclear leukocytes (PMNL). In addition, it was reported that the viability of PMNL cultured with several antibiotics was significantly reduced if it is compared to control cells and that efficient phagocytosis was depressed [10,11]. This was confirmed by other studies, which also showed an immunosuppresive effect of antibiotics, used at *in vivo* relevant concentrations, on

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blood-derived PMNL [12,13]. The effect of commonly used antibiotics on the proper functioning of other important cell types present in the mammary gland, however, has never been studied so far.

Mammary gland stem/progenitor cells (MaSC) are suggested to give rise to all terminally differentiated cells of the mammary gland and as such, are indispensable for maintaining mammary gland homeostasis and for providing the enormous regenerative capacity during successive reproductive cycles [14]. Bovine MaSC (bMaSC) in particular, are being studied with great interest because these cells are considered interesting targets for manipulation to manage cell growth and tissue regeneration, thereby improving dairy cow productivity and udder health [15]. Consequently, it is important to study potential negative effects of products such as antibiotics, which are commonly used in dairy management practice, on proper functioning of bMaSC. Therefore, the goal of the current study was to study the effect of commonly used antibiotics on bMaSC with respect to cell viability, colony forming unit (CFU) and mammosphere formation. Briefly, it was found that antibiotic treatment significantly reduced cell viability and impaired the CFU capacity of bMaSC

MATERIALS AND METHODS

Collection of Mammary Gland Samples and Isolation of Putative Bovine Mammary Stem/progenitor Cells (bMaSC): Samples from slaughtered Holstein-Friesian dairy cows was used to obtain mammary gland tissue samples, conform the guidelines of the Ethical Committee of Veterinary Medicine, Ghent University. All cows (n=3) were apparently healthy and inspected for clinical signs of mastitis. About 5 cm^2 mammary gland tissue was collected next to the median line of the mammary gland compartment and transported to the laboratory in phosphate buffered saline (PBS), supplemented with 1% of penicillin/streptomycin/amphotericine (P/S/A, Sigma), at 4°C.

Isolation of Bovine Mammary Tissue: stem/progenitor cells (bMaSC) from these samples were performed as previously described, with some modifications [16]. Briefly, mammary gland samples were dissociated mechanically with a sterile scalpel, followed by enzymatic digestion with 0.1% collagenase III (Worthington Biochemical Corporation) at 37°C for 60 min. The resulting cell suspensions were sieved sequentially through sterile 100 μm and 40 μm filters to obtain a single cell suspension. Cells were washed twice in PBS with 1% P/S/A by centrifugation at 400 xg and 260 xg for 10 min at room temperature (RT), respectively. Cells were finally resuspended in MaSC medium, consisting of Dulbecco's modified Eagle's medium (DMEM)/F12 medium (50/50) supplemented with 5% fetal bovine serum (FBS, Invitrogen), 2% B27 (Invitrogen), 1% P/S/A, 10ng/mL basic-fibroblast growth factor (BioVision) and 10ng/mL epidermal growth factor (Sigma). Approximately 5 x 10^5 cells were seeded on a 6-well tissue culture dish for 1 h to allow adherence of contaminating fibroblasts and this was repeated once more. The non-adherent cells were collected and seeded at approximately 20,000 cells/cm^2 on 6-well ultralow attachment plates (Corning, Elscolab). Cells were incubated at 37°C and 5% CO_2 and MaSC medium was refreshed twice a week by means of centrifugation of non-adherent cell clusters, named mammospheres, at 230 xg for 6 min. For further experiments, mammospheres were seeded on adhesive tissue culture flasks and plates in MaSC medium and incubated at 37°C and 5% CO_2.

Immunophenotyping of Putative bMaSC: Putative bMaSC were immunophenotyped at passage 2 for expression levels of the mammary stem cell markers CD29, CD44, CD49f and Ki67. Antibodies (Abs) for these markers have been used previously by our laboratory to immunophenotype MaSC [16]. Cells were pelleted in aliquots containing approximately 2 x 10^5 cells per tube and labeled with the primary Abs mouse anti-human CD29-FITC IgG1 (Southern Biotech; clone TDM29, 1:10), rat anti-mouse CD44-APC IgG2b (BD; clone IM7, 1:20), rat anti-mouse CD49f IgG2a (Novus Biologicals; clone GoH3, 1:10) or rabbit anti-human Ki67 IgG (Abcam; ab15580, 1:200). For the latter, cells were fixed with 4% paraformaldehyde for 10 min and subsequently permeabilized with 0.1% Triton X for 2 min, both at RT. Cells were incubated with the primary Abs for 15 min on ice in the dark and washed twice in washing buffer, consisting of DMEM with 1% bovine serum albumin. For CD49f and Ki67, secondary goat anti-rat $Alexa^{488}$ and goat anti-rabbit $Alexa^{647}$-linked Abs (Invitrogen; 1:100), respectively, were used to identify positive cells after 15min of incubation on ice in the dark. Finally, all the cells were washed three times in washing buffer and in addition, viability assessment with 7-amino-actinomycin D (7-AAD, Sigma) was performed on the non-fixed cells. At least 10,000 cells were evaluated by using a FACS Canto flow cytometer (Becton Dickinson Immunocytometry systems) equipped with a 488nm solid state and a 633nm HeNe laser and data were further analyzed with the FACS Diva software.

Antibiotics: Three antibiotics, which are commonly used to treat mastitis, were included in this study which were Cefquinome (Cobactan®; Intervet) [17], penethamate hydriodide (Mammyzine®; Boehringer Ingelheim) [18,19] and marbofloxacin (Marbocyl®; Vetoquinol) [20]. All the drugs were diluted according to the manufacturer's recommendation and were used at a physiologically relevant concentration range (between 10µg/ml and 10000µg/ml). Final dilutions were made in MaSC medium and antibiotics were added 12-18 hours post seeding of the bMaSC on 6-well adherent culture plates (for viability and CFU assays) or on 6-well ultralow attachment plates (for mammosphere assays). All experiments were done in triplicate for the three different bMaSC samples.

Viability Assay: To evaluate viability, 1 x 10^3 bMaSC were seeded in 6-well culture plates using 3 ml of MaSC medium and between 12 to 18 hours later, the three antibiotics were added at their respective concentrations. After 7 days of culture, cells from each well were collected and cell viability was assessed by using the trypan blue exclusion (TBE) assay. The percentage viability was calculated as [1.00 - (number of trypan blue positive cells/number of total cells)] × 100.

Colony Forming Unit (CFU) Assay: For the colony forming unit (CFU) assay, 10 bMaSC were seeded in 6-well culture plates using 3 ml of MaSC medium and between 12 to 18 hours later, the different antibiotics were added at the different concentrations. After 7 days of culture, cells were fixed with 90% ethanol for 10 minutes at -20°C. Crystal violet stainings were performed to visualize CFUs macroscopically and the total number of CFUs/6-well was counted.

Mammosphere Assay: The same protocol was followed as described for the CFU assay, with the exception that ultralow attachment 6-well plates were used instead of adherent culture plates. The formation of mammospheres was recorded on day 4 and 8 post seeding by counting the number of mammospheres/6-well.

Statistical Analysis: The statistical analysis for viability was based on the mixed model using SAS Version 9.3 (SAS/STAT Software, Version 9.3, SAS Institute Inc.). First, antibiotics were compared with the control and amongst each other (averaging over the different doses) using a mixed model with cow as random effect and antibiotics and dose as categorical fixed effects. Then, a separate mixed model was fitted for each antibiotic with cow as random effect and dose as continuous fixed effect to assess the effect of the dose. The statistical analysis for number of CFU/well, a count variable, was based on the Poisson regression model using SAS Version 9.3. Finally, antibiotics were compared with the control and amongst each other (averaging over the different doses) using the Poisson regression model. Next, a separate Poisson regression model was fitted for each antibiotic using dose as continuous fixed effect to assess the effect of the dose. A global significance level of 5% was used and the significance level for multiple comparisons was adjusted by the Tukey's multiple comparisons technique.

RESULTS AND DISCUSSION

The objective of this study was to determine the effect of three antibiotics, commonly used for the treatment of bovine mastitis, on functional properties of bovine stem/progenitor cells of the mammary gland (bMaSC). For this purpose, bMaSC were isolated from mammary gland tissue from Holstein-Friesian slaughterhouse cows, using the protocol that was recently established in our lab to isolate MaSC from mares [16]. Immunophenotpying was then used to characterize the bMaSC and the cells were positive for the stem cells markers CD29, CD44, CD49f and Ki67 (Figure 1). The markers CD29 and CD49f are generally accepted as markers to identify bMaSC [21]. CD44 is a marker commonly used to immunophenotype human and murine MaSC [15] and was shown in this present study to also be expressed on bMaSC (Figure 1). The proliferation marker Ki67 is frequently used as an indicator for the self-renewal rate of MaSC and other highly proliferating (stem) cells [16]. Next, bovine MaSC were cultured in the presence or absence of antibiotics, commonly used to treat bovine mastitis, to study any potential negative effect of these drugs on MaSC growth and proliferation.

Effect of Antibiotics on the Viability of bMaSC: In the current study, it was found that all antibiotics had a significant negative impact on the viability of bMaSC compared to non-treated control cells (Table 1). Viable cells were no longer observed when treating bMaSC with (i) Marbocyl at concentrations 1000µg/ml and 10000µg/ml and (ii) Cobactan and Mammyzine at a concentration of 10000µg/ml (Figure 2). From the three antibiotics tested, cobactan showed the least detrimental effect on cell viability compared to the other two, although incubation of bMaSC with high doses of this antibiotic also resulted in a 100% cell death (Figure 2).

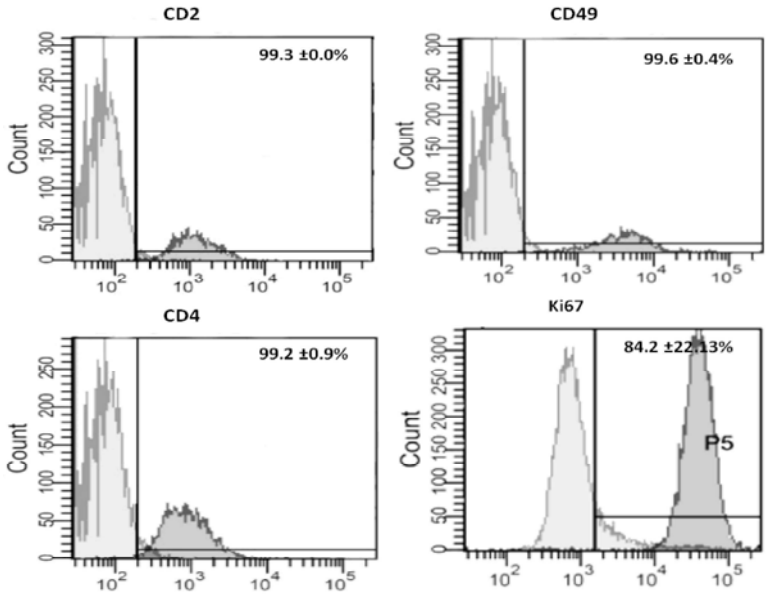

Fig. 1: Immunophenotypic characterization of bMaSC. Flow cytometry was performed using the markers CD29, CD44, CD49f and Ki67. Histograms show relative numbers of cells versus isotype control staining (light grey) and marker antibody staining (darker grey). Data represent the mean percentage of 3 experiments ± standard deviations.

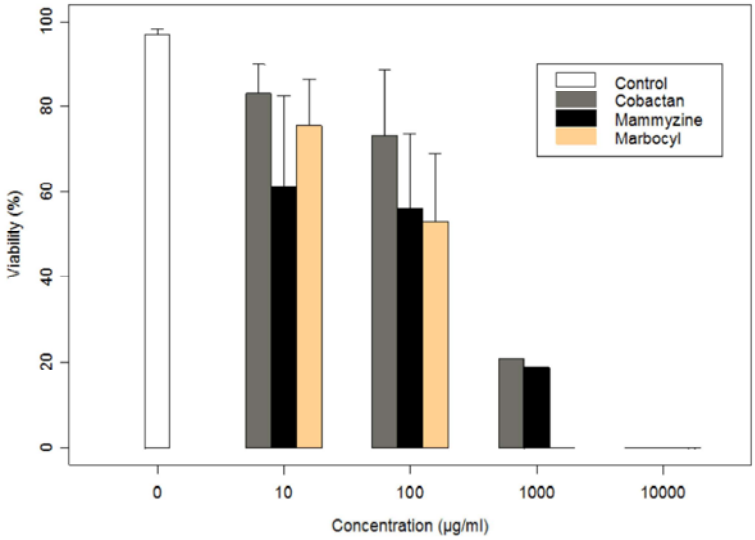

Fig. 2: Effect of antibiotics on bMaSC viability. The bMaSC were seeded on attachment plates and cultured with the three different antibiotics at different concentrations for 7 days. Viability is expressed as the mean and standard error of the percentage viable cells

Table 1: Descriptive statistics of the effects of antibiotic treatment (average of three concentrations: 10, 100 and 1000 µg/ml) on viability and CFU capability of bMaSC.

Treatment	Viability (standard error)	Number of CFU (standard error)
Control	97.2 % (3.2)[a]	4.8 (0.9)[a]
Cobactan	44.4 % (16.9)[b]	3.1 (0.4)[ab]
Mammayzine	32.2 % (27.8)[b]	3.6 (0.4)[ab]
Marbocyl	34.1 % (20.2)[b]	2.6 (0.4)[b]

a,b: Means within the same column sharing the same superscript do not differ significantly from each other.

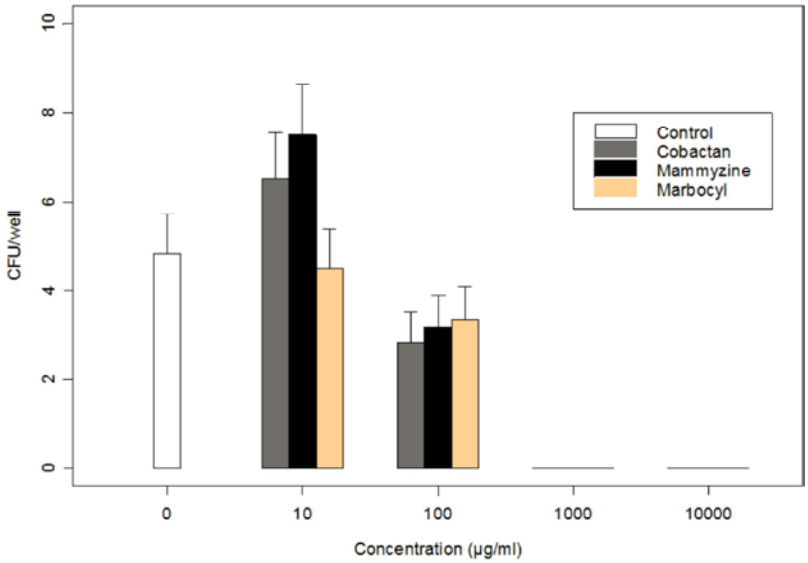

Fig. 3: Effect of antibiotics on the colony-forming unit (CFU) capability of bMaSC. The bMaSC were seeded at a concentration of 10 cells/well and cultured with the three different antibiotics at different concentrations for 7 days. The CFU are expressed as the mean number and standard error of colonies per 6 well culture plates.

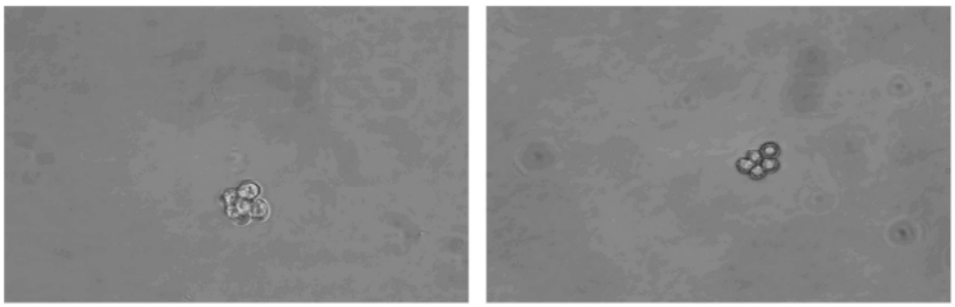

Fig. 4: Representative pictures of mammopsheres. Bovine MaSC, non-treated (A) or treated with antibiotics (B) were seeded on ultralow attachment plates and inspected for mammopshere formation. Magnification 40x.

Effect of Antibiotics on the Colony-forming Unit (CFU) Capability of bMaSC: Colony forming unit (CFU) assays are used to monitor and compare the clonogenic expansion of MaSC from different animal species [16,22,23]. For this purpose, a limited number of cells was seeded at clonal density on a large surface and after a cultivation period 7 days, colonies were counted. In general, antibiotic treatment resulted in a significantly reduced number of CFU compared to the control (Table 1), which was mainly due to the complete absence of colonies when bMaSC were cultured with antibiotics at a concentration of 1000µg/ml or higher (Figure 3).

Effect of Antibiotics on Mammosphere Formation of bMaSC: When seeding the bMaSC on non-adherent, ultralow attachment plates, mammosphere formation was observed in only one out of the three bMaSC cultures. These mammospheres typically consist of non-adherent spherical clusters of cells, which are a mixture of stem cells, their progeny and non-stem progenitor cells and are in general initiated by the stem cell population present, reflecting the self-renewal capacities of these primitive stem cells [22,24]. A potential explanation for the lack of mammosphere formation in the two other samples could indicate that the bMaSC present in these samples were already differentiating during culture on adherent plates, making it more difficult for these cells to initiate mammospheres when subsequently cultured on ultralow attachment plates. In the successful sample, mammopsheres were readily visible starting at 4 days post seeding in the non-treated group (Figure 4). Mammospheres were also visible, however, in the groups treated with 10 and 100 µg/ml and this for all three antibiotics tested (Figure 4). This indicates that antibiotic

treatment does not negatively influence the self-renewal capacity of bMaSC, at least not at these concentrations. On the other hand, mammospheres were no longer formed when bMaSC were treated with the antibiotics at a concentration of 1000 µg/ml and above.

CONCLUSIONS

Combining all the results obtained in the present study, we can conclude that antibiotics might indeed have a negative effect on the proper functioning of bMaSC and their more differentiated progeny. Even though these experiments were done *in vitro*, we tested a concentration range of the antibiotics that are relevant *in vivo*.

Competing Interests: The authors declare that there will be no potential conflicts of any interest.

Authors' Contributions: BB and LB were involved in conception and design, sample collection, laboratory analysis and manuscript writing. YG was involved in data analysis and interpretation. LD and GVdW were involved in conception and design, data analysis and interpretation and manuscript writing. All authors read and approved the final manuscript.

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