Human colostral phagocytes eliminate enterotoxigenic *Escherichia coli* opsonized by colostrum supernatant

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**Abstract**

Background: Several elements in colostrum and human milk, including antibodies and nonspecific factors with bactericidal and antiviral activity, may play an important anti-infectious and protective role. In developing countries, enterotoxigenic *Escherichia coli* (ETEC) is the main etiological agent of diarrhea in low-socioeconomic level children. In the present work, we studied the functional activity of mononuclear (MN) and polymorphonuclear (PMN) phagocytes of human colostrum against ETEC, as well as the interactions between these cells and colostral or serum opsonins.

Methods: Colostrum samples were collected from 33 clinically healthy women between 48 and 72 hours postpartum. We verified superoxide release in colostral MN and PMN using cytochrome C reduction methods, phagocytosis, and bactericidal activity using acridine orange methods and superoxide dismutase (SOD) in the colostrum supernatants.

Results: Colostral MN and PMN phagocytes exposed to ETEC opsonized with colostrum supernatants caused a significant increase (p < 0.05) in superoxide release. Phagocytosis by colostral PMN cells increased significantly (p < 0.05) when the phagocytes were incubated with both sources of opsonins (sera and colostrum). Increases in superoxide release in the presence of opsonized bacteria triggered the bactericidal activity of the phagocytes. Phagocyte treatment with SOD decreased their ability to eliminate ETEC. Colostrum supernatant had higher SOD concentrations (p < 0.05) compared with normal human sera.

**Keywords**

Colostrum; Enterotoxigenic *Escherichia coli*; Microbicidal activity; Phagocytes
Introduction

Colostrum is a rich source of nutrients containing several immunological components, antioxidants, and bioactive substances that play an important role in infant protection against gastrointestinal and respiratory infections. Acute diarrhea is the second main cause of death in low-socio-economic level infants in developing countries. Enterotoxigenic Escherichia coli (ETEC) is a major causative agent of death from diarrhea in children under five years of age.

Many studies have shown that colostrum and human milk contain important protective factors that combat pathogens in children. Human milk is particularly rich in secretory immunoglobulin A (sIgA), which blocks bacterial adherence to human epithelial cells; neutralizes toxins; prevents viral infections; and acts as an opsonin by increasing free radicals, phagocytosis, and the microbicidal activity of colostral cells. The protective role of colostral IgA and human milk has been shown for various microorganisms.

In addition to antibodies, soluble bioactive components, and anti-infectious factors, human colostrum contains large amounts of viable leukocytes (1 × 10⁹ cells/mL in the first days of lactation), especially macrophages and neutrophils. In addition to circulating leukocytes, these cells produce free radicals and have phagocytic and bactericidal activity. In bacterial infections, phagocytes are known to be the main cell lineage in host defense.

Colostral macrophages have phagocytic activity, express IgA receptors (FcζR; CD89), and produce oxygen-free radicals. The bactericidal activity of colostral mononuclear (MN) phagocytes after opsonization with sIgA is equivalent to that of MN and polymorphonuclear (PMN) phagocytes from peripheral blood. Colostral neutrophils, in turn, have lower phagocytic and bactericidal activity compared with the peripheral neutrophils. The IgA receptors that the neutrophils express are free of gamma chain associations (γ-less FcζR) and may mediate the noninflammatory effects of sIgA. Therefore, the main role of sIgA is likely related to maternal protection.

Human milk contains antibodies against a variety of bacteria, and colostrum inhibits the adherence of different ETEC serotypes. Components other than immunoglobulins (i.e. oligosaccharides, glycoproteins, and glycolipids) also seem to prevent the adherence of ETEC strains to host cells. Phagocytes in human colostrum possibly act in conjunction with soluble components, thereby providing an additional protection mechanism against enterobacterial infection. However, studies on the functional activity of colostral MN and PMN phagocytes and their interactions with the bioactive components of colostrum are only partially understood. Considering that ETEC accounts for child diarrhea worldwide, this study aimed at evaluating the potential of colostral MN and PMN phagocytes for eliminating ETEC as well as the interaction between this pathogen and soluble elements of colostrum.

Materials and methods

Subjects

After an informed consent was obtained, around 15 mL of colostrum was collected from clinically healthy women, 18–35 years of age, between 48 and 72 hours postpartum at the Human Milk Bank of the Center for Female Care, Araxá, Minas Gerais (n = 23) and at the Health System Program of Barra do Garças, Mato Grosso (n = 10). All the procedures were analyzed and approved by the Research Ethics Committee of the "Centro Universitário do Planalto de Araxá", Araxá, Minas Gerais, Brazil.

Separation of colostral cells

Colostrum samples were collected manually in sterile plastic tubes and centrifuged (160×g, 4°C) for 10 minutes. Centrifugation separated colostrum into three different phases: cell pellet, an intermediate aqueous phase, and a lipid-containing supernatant, as described by Honorio-França et al. Cells were separated by a Ficoll-Paque gradient (Pharmacia, Upsala, Sweden), producing preparations with 95% of pure PMN cells and 98% of pure MN cells, analyzed by light microscopy. Purified neutrophils and macrophages were resuspended independently in serum-free medium 199 at a final concentration of 2 × 10⁶ cells/mL.

Escherichia coli strain

The ETEC used was the ETEC 14–057:H7 serotype, provided by the Laboratory of Immunology of Intestine Mucosa of the Biomedical Sciences Institute of the University of São Paulo, Brazil. The stock culture was cultivated in tryptic soy broth (Difco Laboratories, Detroit, MI, USA) for 18 hours at 37°C. Bacteria were washed twice in phosphate-buffered saline and adjusted to an approximate concentration of 1 × 10⁸ bacteria/mL as measured by turbidimetry at 540 nm, using a spectrophotometer (Femto, São Paulo, Brazil). This bacterial concentration was previously determined by colony unit counting on tryptic soy agar (Difco Laboratories, Detroit, MI, USA).

Opsonin sources

Colostrum supernatant—a pool of 10 colostral samples [immunoglobulin concentration (g/L): IgA = 7.4, IgG = 0.15,
and IgM = 0.37] was defatted by repeated centrifugation at 160×g for 10 minutes, at 4°C and used for ETEC opsonization. Sera—a pool of normal human serum samples from 10 volunteer donors [immunoglobulin concentration (g/L): IgA = 2.64, IgG = 14.0, and IgM = 2.0] was prepared and used for ETEC opsonization.

**Bacterial opsonization**

The opsonization of ETEC was achieved according to the technique described by Bellinati-Pires et al.20 Colostrum supernatants and sera from 10 individuals were collected, pooled, and frozen at −70°C. Immediately before use, Colostral and serum aliquots were thawed and mixed with appropriate volumes of bacterial suspension to a final concentration of 2 × 10⁷ bacteria/mL in 10% of the opsonin sources. Another bacterial suspension prepared at the same concentration in medium 199 without opsonin was used as an untreated bacterial control. Both bacterial suspensions were incubated for 30 minutes at 37°C and used in the bactericidal assays.

**Release of superoxide anion**

Superoxide release was measured by determining cytochrome C (Sigma, St Louis, USA) reduction as previously described.4,21 Briefly, MN and PMN phagocytes and bacteria, opsonized or not, were mixed and incubated for 30 minutes for phagocytosis. Cells were then resuspended in phosphate-buffered saline containing 2.6 mM CaCl₂, 2 mM MgCl₂, and cytochrome C (2 mg/mL). Phorbol myristate acetate stimulation was performed as control at 0.5 μg/mL. The suspensions (100 μL) were incubated for 60 minutes at 37°C on culture plates. The reaction rates were measured by absorbance at 550 nm and the results were expressed as nmol/O₂. All the experiments were performed in duplicate or triplicate.

**Bactericidal assay**

Microbicidal activity and phagocytosis were evaluated using the acridine orange method described by Bellinati-Pires et al.20 Equal volumes of bacteria and cell suspensions were mixed and incubated for 30 minutes at 37°C under continuous shaking and in the presence or absence of superoxide dismutase (SOD; 140 units).22,23 Phagocytosis was stopped by incubation in ice. To eliminate extracellular bacteria, the suspensions were centrifuged twice (160g, 10 minutes, 4°C), and the cells were resuspended in serum-free medium 199 and centrifuged. The supernatant was discarded and the sediment dyed with 200 μL of acridine orange (14.4 g/L) for 1 minute. The sediment was resuspended in cold culture 199, washed twice, and observed under immunofluorescence microscope at 400× and 1,000× magnification. The phagocytosis index was calculated by counting the number of cells ingesting at least three bacteria in a pool of 100 cells. To determine the bactericidal index, we stained the slides with acridine orange and counted 100 cells with phagocytized bacteria. The bactericidal index is calculated as the ratio between orange stained (dead) and green stained (alive) bacteria × 100.20 All the experiments were performed in duplicate or triplicate.

**CuZn-SOD concentration (CuZn-SOD—E.C.1.15.1.1)**

The CuZn-SOD enzyme concentration was determined in sera (n = 10) and Colostrum (n = 10) samples using the nitroblue tetrazolium test (NBT; Sigma, St Louis, USA.) and spectrophotometrically read at 560 nm.24,25 A 0.5 mL volume of sera or Colostrum samples was placed in test tubes, and 0.5 mL of standard hydroalcoholic solutions (1:1 v/v) were prepared in other tubes. Both samples of sera/colostrum and the standard solutions were added to 0.5 mL of chloroform-ethanol solution (1:1 ratio) followed by 0.5 mL of a reactive mixture of NBT and diaminoethanetetraacetic acid at a 1:1.5 ratio (v/v). After 2.0 mL of carbonate buffer plus hydroxylamine were added, pH increased to 10.2.24 The tubes remained at room temperature for 15 minutes and underwent spectrophotometrical reading. The reactive mixture reached zero values (for 3.5 mL). The SOD was calculated by the following relationship: SOD = (Ab standard–Ab sample/Ab standard) × 100 = % reduction of NBT/CuZn-SOD. The results were expressed in international units of CuZn-SOD.

**Statistical analysis**

We used analysis of variance to evaluate superoxide anion release, phagocytosis, and the bactericidal index according to the presence or absence of opsonized ETEC, in phagocytes with or without SOD treatment and in MN and PMN phagocytes. Student’s t test was used to analyze SOD enzyme concentration for two independent samples. We considered p values < 0.05 to be statistically significant.

**Results**

**Superoxide release by colostral phagocytes in the presence of ETEC opsonized with Colostrum supernatant**

Colostrum MN and PMN phagocytes exposed to ETEC opsonized with Colostrum supernatant increased superoxide release compared with phagocytes exposed to bacteria opsonized with normal human sera and phorbol myristate acetate-stimulated cells (Table 1). When phagocytes were incubated with nonopsonized ETEC, the superoxide released by MN phagocytes was higher than that of PMN and equal to those released as phagocytes when exposed to bacteria opsonized by human sera. PMN phagocytes exposed to nonopsonized ETEC had levels of superoxide release similar to those of spontaneous release (Table 1).

**Phagocytosis activity of Colostrum MN and PMN Cells against ETEC opsonized by Colostrum Supernatant**

Colostrum MN and PMN phagocytes have phagocytic activity for ETEC. In the presence of opsonins (colostrum supernatant or normal sera), phagocytosis increased significantly. Both sources of opsonins induced equivalent phagocytosis rates by Colostral MN and PMN (Fig. 1A and B). To verify the effects of superoxide release on the phagocytosis of ETEC, the phagocytic activity of Colostral MN and PMN was
analyzed in the presence of SOD, an enzyme that metabolizes anion superoxide. As shown in Fig. 1A and B, phagocytosis index did not change when MN or PMN phagocytes were incubated in the presence of SOD.

Elimination of ETEC opsonized with colostrum supernatant by colostral MN and PMN phagocytes

Colostral MN and PMN phagocytes exerted bactericidal activity against ETEC. When the bacteria were opsonized with colostrum supernatant or normal sera, cell activity enhanced significantly \((p < 0.05)\). Both sources of opsonins (colostrum supernatant or normal sera) induced equivalent ETEC elimination rates (Fig. 2). When the cells were preincubated in the presence of SOD, decreased bactericidal activity (nonopsonized bacteria) was observed in both types of cell. ETEC opsonized with colostrum supernatant in the presence of MN or PMN phagocytes and preincubated with SOD had lower bactericidal activity. No effects of SOD were observed in ETEC elimination by PMN phagocytes using serum-opsonized bacteria (Fig. 2).

CuZn-SOD concentration in human colostrum supernatant

CuZn-SOD concentrations in colostrum supernatant increased significantly \((p < 0.05)\) compared with CuZn-SOD levels in sera (Fig. 3).

Discussion

In the present study, we show that colostral phagocytes significantly eliminate ETEC and that this activity is dependent on prooxidative cell metabolism. ETEC is the main cause of diarrhea in developing countries, accounting for 800 million deaths a year, mainly in children under the age of 5 years.²⁶

Breastfeeding has been described as an effective intervention for infant protection, especially against respiratory and gastrointestinal infections.²⁷ Several studies have shown that the phagocytic and microbicidal activity of colostrum phagocytes¹² is comparable with that of blood phagocytes,¹³ with similar rates of phagocytosis and bactericidal function.⁴ Earlier studies have shown that colostrum supernatant contains components that activate the prooxidative mechanisms of cells.⁴ The microbicidal activity of

### Table 1

<table>
<thead>
<tr>
<th>Phagocytes</th>
<th>Superoxide release (nmol/O₂)</th>
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<tbody>
<tr>
<td></td>
<td>MN</td>
</tr>
<tr>
<td>Spontaneous (without bacteria)</td>
<td>11.5 ± 0.7</td>
</tr>
<tr>
<td>PMA (without bacteria)</td>
<td>15.8 ± 0.9**</td>
</tr>
<tr>
<td>Bacteria incubated with medium 199</td>
<td>15.0 ± 1.1**</td>
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<tr>
<td>Bacteria opsonized with colostrum pool</td>
<td>21.7 ± 0.6**</td>
</tr>
<tr>
<td>Bacteria opsonized with a serum pool</td>
<td>15.7 ± 0.8**</td>
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</table>

Bacteria were opsonized with a colostrum supernatant pool or a normal serum pool. In control assays, MN and PMN cells were preincubated with medium 199. The results represent the mean ± standard deviation of 8 experiments with cells from different individuals.

\*\(p < 0.05\) comparing cell type, considering the same opsonin source.

\**\(p < 0.05\) comparing the treated groups with spontaneous superoxide released, considering the same cell type.

ETEC = enterotoxigenic *Escherichia coli*; MN = mononuclear; PMN = polymorphonuclear.
Colostral phagocytes has been linked to the activation of cellular oxidative metabolism and release of large amounts of free radicals, an extremely important phenomenon during immune responses and inflammatory reactions.28 The results of this study confirm the importance of superoxide anion for bacterial death. The increase in superoxide release affects phagocytic and bactericidal activity because SOD-treated phagocytes decrease ETEC elimination. Other studies found that heat-inactivated SOD does not affect the microbicidal activity of phagocytes. Similarly, heat-inactivated SOD fails to block peroxidase inhibitors.29

Superoxide anion production, phagocytosis, and the microbicidal activity of MN and PMN phagocytes was higher in the presence of bacteria previously opsonized with colostrum supernatant or normal sera. The immunoglobulins and their complements, in particular IgG and C3b, which are the main opsonins in the blood,30 are also found in smaller amounts in human milk and colostrum.31 Other studies have already reported the elimination of opsonized enteropathogenic Escherichia coli by MN phagocytes but not by PMN phagocytes.4 In the case of MN phagocytes, microbicidal activity was stimulated only in the presence of opsonins or other immunomodulatory agents.4

The functional activity of colostral PMN has been shown to be lower than that of both human blood PMN and colostral MN.32 But in the present study, PMN and MN phagocytes had similar responses against ETEC previously opsonized with colostrum supernatant or sera. Although IgG is the predominant antibody type in sera, it is found in lower amounts in colostrum, as are proteins from the complement system.17

Both colostral MN and PMN phagocytes had higher superoxide release and microbicidal activity in the presence of ETEC previously opsonized with colostrum supernatant. Because IgA is the predominant antibody class in colostrum,6 this finding suggests that IgA is the opsonin that stimulates microbicidal activity by both types of phagocytes in human colostrum. The significance of the biological activity of IgA, such as opsonin in colostrum secretion, is likely associated to the formation of a complete microenvironment in which soluble and cellular components act together.4

An interesting point is that colostrum phagocytes exposed to nonopsonized ETEC had low bactericidal activity. Some studies report that these cells have decreased bactericidal activity because they lack nonspecific receptors on their surface. Hence, bacteria that are incubated with phagocytes may not be phagocytized and thus remain outside these cells.4 The fact that colostral MN phagocyte activity is opsonin dependent strengthens the hypothesis of an interaction between soluble and cellular components from colostrum, with phagocytes playing a fundamental role in this interaction.

Figure 2. Bacterial elimination by collostral (A) MN or (B) PMN cells. Action of SOD on ETEC elimination by collostral phagocytes. Bacterial elimination by MN or PMN cells from colostrum was determined using acidine orange methods in the presence or absence of SOD (140 units). Results represent the mean ± standard deviation of five experiments with cells from different individuals. *p < 0.05 comparing the opsonized groups to the control groups, using a same SOD treatment or not; **p < 0.05 comparing the SOD-treated group to the same opsonin source. ETEC = enterotoxigenic Escherichia coli; MN = mononuclear; PMN = polymorphonuclear; SOD = superoxide dismutase.

Figure 3. Concentration (mean ± standard deviation) of CuZn-SOD in human sera and colostrum obtained in eight experiments. SOD = superoxide dismutase.
According to our data, the release of superoxide anion was higher in MN phagocytes than in PMN phagocytes, and this difference was more marked when ETEC was previously opsonized with colostrum supernatant. The bactericidal activity of MN phagocytes was also higher in the presence of ETEC previously opsonized with colostrum supernatant, as shown in Fig. 2. Similar results were found for the MN cells of Giardia lamblia\textsuperscript{22} and enteropathogenic Escherichia coli,\textsuperscript{4} whose phagocytic ability increased after exposure to a large amount of opsonins, suggesting that this cell type is functionally more active, especially after opsonization.\textsuperscript{6}

The activation mechanisms of human colostrum phagocytes may depend on stimulatory signals generated by complement proteins and antibodies in milk, especially IgA, complement receptor, and Fc receptor.\textsuperscript{15–35} The combined action of these factors mediates signals that lead to degranulation, oxygen radical production, and phagocytosis.\textsuperscript{35}

Human colostrum and milk are rich in biologically active molecules that are essential for antioxidant functions. A number of milk enzymes are important as immune protectors.\textsuperscript{9} In the present study, cellular activation produced a significant amount of superoxide, which is essential for microbicidal activity. SOD concentration in colostrum was higher than in human sera. Their soluble and cellular components act in a child’s gut without, however, provoking an inflammatory response.\textsuperscript{36} The activity of SOD in human milk appears to be 10–25 times higher than in sera\textsuperscript{5} and may change significantly during lactation to meet the different needs of newborn development.\textsuperscript{37} Other studies show that the highest activity of this enzyme in milk occurs at three weeks of lactation and that it decreases after 4 months of lactation.\textsuperscript{37} The antioxidant capacity of colostrum, which is vital for the newborn in the first days of life, is higher than that of transition and mature milk.\textsuperscript{1}

The activation of human colostral phagocytes by opsonins and consequent release of superoxide anion is an important mechanism for child protection. This secretion exhibits high SOD enzyme content, which likely underlies phagocyte action and avoids inflammatory reactions in a child’s intestine. The lack of antioxidant defenses may trigger an intensification of oxidative actions that culminate in a series of diseases, especially in premature infants.\textsuperscript{38}

Owing to the immaturity of the immune system of newborns, many immunological components are not completely developed.\textsuperscript{5} Human milk not only provides passive immunity but can also directly modulate the immune development of the child.\textsuperscript{39} Because of the non-invasive characteristics of ETEC, a protective immune response to the infection caused by this pathogen is likely mediated by the mucosal immune system.\textsuperscript{40} There are many significant immune components in human milk, and these factors interact with each other, acting synergistically and providing protection to the newborn.\textsuperscript{5,14} The number of viable cells and bioactive substances in the colostrum suggests that this secretion is important to protect the child. Because of the immature gastrointestinal system in newborns, cells received through the colostrum are not eliminated by digestive enzymes and remain intact in the intestinal mucosa for 4 hours, participating actively in bacteria elimination.\textsuperscript{41,42}

Colostrum phagocytes exert bactericidal activity against ETEC through interactions with soluble factors in colostrum. The functional activity of phagocytes described in this work reinforces the findings of other studies that demonstrate the importance of breastfeeding in combating these enterobacteriaceae.\textsuperscript{8,43} ETEC diarrhea affects mainly children under the age of 2 years, and its incidence decreases over time.\textsuperscript{44}

The ability of phagocytes to eliminate ETEC depends on the activation of cellular oxidative metabolism; in addition, activation of colostral phagocytes is likely an additional breast-feeding protection mechanism for infants against intestinal infections and may represent an important source of protection against infections during this phase of life and may persist until maturity of the infant’s immune system is well developed.

**Acknowledgments**

The authors are very grateful to the Postdoctoral Program of São Paulo State University. This research was supported by FAPESP- The State of São Paulo Research Foundation (grant no. 2008/09187-8) and FAPEMAT- The State of Mato Grosso Research Foundation (grant no. 735593/2008 and No453387/2009).

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