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Release of hydrogen peroxide by phagocytes from bovine colostrum in the *peripartum* period

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Abstract

Changes in the composition of colostrum in the *peripartum* period focused on the neonate's immunity can minimize the response of the mammary gland before natural exposure to mastitis pathogens. This research evaluated the oxidative activity of mononuclear and polymorphonuclear phagocytes of bovine colostrum. Bacteriologically negative (n = 171) bovine colostrum samples of first and second milking *postpartum* were evaluated. The oxidative activity was assessed by the production of hydrogen peroxide (H_2O_2) released either spontaneously or upon stimulation by enterotoxigenic *Escherichia coli* (ETEC). For this, three treatments were used: control (C) – only cells; non-opsonized (NO) - cells and ETEC non-opsonized; and opsonized (O) – cells and ETEC opsonized. Median values of concentrations (nmol/10^6) of H_2O_2 produced by cells obtained from the first and second milking treatments C, NO and O were 149.1 and 39.9 (P = 0.0007); 125.5 and 49.1 (P = 0.0007); 102.4 and 54.4 (P = 0.008), respectively. No differences were observed between the treatments at each milking. The amount of H_2O_2 produced at first milking exceeded the values found thereafter. Furthermore, the presence of bacteria did not determine the increased amount of H_2O_2 released by phagocytes. Thus, we concluded that the mammary gland's events during the *peripartum* period have an effect on the proportion and activity of phagocytes, which can cause injuries to the breast parenchyma by the large amount of free radicals produced. The high frequency of bovine mastitis during this period indicates the need for studies of the immunity of the mammary gland and research of susceptibility factors for bacterial infections.

Bovine colostrum, cell, bactericidal activity, cow, mammary secretion

Changes in the composition of colostrum in the *peripartum* period focused on the neonate's immunity can minimize the response of the mammary gland before its natural exposure to mastitis pathogens. The immune components of colostrum include viable cells, enzymes, immunoglobulins, and cytokines (Tóthová et al. 2007; Stelwagen et al. 2009).

The proportion of colostrum cell components varies with the stage of lactation. It is noteworthy that colostrum in the immediate *postpartum* period shows a large percentage of phagocytes, especially macrophages and epithelial cells (69.5 ± 17.4%), on the other hand, neutrophil population is very small (13.3 ± 19.4%) (Gomes et al. 2011a). These cells are involved in the non-specific immunity of the mammary gland, and release chemotactic factors and pro-inflammatory cytokines which are responsible for the recruitment of polymorphonuclear leukocytes (PMN) from the blood stream to the udder. Recognition and phagocytosis of the bacteria by PMN cells are made easier by the opsonization of the microorganism with immunoglobulins (IgG2 and IgM), and factors of the complement system (C3b). After phagocytosis, bacteria are destroyed by dependent and independent oxygen mechanisms (Paape et al. 2002, 2003).

Study of the peculiarities of the innate immune response in the immediate *postpartum* period is limited by the difficulty of isolating colostrum mononuclear leukocytes (Meganck et al. 2014). Thus, the knowledge available comes from studies that were carried out with

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phagocytes obtained from blood or milk. The high frequency of bovine mastitis during the colostrogenesis and immediate *postpartum* period (Hogan and Smith 2012) indicates the need for studies of the immunity of the mammary gland and research of susceptibility factors for bacterial infections. The main pathogens isolated from colostrum are coagulase-negative *Staphylococcus* and *Streptococcus agalactiae*. However, higher incidence of clinical mastitis in the *postpartum* period has been associated with infections caused by *Escherichia coli* (Dingwell et al. 2002; Rajala-Schultz et al. 2005; Molina et al. 2013).

The hypothesis of this research is that the disharmony between the proportions and cell types of the mammary gland during the *peripartum* compromise the innate defense mechanisms of tissue, such as phagocytosis, generation of free radicals, and bactericidal activity of phagocytes. Thus, the objective of this study was to evaluate the indirect bactericide activity of the mononuclear (MN) and PMN cells from bovine colostrum by spontaneous release of H_2O_2 or after stimulation with *Escherichia coli.*

Materials and Methods

Animals and samples

Unpaired bacteriologically negative samples of colostrum (n = 171 to each treatment) were obtained from udder quarters of cows and heifers that were separated from their calves immediately after parturition. These samples were collected in the beginning of the first and second milking, at most 12 h after delivery, with milking breaks at most 8 h. Prior to collection of the samples, the cows were examined according to the criteria established by Dirksen et al. (1993), obtaining the samples of colostrum only from mammary quarters free of clinical and subclinical mastitis. The udders were cleaned with a 2% water and hypochlorite solution; then they were dried, and teat orifices were disinfected with 70% ethanol.

We harvested three aliquots of each sample of whole colostrum, as follows: (1) the first aliquot (80 ml) was added in sterile plastic bottles containing 80 ml of phosphate buffered saline (PBS), (2) the second aliquot consisted of 20 ml of pure colostrum, and (3) the third aliquot (3 ml) was stored in sterile glass vials.

Obtaining of colostrum cells

To obtain and separate cells, the first aliquot was centrifuged at 600 × *g* for 15 min at 4 °C. The centrifugation step enabled the separation of colostrum into three distinct phases: cell pellet, an intermediate fluid portion, and a fat layer. The upper portion, composed of fat and fluid, was discarded. The cell pellet was resuspended in 10 ml of cell culture medium (RPMI 1640 medium, Sigma™) and centrifuged at 600 × *g* for 15 min at 4 °C. This procedure was repeated × 3 to wash the cells. Cell viability in the pooled suspension was assessed by Trypan blue exclusion test, and cells were diluted to 2×10^6 cells/ml.

Escherichia coli opsonization

The second aliquot of colostrum was used to obtain colostral serum, which was used for the opsonization of ETEC in indirect bactericide activity trials of phagocytes. Enterotoxigenic *E. coli* (F5) was cultured in Tryptic Soy Broth (TSB, Difco™, Detroit, USA) for 18 h at 37 °C, washed twice in PBS, and cell concentration was adjusted to approximately 2×10^8 cells/ml, as measured by turbidimetry at 540 nm on a Coleman spectrophotometer (Celm, Brazil). This bacterial concentration was previously determined by colony counts on Tryptic Soy Agar (TSA, Difco™, Detroit).

To obtain opsonins, the second colostrum aliquot of 10 ml was centrifuged at 2 000 × *g* for 1 h. After centrifugation, whey was separated and aliquots were placed in 3 ml microtubes to assess bacterial opsonization and to evaluate bactericide activity of the cells in each sample, according to the method previously described by Bellinati-Pires et al. (1989).

For the opsonization of *E. coli*, colostrum aliquots were thawed immediately before use and mixed with appropriate volumes of bacterial suspension to a final concentration of 2×10^7 bacteria/ml (Meynell and Meynell 1965), in a 10% opsonin source. As an untreated bacterial control, another bacterial suspension was prepared at the same concentration in cell culture medium without opsonins. Both bacterial suspensions were incubated for 30 min at 37 °C and used in the bactericide treatments.

Evaluation of oxidative activity

Three treatments were performed to evaluate the oxidative activity of the cells in bovine colostrum, as follows: Treatment 1 measured spontaneous H_2O_2 release by the cells (Control); Treatment 2 measured H_2O_2 release following the stimulation of the cells with non-opsonized *E. coli* (non-opsonized - NO); and Treatment 3 measured H_2O_2 release following the stimulation with *E. coli* that was opsonized with 10% delipidated colostrum supernatant (opsonized - O).

Horseradish peroxidase-dependent oxidation of phenol red, which is assessed by its increased absorbance at 600 nm (Pick and Mizel 1981; Russo et al. 1989), is a sensitive method to measure H_2O_2 release from phagocytic cells. Briefly, suspensions of cells (2×10^6 cells/ml) were mixed with overnight *E. coli* cultures (2×10^7 bacteria/ml) that were inactivated for 1 h at 60 °C; the procedure was performed in duplicate, and the co-cultures were stirred at 37 °C for 30 min. Phagocytosis was halted by incubating co-cultures in an ice bath. To eliminate any extracellular bacteria, suspensions were centrifuged twice ($600 \times g$ for 10 min at 4 °C).

Cells were resuspended in a phenol red solution consisting of 140 mM NaCl, 10 mM potassium phosphate, pH 7.0, 5.5 mM dextrose, 0.56 mM phenol red and 0.01 mg/ml type II horseradish peroxidase (Sigma™). Then, 0.1 ml of each sample was transferred to a flat-bottomed, 96-well culture plate (Corning™). Spontaneous H_2O_2 release by the cells was measured in the samples that contained cells alone. The samples were incubated for 1 h at 37 °C in humidified atmosphere, and the reaction was terminated by adding 10 mL of 1 N NaOH.

Absorbance was measured by enzyme-linked immunosorbent assay in an automatic photometer that was equipped with a 630-nm filter. Results were expressed as nM H_2O_2 per 2×10^6 cells, calculated based on a standard curve that consisted of known molar H_2O_2 concentrations in buffered phenol red.

Bacteriological examination

The third aliquot was aseptically obtained for bacteriological examination and cultured on Petri dishes with 5% sheep blood agar and Sabouraud agar. The cultures were kept at 37 °C for 24 to 48 h. Bacterial microorganisms were characterized according to morphological, staining and biochemical cultivation. Samples that were positive for any microorganism in the bacteriological analysis were excluded from the study (Lennette 1985).

Statistical analysis

Due to the difference in physico-chemical properties of the milk secretion obtained before the first and second milking *postpartum*, it was decided to distribute them into groups and analyze them separately. The quantity of H_2O_2 showed a nonparametric distribution by the Kolmogorov-Smirnov test. The median of the quantity of H_2O_2 produced was calculated and differences among the C, NO and O treatments were analyzed by Mann-Whitney test.

Results

Median values of hydrogen peroxide released by phagocytes from colostrum, stimulated or non-stimulated by *Escherichia coli* bacteria, are shown in Table 1.

Table 1. Oxidative activity (nmol/10^6) of cells found in bovine colostrum, according to the different treatments, in samples collected before the first and second milking.

Treatments	C		NO		O	
Milking order	1st	2nd	1st	2nd	1st	2nd
Mean	134.5	71.4	127.0	68.8	131.0	75.5
Standard deviation	87.9	65.1	80.8	58.6	94.6	64.8
Median	149.1[a]	39.9[b]	125.5[a]	49.1[b]	102.4[a]	54.4[b]
Minimum	8.1	3.1	13.2	4.5	17.8	4.5
Maximum	273.2	264.4	297.3	325.7	296.0	273.6
P value	0.0007		0.0007		0.008	

C – Control; NO - Non-opsonized; O – Opsonized

[a,b] - Values with different lower case letters in the same row indicate statistical differences between them ($P < 0.05$)

The median values of H_2O_2 (nmol/10^6) produced were higher in the first milking independent of the assay performed. Values of 149.1 and 39.9 ($P = 0.0007$), 125.5 and 49.1 ($P = 0.0007$), and 102.4 and 54.4 ($P = 0.008$), were obtained in the first and second milking *postpartum*, respectively, in C, NO and O treatments.

The median value of hydrogen peroxide produced between C, NO and O assays was similar ($P > 0.05$), independent of the number of milking *postpartum*. However, increasing values of H_2O_2 (nmol/10^6) were observed in C, NO and O assays only in the second milking *postpartum*.

Discussion

Phagocytosis, oxidative burst, and bacterial death are among the innate defense mechanisms that are employed by phagocytic cells in the host's defense stimulated by microorganisms (Paape et al. 2003). This study evaluated the oxidative activity of phagocytes in bovine colostrum by measuring H_2O_2 released either spontaneously or after stimulation with *E. coli*, which is an important agent of bovine mastitis.

Spontaneous release of H_2O_2, which demonstrates the activation status of cells, was observed in bovine colostrum cells. Median H_2O_2 concentrations in this study were greater than those found by Pontes (1999) in human colostrum using the same colorimetric technique for the measurement of H_2O_2 by colostrum phagocytes. It should be noted that human colostrum is significantly different than bovine colostrum. Human colostrum has a lower somatic cell count (SCC), predominance of IgA, and greater proportion of neutrophils (60%), whereas bovine colostrum has higher cell concentration, about 1×10^6 cells/ml of colostrum, with the predominance of macrophages.

The amount of H_2O_2 produced at first milking exceeded the values found thereafter. H_2O_2 is released by macrophages and PMNs after phagocytosis. Macrophages are the predominant cells in bovine colostrum (Gomes et al. 2011a); they may locate both opsonized and non-opsonized particles and phagocyte and destroy them intracellularly (Pick and Mizel 1981). Unspecific phagocytosis by bovine colostrum macrophages may have contributed to the lack of significant differences between the treatments in this study, because colostrum contains a high concentration of fat globules and other substances, which may have been engulfed and could, thus, stimulate the release of H_2O_2 in the absence of *E. coli*.

The presence of bacteria did not determine the increased amount of H_2O_2 released by phagocytes. However, a trend was observed in the amplification of the colostrum phagocytes response obtained in the 2nd milking *postpartum*, in the presence of *Escherichia coli* compared to bacterial assays without stimulation. We believe that the macrophages that are present in bovine colostrum (69.5 ± 17.4%) before the first milking (Gomes et al. 2011a) have a lower microbicidal activity toward bacteria compared to neutrophils. Therefore, the latter cells depend on the opsonization of bacteria with IgG_2 and IgM for optimum phagocytosis, but the major immunoglobulin of bovine colostrum is the IgG_1 (Gomes et al. 2011b).

Smaller proportion of PMN in the first milking *postpartum* is due to a decreased expression of the L-selectin molecule, responsible for the migration of PMN from blood to udder tissue (Paape et al. 2002, 2003), which could justify the higher proportion of neutrophils in the second milking colostrum reported in a previous research by our team (Gomes et al. 2011a). This is an important factor that may be related to the greater prevalence of bovine mastitis in the *peripartum* period (Burvenich et al. 1994).

We conclude that the low activity of phagocytes for *E.coli* might represent a susceptibility factor for the mammary gland to bacterial infections in the immediate *postpartum* period. Furthermore, the large amount of free radicals produced can cause injuries to the udder parenchyma.

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