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Research Article

Modulation of Human Colostrum Phagocyte Activity by the Glycine-Adsorbed Polyethylene Glycol Microspheres

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Colostrum is a secretion that contains immunologically active components, including immunocompetent cells and glycine, which has anti-inflammatory, immunomodulatory, and cytoprotective effects. The aim of this study was to evaluate the adsorption of glycine onto polyethylene glycol (PEG) microspheres and to verify the immunomodulatory effect of this nanomaterial on human colostrum phagocytes. The PEG microspheres were evaluated by fluorescence microscopy. The effects of PEG microspheres with adsorbed glycine on viability, superoxide release, phagocytosis, microbicidal activity, and intracellular calcium release of mononuclear (MN) and polymorphonuclear (PMN) colostrum phagocytes were determined. Fluorescence microscopy analyses revealed that glycine was able to be adsorbed to the PEG microspheres. The PMN phagocytes exposed to glycine-PEG microspheres showed the highest superoxide levels. The phagocytes (both MN and PMN) displayed increased microbicidal activity and intracellular calcium release in the presence of PEG microspheres with adsorbed glycine. These data suggest that the adsorption of PEG microspheres with adsorbed glycine was able to stimulate the colostrum phagocytes. This material may represent a possible alternative therapy for future clinical applications on patients with gastrointestinal infections.

1. Introduction

Polymers are natural or synthetic versatile macromolecules that represent a class of materials used as excipients for pharmaceutical preparations [1, 2]. The use of polymers for the development of microspheres (matrix systems) has been shown to be an important strategy for studies on health sciences. There are a variety of polymers, including polyethylene glycol (PEG), that have the capacity to form more porous structures, making it useful in improving the biological activity of substances. Furthermore, these polymers have the capacity to adsorb other organic compounds to improve its biological function [3–6].

Glycine is a simple nonessential amino acid that consists of a carbon molecule linked to an amino group and a carboxyl group [7, 8]. Interactions between amino acids and immunocompetent cells have been reported in the literature. It is known that glycine has anti-inflammatory, immunomodulatory, and cytoprotective effects [7].

Several works have reported that colostrum contains soluble immunological components and large amounts of viable leukocytes (1×10^9 cells mL^{-1} in the first days of lactation), especially phagocytes, such as neutrophils and macrophages [9]. Phagocytes represent 80–100% of the cells of colostrum, but the proportion of mononuclear (MN) and polymorphonuclear (PMN) phagocytes vary [9]. The role of these cells in colostrum remains controversial.

Colostrum is also rich in bioactive compounds with anti-inflammatory effects, such as glycine [10–12]. This amino acid in colostrum [9, 10] is associated with clinical and

epidemiological evidence that breastfeeding protects children against acute respiratory and gastrointestinal infections [13–15]. Studies have shown that the adsorption of hormones onto PEG microspheres can immunomodulate human colostrum phagocytes, and it is reported as a possible neomaterial to treat children with infections or inflammatory diseases [5].

Considering that colostrum is a secretion that contains immunologically active components, including immunocompetent cells that act in the neonatal intestine without causing inflammation, and that glycine is an anti-inflammatory molecule, it is possible that PEG microspheres with adsorbed glycine can modulate colostrum phagocytes and trigger important therapeutic processes, especially during gastrointestinal infection and/or inflammation. The aim of this study was to evaluate PEG microspheres with adsorbed glycine and to verify the immunomodulatory effects of this nanomaterial on human colostrum phagocytes.

2. Materials and Methods

2.1. Subjects. Approximately 15 mL of colostrum was collected from clinically healthy women aged 18–35 years at the Health System Program of Barra do Garças, Mato Grosso, Brazil, upon informed consent ($N = 67$). All of the mothers had given birth to healthy full-term babies. The colostrum samples were collected into sterile plastic tubes between 48 and 72 hours postpartum. All of the procedures were submitted for ethical evaluation and received institutional approval.

2.2. Polyethylene Glycol (PEG) Microsphere Preparation. The microspheres were obtained from polyethylene glycol (PEG) 6000 (Synth, São Paulo, Brazil) using a modification [4, 5] of a previously described protocol [3]. Briefly, 20 g of PEG 6000 was resuspended in 100 mL of a 2% sodium sulfate solution in phosphate-buffered saline (PBS) and incubated at 37°C for 45 min. After incubation, the PEG microspheres were diluted 3 : 1 in PBS and washed twice in PBS (500 ×g, 5 min). The PEG microspheres were resuspended in PBS. The formation of the microspheres was thermally induced by subjecting the solution to 95°C for 5 min. For adsorption, the suspensions of PEG microspheres in PBS were incubated with glycine (Vetec, RJ, Brazil, concentration 100 ng mL^{-1}) at 37°C for 30 minutes. The PEG microspheres with or without adsorbed glycine were fluorescently labeled overnight at room temperature with a solution of Dylight-488 (Pierce Biotechnology, Rockford, USA; 10 $\mu g\, mL^{-1}$) in dimethylformamide (Sigma, St. Louis, USA, at a 100 : 1 molar ratio of PEG : Dylight). The samples were then analyzed by fluorescence microscopy.

2.3. Separation of Colostrum Cells. Approximately 15 mL of colostrum was collected in sterile plastic tubes from each woman. The samples were centrifuged (160 ×g, 4°C) for 10 min, which separated the colostrum into three different phases: a cell pellet, an intermediate aqueous phase, and a lipid-containing supernatant, as described by Honorio-França et al. [13]. The cells were separated by a Ficoll-Paque gradient (Pharmacia, Upsala, Sweden), producing preparations with 95% of pure polymorphonuclear cells and 98% of pure mononuclear cells analyzed by light microscopy. The purified macrophages and neutrophils were resuspended independently in serum-free medium 199 at a final concentration of 2×10^6 cells mL^{-1}.

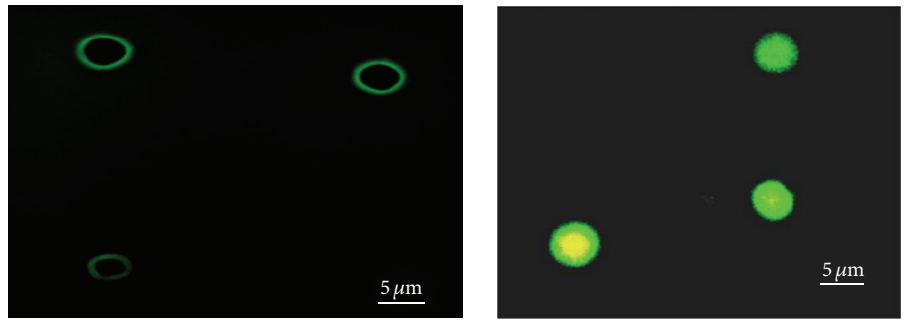
2.4. E. coli Strain. The enteropathogenic *Escherichia coli* (EPEC) was isolated from the stools of an infant with acute diarrhea (serotype 0111:H^-, LA^+, eae^+, EAF^+, bfp^+). This material was prepared and adjusted to 10^7 bacteria mL^{-1}, as previously described by Honorio-França et al. [13].

Treatment of Colostral Phagocytes with PEG Microspheres with Adsorbed Glycine. To assess the effects of PEG microspheres with adsorbed glycine on superoxide anion release, as well as on phagocytic and microbicidal activity, phagocytes (2×10^6 cells/mL) were incubated with 50 μL glycine (Sigma, St. Louis, USA, final concentration of 100 ng/mL, with 50 μL of PEG microsphere and with 50 μL of PEG microspheres with adsorbed glycine) for 1 h at 37°C.

The phagocytes were then washed once with medium 199 at 4°C and immediately used in the assays developed to measure superoxide release, phagocytosis and bactericidal activity.

2.5. Release of Superoxide Anion. Superoxide release was determined by cytochrome C (Sigma, St. Louis, USA) reduction [13, 16]. Briefly, mononuclear phagocytes and bacteria were mixed and incubated for 30 min to allow phagocytosis. The cells were then resuspended in PBS containing 2.6 mM $CaCl_2$, 2 mM $MgCl_2$, and cytochrome C (Sigma, St. Louis, USA, 2 mg mL^{-1}). The suspensions (100 μL) were incubated for 60 min at 37°C on culture plates. The reaction rates were measured by their absorbance at 630 nm, and the results were expressed as nmol/O_2^-. All of the experiments were performed in duplicate or triplicate.

2.6. Bactericidal Assay. Phagocytosis and microbicidal activity were evaluated by the acridine orange method [14, 17]. Equal volumes of bacteria and cell suspensions were mixed and incubated at 37°C for 30 min under continuous shaking. Phagocytosis was stopped by incubation on ice. To eliminate extracellular bacteria, the suspensions were centrifuged twice (160 ×g, 10 min, 4°C). The cells were resuspended in serum-free 199 medium and centrifuged. The supernatant was discarded, and the sediment was dyed with 200 μL of acridine orange (Sigma, St. Louis, USA; 14.4 g L^{-1}) for 1 min. The sediment was resuspended in cold 199 medium, washed twice and observed under an immunofluorescence microscope at 400x and 1000x magnification. The phagocytosis index was calculated by counting the number of cells ingesting at least 3 bacteria in a pool of 100 cells. To determine the bactericidal index, the slides were stained with acridine orange, and 100 cells with phagocytized bacteria were counted. The bactericidal index was calculated as the ratio between orange-stained (dead) and green-stained (alive) bacteria × 100 [14]. All of the experiments were performed in duplicate or triplicate.



(a) (b)

Figure 1: Fluorescence microscopy image of the PEG microspheres stained with Dylight-488 (100x—panel (a)). (a) Represent the PEG microsphere and (b) PEG microspheres with adsorbed glycine. Experiments were repeated five times and the results were comparable.

2.7. Intracellular Ca^{2+} Release Determined by Fluorescence and Flow Cytometry. Immunofluorescence staining was performed at the FACS Calibur (BD, San Jose, USA) to assess intracellular Ca^{2+} release in colostrum phagocytes. Cells were loaded with the fluorescent radiometric calcium indicator Fluo3-Acetoxymethyl (Fluo3-AM, Sigma, ST. Louis, USA). Cell suspensions are pretreated with 50 μL of glycine (Sigma, St. Louis, USA, final concentration of 100 ng/mL), PEG (Synth, São Paulo Brazil, final concentration of 100 ng/mL), or PEG microspheres with adsorbed glycine (final concentration of 100 ng/mL), mixed, and incubated at 37°C for 30 min under continuous stirring to 50 revolutions per minute (RPM). Suspensions were centrifuged twice (160 ×g, 10 min, 4°C) and resuspended in PBS containing BSA (Sigma, St. Louis, USA, 5 mg/mL). This suspension was incubated with 5 μL of Fluo-3 (1 μg/mL) for 30 min at 37°C. After incubation, cells were washed twice in PBS containing BSA (5 mg/mL, 160 ×g, 10 min, 4°C) and then analyzed by flow cytometry. Calibration and sensitivity were routinely checked using CaliBRITE 3 Beads (BD Cat., no. 340486 USA). Fluo-3 was detected at 530/30 nm filter for intracellular Ca^{2+}. The rate of intracellular Ca^{2+} release was expressed in geometric mean fluorescence intensity of Fluo-3. The experiments were repeated on several occasions, and the data showed in the figures correspond to one of several trials performed.

2.8. Statistical Analysis. An analysis of variance (ANOVA) was used to evaluate superoxide, phagocytosis, the bactericidal index, and intracellular calcium in the presence or absence of PEG microspheres adsorbed to glycine. Statistical significance was considered when $P < 0.05$.

3. Results

3.1. Characterization of PEG Microspheres. The fluorescence microscopy image (Figure 1(a)) shows the PEG microspheres produced in PBS. This result confirmed that our method successfully produced microspheres that are easily separated in suspension, regularly sized, stable, and spherical (Figure 1(a)). Fluorescence microscopy showed that the PEG microspheres were able to adsorb the glycine and distribute it throughout their surface (Figure 1(b)).

3.2. General Characteristics of Colostrum Components. A total of $1.51 \pm 0.72 \times 10^6$ mononuclear cells mL^{-1} and $2.69 \pm 0.91 \times 10^6$ polymorphonuclear cells mL^{-1} were isolated from the colostrum, and the viability (%) was 94.5% ± 2.2 for mononuclear cells and 92% ± 1.4 for polymorphonuclear cells.

3.3. The Effect of PEG Microspheres Adsorbed to Glycine on Superoxide Release. Glycine increased the superoxide release of both MN and PMN colostrum phagocytes compared to spontaneous release. When the phagocytes were incubated with bacteria, MN cells displayed increased superoxide release. PMN phagocytes incubated with glycine and bacteria displayed decreased superoxide release compared to cells not incubated with bacteria. In the presence of bacteria, MN phagocytes incubated with PEG microspheres showed increased superoxide release, whereas PMN phagocytes showed decreased release. Phagocytes incubated with glycine adsorbed to PEG microspheres had increased superoxide release. In the presence of bacteria, PMN phagocytes showed decreased anion release. Glycine adsorbed to PEG microspheres did not increase the release of superoxide compared to MN phagocytes exposed to the PEG microspheres alone. The highest superoxide release was observed by PMN phagocytes when exposed to glycine and bacteria (Table 1).

3.4. The Effects of PEG Microspheres Adsorbed to Glycine on the Phagocytosis of Colostrum Cells. Colostrum phagocytes displayed some phagocytic activity in response to EPEC. Phagocytosis, independent of the cell type, increased significantly in the presence of glycine. Phagocytosis was generally similar between cells exposed to glycine-adsorbed PEG microspheres and PEG microspheres alone (Table 2).

3.5. The Effects of PEG Microspheres Adsorbed to Glycine on the Bactericidal Activity of Colostrum Phagocytes. The colostrum phagocytes that were not stimulated had some bactericidal activity against EPEC. Regardless of the cell type, phagocytes

Table 1: Superoxide release by colostrum mononuclear (MN) and polymorphonuclear (PMN) cells (mean ± SD, $N = 10$ in each treatment).

Treatment	EPEC	Superoxide release (nmol)	
		MN	PMN
Cells	No	0.47 ± 0.16	$1.65 \pm 0.19^{\dagger}$
	Yes	$1.86 \pm 0.41^{*+}$	1.95 ± 0.82
Glycine	No	$1.43 \pm 0.27^{*}$	$2.84 \pm 0.66^{*\dagger}$
	Yes	$1.14 \pm 0.22^{*}$	$1.05 \pm 0.46^{*+}$
PEG	No	$1.47 \pm 0.24^{*}$	$1.85 \pm 0.38^{*}$
	Yes	$2.62 \pm 0.57^{*+}$	$0.43 \pm 0.14^{*+\dagger}$
PEG glycine	No	$1.66 \pm 0.13^{*}$	$2.21 \pm 0.27^{*\dagger}$
	Yes	$1.29 \pm 1.04^{*}$	$1.34 \pm 0.82^{*+}$

* Indicates differences between each treatment with control (cells), considering the same types of phagocytes (ANOVA, $P < 0.05$). $^{+}$ Indicates differences between cells incubated with bacteriaand cells without bacteria, considering the same treatment and types of cells. † Indicates differences between types of phagocytes, considering the same treatment.

incubated with glycine showed higher bactericidal activity. Both types of phagocytes showed increased microbicidal activity in response to EPEC when incubated with PEG microspheres adsorbed to glycine (Table 2). Glycine adsorbed to PEG microsphere mediated bacterial killing by colostral phagocytes is shown in Figure 2.

3.6. Effect of PEG Microspheres Adsorbed to Glycine on the Intracellular Ca^{2+} Release of Colostrum Phagocytes. Figure 3 shows the rate of intracellular Ca^{2+} release of colostrum phagocytes as measured by Fluo-3 fluorescence intensity. The MN colostrum phagocytes had increased intracellular Ca^{2+} levels when compared to PMN phagocytes. The PEG microspheres increased the intracellular Ca^{2+} levels in both types of cells. The highest intracellular Ca^{2+} release, independent of cell type, was found with the phagocytes treated with PEG microspheres adsorbed to glycine (Table 3).

4. Discussion

This study verified that glycine can be adsorbed to PEG microspheres, and this material provides immunomodulatory effects on the microbicidal activity of human colostrum phagocytes.

Microsphere-based polymeric substances can be used for intelligent and controlled drug delivery [18]. The PEG microspheres are a type of copolymer that are used in the clinical administration of drugs because of their incorporation capacity [19], their ability to increase the duration of drug exposure or other products such as enzymes, and their role as an important signaling vehicle in immunity [20]. The rate of drug release is controlled by two factors, and it is important to understand the physical and chemical properties of the releasing medium [21]. By modulating the size of the pores and the drug concentration, PEG microsphere formulations may allow controlling the speed by which the drug is released from the polymer matrix [22]. Microsphere of PEG is a potential carrier system for hormones [5, 23] and other substances [4, 6], because PEG has biocompatible characteristics and its degradation products do not present toxicity and are easily metabolized and excreted by normal physiological pathways [24].

In this study, analysis by fluorescence microscopy showed the PEG microspheres to be easily separated from a suspension and readily able to adsorb glycine. The PEG microspheres have previously been characterized, and those studies have shown that the polymer is approximately 5.8 μm in diameter and able to bind to various bioactive components [4–6].

The effectiveness of associating bioactive substances with the polymeric matrix has increased the ability to obtain new formulations and activate the immune system [5]. There is a growing awareness of the interaction of food constituents with the immune system [25]. Considering that full-length glycine is present in milk at concentrations of approximately 200 mg/g [10], the interactions between this amino acid and the milk cells form a natural environment and are an ideal experimental model. Glycine is the smallest of the amino acids and consists of a single carbon molecule attached to an amino group and a carboxyl group. The simplicity of the glycine structure led to the assumption that glycine is metabolically inert, and this amino acid can cause metabolic and immunological alterations [26].

Human colostrum and milk are rich in biologically active molecules that are essential for prooxidative functions. Their soluble and cellular components act together within the infant gut without provoking an inflammatory response [17, 27, 28]. The mechanisms of human colostrum phagocyte activation may depend on stimulatory signals generated by active molecules [29]. The combined action of these factors mediates signals that lead to degranulation, the production of oxygen free radicals and phagocytosis [29]. The generation of free radicals is considered an important mechanism of immune protection during infectious processes [17, 30]. Several bioactive compounds have shown the ability to induce immune activation by the release of free radicals, providing protection from a variety of diseases [21, 31].

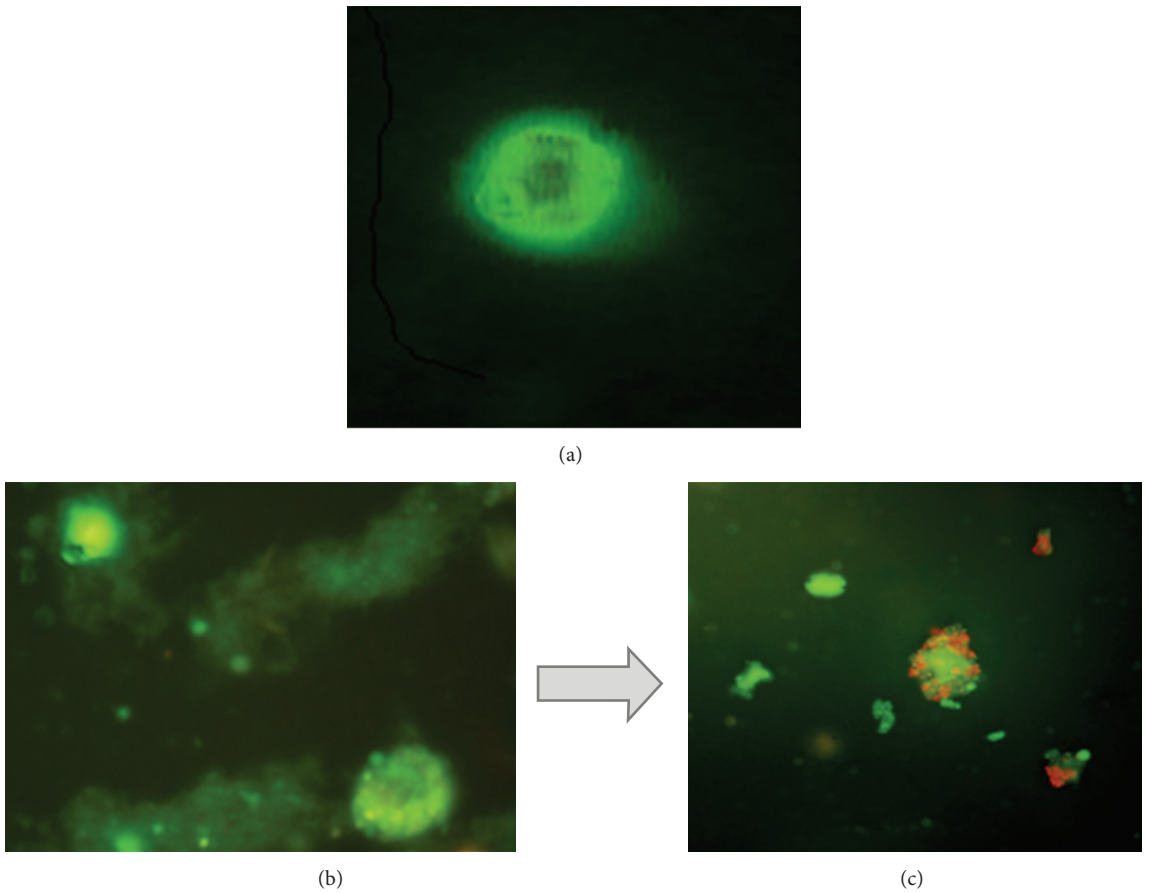
In this study, PEG microspheres with adsorbed glycine modulate the functional activity of phagocytes in human colostrum. The literature reports that soluble components present in colostrum interact with the cells, and the superoxide concentration has been associated with increased phagocytic and microbicidal activities [14, 32]. In this work, we identified a positive correlation between superoxide release and microbicidal activity in both MN and PMN phagocytes.

Microbicidal activity and the products derived from oxidative metabolism, both promoted by glycine in phagocytes, may have important clinical implications. It is unclear how glycine modulates the phagocytes activity [33]. Glycine increases the influx to Na^{+} [34], and this leads to a depolarization of cell membrane, which in turn will augment the Ca^{2+} influx [35]. The resulting increase in Ca^{2+} could in turn activate Ca^{2+}-dependent pathways, which are involved in the phagocytosis [36] and microbicidal activity [5, 37],

Table 2: Bacterial phagocytosis and bactericidal activity index by mononuclear (MN) and polymorphonuclear (PMN) cells (mean ± SD, $N = 10$ in each treatment).

EPEC	Phagocytosis (%)		Bactericidal index (%)	
	MN	PMN	MN	PMN
Cells	34.40 ± 4.5	39.00 ± 3.5	34.90 ± 9.5	36.00 ± 4.6
Glycine	75.33 ± 3.5*	69.00 ± 5.6*	53.81 ± 5.1*	55.00 ± 4.9*
PEG	82.00 ± 5.5*	74.00 ± 6.3*	57.6 ± 5.6*	67.00 ± 5.6*
PEG glycine	72.00 ± 6.7*	63.00 ± 6.0*	52.32 ± 3.4*	58.00 ± 4.9*

Bacterial phagocytosis and elimination index by mononuclear cells from colostrum were determined with the acridine orange method. *Indicates differences between each treatment with control (cells), considering the same kind of phagocytes (ANOVA, $P < 0.05$).



(a)

(b)

(c)

Figure 2: Bacterial killing by colostral phagocytes. Phagocytes with PEG microspheres with adsorbed glycine internalized (panel (a)). The phagocytes were incubated with bacteria on a shaker 50 rpm for 30 min at 37°C. After washing at 4°C, cells were stained with acridine orange and analyzed by fluorescent microscopy. Orange-stained bacteria (dead) and green-stained bacteria (alive). Phagocytosis of bacteria (panel (b)) and bacterial killing (panel (c)).

and increased superoxide levels modify intracellular Ca^{2+} and phosphorylation events during oxidative metabolism [38]. Here we show that PEG microspheres with adsorbed glycine have direct effects on colostrum phagocytes and are able to increase their release of intracellular calcium. Therefore, the results supported that PEG microspheres with adsorbed glycine change the intracellular Ca^{2+} level, which facilitate the microbicidal activity of cells. PEG is known to induce oligomeric association of the cellular membrane protein enhancing transport activity of calcium or influences the microenvironment of the phospholipids of the calcium pump in the membrane [39]. Probably the increased intracellular Ca^{2+} levels observed in both types of cells by PEG microsphere might be associated with multiple interactions of PEG with proteins in the cellular membrane.

On the other hand, the literature has reported that colostrum phagocytes are present in two cell populations that differ in functional activity. When stimulated, mononuclear phagocytes have microbicidal activity against enteropathogenic *Escherichia coli* (EPEC), while polymorphonuclear phagocytes have lower microbicidal capacity against this bacterium [13, 31, 40]. This muted response of

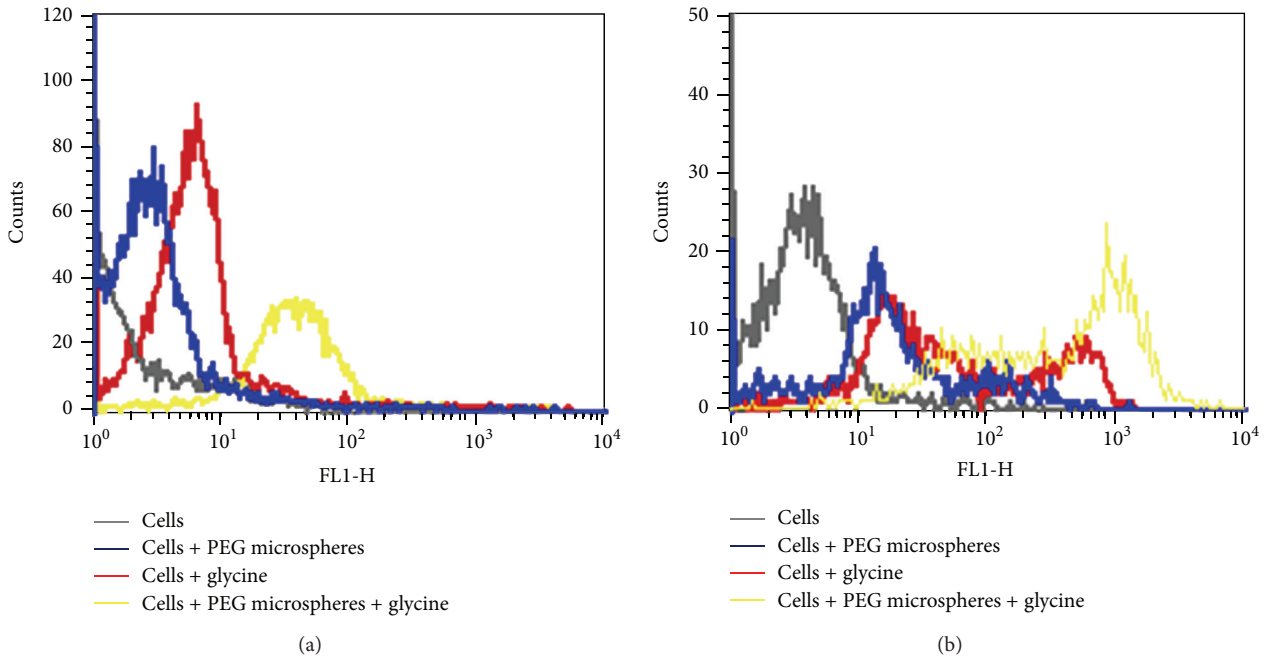


Figure 3: Colostrum mononuclear (MN—panel (a)) and polymorphonuclear (PMN—panel (b)) phagocytes stimulated with glycine were staining with Fluo-3 to assess intracellular Ca^{2+} release as described in Section 2. Immunofluorescence analyses were then carried out by flow cytometry (FACScalibur, Becton Dickinson, San Jose, USA).

Table 3: Intracellular Ca^{2+} release by mononuclear (MN) and polymorphonuclear (PMN) colostrum phagocytes in the presence of glycine adsorbed to PEG microsphere indicated by fluorescence intensity.

Colostrum phagocytes	Intensity of fluorescence (%; mean ± sd)	
	MN	PMN
PBS	13.8 ± 0.6	6.8 ± 1.5†
Glycine	30.1 ± 9.5*	24.6 ± 9.0
PEG microsphere	18.9 ± 0.9*	24.5 ± 8.9*
PEG glycine	36.1 ± 7.4^{*+}	38.9 ± 2.8^{*+}

The results represent the mean and SD of five experiments with cells of different individuals. $^{*}P < 0.05$ compared the treated cells with cells nontreated, considering the same kinds of phagocytes. $^{+}P < 0.05$ compared the different treatment, considering the same kinds of phagocytes. $^{\dagger}P < 0.05$ indicates differences between types of phagocytes, considering the same treatment.

colostrum neutrophils has been attributed to the surface expression of receptors that may mediate anti-inflammatory effects [32]. In this study, PEG microspheres with adsorbed glycine revealed similar functional activities between the colostrum phagocytes. The biological activity of glycine is of great importance because colostrum secretion contains high levels of this amino acid. Moreover, colostrum represents a complete microenvironment in which both the soluble and cellular components act together [13, 32].

The interaction of amino acid associated with controlled release systems and colostrum cells may be of fundamental importance for newborn. Because of the immaturity of the digestive function of the neonate, the cells received through colostrum are not destroyed by digestive enzymes and other factors and are likely to remain intact in the upper portions of the intestine; thus, they can interact with other components and protect the mucosa. Studies have suggested that colostrum cells remain viable in the intestinal mucosa for a period of 4 hours [41, 42] and that they may have microbicidal activity in the newborn.

The complex relationship between the immune system and anti-inflammatory or infectious processes indicates the importance of glycine in therapy for a variety of diseases, since that glycine has immunoregulative properties and can cause metabolic and even immunological actions, especially considering the cost-effectiveness of combination therapy drug delivery systems. The controlled delivery system of glycine adsorbed to PEG microspheres may be an additional mechanism for activating colostrum cells and thus an additional avenue for the protection and treatment of gastrointestinal infections of newborns. Thus, the prooxidative effect resulting from the interaction between phagocytes and glycine-adsorbed PEG microspheres may be an alternative tool for treating infections.

5. Conclusion

These data suggest that PEG microspheres with adsorbed glycine can stimulate colostrum phagocytes. Contact between this material and phagocytes may protect children from gastrointestinal infections and could be a possible alternative therapy for future clinical applications.

Conflict of Interests

The authors declare no conflict of interests and nonfinancial conflict of interests.

Acknowledgments

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