https://dx.doi.org/10.22161/ijaers.5.6.3 ISSN: 2349-6495(P) | 2456-1908(O)

Polyethyleneglycol nanoparticles adsorbed to glycine as a bioengineered neomaterial for application in inflammatory processes

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***Abstract*—** *Polyethylene glycol nanoparticles (NP-PEG) have good adsorption in bioactive compounds and are considered promising vehicles. Several studies have reported the importance of the amino acid for several treatments, among them glycine that has immunomodulatory, cytoprotective and anti-inflammatory effects. The objective of this work was to evaluate the efficiency of the synthesis of polyethylene glycol (PEG) nanoparticles adsorbed with glycine (NANO-PEG/GLY) on functional activity of colostrum macrophages. Human colostrum cells were obtaining from 18 clinically healthy women and used for bioassays of cell viability, phagocytosis, microbicidal activity and cytokines concentration. It was observed that the cell viability index in the presence of NANO-PEG/GLY was above 85%. Phagocytosis rates in colostrum cells treated with glycine and in the presence of EPEC, wheareas the higest microbicidal index were observed in macerophages treated with PEG-NANO-GLY. IL-1β and TNF-α levels increased in GLY and NANO-PEG/GLY groups. The levels of IL-12 and IL-17 also increased in the macrophages cultures under the NANO-PEG/GLY treatment. In the supernatant cell culture IL-8 and IFN-γ levels were similar among the treatments. The date suggest that NANO-PEG particles produced were able to adsorb the amino acid glycine, and this new bioengineered material is capable of modulating the functional activity of human colostrum macrophages and represents an alternative route for the treatment of inflammatory deseases..*

***Keywords*— Colostrum, Glycine, Inflammation, Macrophages, Polyethylene glycol.**

I. INTRODUCTION

The inflammatory processes are part various pathologies which involves leukocyte infiltration, enzymes release and formation of free radicals derived from oxygen [1,2]. Macrophages are cells important in the regulation of the immune response during the inflammation [3]. Some works have reported that colostrum presents soluble immunological components and contains large amounts of viable leukocytes, especially macrophages [4]. On the other hand, other studies have revealed that many nutrients and metabolites provided by food exert an antioxidant effect, useful in protecting human health, preventing diseases [5,6]. There are some amino acids, such as glycine, which is being used in the treatment of patients suffering from enterocolitis because they have cytoprotective and anti-inflammatory action [7,8,9,10]. Glycine is a simple, non-essential amino acid known as a functional supplement used in the pharmaceutical industry, nutritional supplementation [10] and is bioactive compounds with anti-inflammatory activity present in colostrum [11,12,13]. Previous work showed that the adsorption of glycine onto Polyethylene glycol (PEG) microsphere was able to stimulate the colostrum macrophages and suggest that the controlled delivery system of glycine may be an additional mechanism of protection and treatment of patients with infections and/or inflammation gastrointestinais, as well as for the functioning of colostrum cells [7]. Considering that the PEG is a biodegradable and biocompatible component [14] and that this particles have the capacity not to be recognized by the immune system, circulate for longer periods in the body [15,16,17]] and has the ability to adsorb organic compounds, it is possible that this carrier of biological components to be used in the treatment of

gastrointestinal inflammation [7,18,19,20]. The aim of this study was to evaluate the efficiency of the synthesis of polyethylene glycol (PEG) nanoparticles adsorbed with glycine (NANO-PEG/GLY) on functional activity of colostrum macrophages.

II. MATERIAL AND METHODS

2.1 Synthesis and Preparation of PEG Nanoparticles

The nanoparticles were obtained from Polyethylene glycol 6000 (Sigma-Aldrich Brasil Ltda.) PH = 6.8 using a modification of the described method [18,19,20]. For the synthesis of polyethylene glycol (PEG) nanoparticles (NP-PEG) with glycine adsorbed for the complex formation (NANO-PEG/GLY) PEG 6000 (10g) at the concentration of 0.016 mol/L, was diluted in 100 mL of phosphate buffered saline (PBS), incubated for 45 min at 45ºC. Thereafter, 100 µL of a 2% sodium sulfate solution in PBS was added by dropwise. After incubation, the PEG solution was 3: 1 in PBS under rigorous stirring by adding 100 µL of sodium sulfate (UltraTurrax IKA T25/10 min Ultrasound Probe). Shortly thereafter, heat treatment was carried out for 30 minutes at 45ºC to favor sorting in size. After this period the PEG solution was centrifuged for 15 minutes at 15,000 rpm and washed (2x); Diluted 10 times (PEG + sodium sulfate 100µL 0.45mM) in PBS to thermally induce the formation of nanoparticles for 7 minutes at 97 °C. Subsequently, the pH was adjusted to 6.4 and incubated volume to volume the glycine solution (GLY - concentration of 10^{-3} mol /L - Dynamic® - Diadema, São Paulo, Brazil) and PEG nanoparticles at 37 ° C for 30 minutes. The experiments were repeated 20 times and the results were analyzed and compared for hydrodynamic diameter and surface charge by means of dynamic light scattering (DLS) and Zeta potential (ζ).

2.2 Dynamic light scattering (DLS)

GLY, NP-PEG and NANO-PEG/GLY systems were prepared for analysis by DLS technique. It was then possible to investigate the hydrodynamic diameter of the dispersed solid. DLS analyzes were obtained from the Zetasizer Nano Z90 equipment (Malvern Instruments, Malvern, Worcestershire, United Kingdom), with excitation at 632.8 nm.

2.3 Effect of GLY, NP-PEG and NANO-PEG/GLY on the functional activity of phagocytes

After obtaing written consent, about 15 ml of colostrum were collected from 18 clinically healthy women (18 to 30 years), all registered in the health system of Barra do Garças, Mato Grosso, Brazil. All mothers had given birth to healthy newborns. Colostrum samples were collected in sterile plastic tubes between 48 and 72 hours postpartum. All procedures were submitted to the ethics committee for evaluation and received approval.

2.4Macrophages from Human Colostrum

After collection in sterile plastic tubes of each donor, the samples were centrifuged at (160 × g, 4 ° C) for 10 minutes, where the colostrum was separated into three distinct phases: cell pellet, an intermediate aqueous phase, and a lipid-containing supernatant as described by Honorio-França et al. [21]. Cells were separated by a Ficoll-Paque gradient [Pharmacia, Upsala, Sweden]. This procedure generated 98% pure mononuclear cell preparations as analyzed by light microscopy. Purified macrophages were resuspended independently in serum-free 199 medium at a final concentration of 2×10^{6} L^{-1} cells. After this period the cells were washed twice and used for the cell viabilty, phagocitosis and microbicidal assays and the culture supernatant used for quantification of cytokines by the flow cytometric assay.

2.5 *Escherichia coli* strain

The enterophatogenic *Escherichia coli* (EPEC) used was isolated from stools of an infant with acute diarrhea (serotype 0111: H- AL-, eae+, eaf+, bfp+). This material was prepared and adjusted to 10^{7} bacteria/ml, as previously described Honorio-França et al. [21].

2.6 Cell viability determination by acridine Orange and MTT method

In order to determine the cellular viability of GLY, NP-PEG and NANO-PEG/GLY after treatments in the cultures by acridine orange the experiments were performized according to Belinatti-Pires et al. [22]. The cells were stained with 200 µl of acridine orange (Sigma, St. Louis, USA; 14.4 g L-1) for 1 min. The pellet was resuspended in cold medium, washed twice and observed under an immunofluorescence microscope at magnification of 40x and 100x. The viability index was calculated by counting the number of orange- stained [dead] and green- stained [alive] cells out of 100 [23]. All experiments were performed in triplicate.

To verify the cytotoxicity of the NANO-PEG / GLY system using the colorimetric method Tetrazoylium bromide 3- [45-Dimethylthiazol-2-yl] 25- Diphenyl Tetrazolium bromide [MTT Sigma St Louis USA], colostrum macrophages (5×10^{5} cells) were distributed into plate wells and incubated with their respective treatments in humidified chamber with 95% air and 5% CO_2 at 37° C for 2h30min. After incubation the supernatant was removed and each well was added 40 µL of 5 mg.mL-1 MTT and 360 µL of complete culture medium. The plate was then incubated for 3 h in the same humidified chamber. The supernatant was then discarded and 150 µL of DMSO (Dimethylsulfoxide) was added to solubilize the Formazan crystals. Optical density was measured in a plate spectrophotometer using an interference filter at 492-630 nm.

2.7 Bactericidal assay

Table 1. Stability of the NANO-PEG/GLY system in differents periods (days).

Samples	0		15		30		45		60	
	D_h/nm	ξ/mV	D_h/nm	ξ/mV	D_h/nm	ξ/mV	D_h/nm	ξ/mV	D_h/nm	ξ/mV
PEG	191±11	-1.2	197±11	-3.7	321±20	-1.5	349±22	-1.3	549±34	-1.9
NANO-PEG/GLY (1Molar)	209±12	-1.6	214±14	-2.9	362±27	-1.8	355±28	-1.9	557±32	-1.4
NANO-PEG/GLY (2Molar)	213±14	-11.9	218±16	-14.7	340±44	-13.6	371±47	-10.1	571±37	-8.9
NANO-PEG/GLY (3Molar)	229±14	-9.5	234±16	-11.4	356±24	-10.9	391±25	-9.2	599±45	-9.7

Biocompatibility and toxicity studies of the GLY, NP-PEG and NANO-PEG/GLY complex were evaluated prior to a potential biomedical use, the cytotoxicity of each stimulus was examined by acridine orange and MTT assays. Cell viability was determined as shown in figure 1. The results indicate the high biocompatibility of GLY, NP-PEG and NANO-PEG/GLY. The cell viability index in the presence of Gly, NP-PEG and NANO-PEG/GLY was above 85% (Figure 1A and 1B).

Phagocytosis and microbicidal activity were evaluated using the acridine orange method [22]. 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 x g, 10 min, 4°C). Cells were resuspended in serum-free 199 medium and centrifuged. The supernatant was discarded, and the sediment was dyed with 200 µL acridine orange (Sigma, ST Louis, USA; 14.4 g/L) for 1 min. The sediment was resuspended in cold 199 medium, washed twice and observed under immunofluorescence microscopy at 400x and 1000x magnification.

The phagocytosis index was calculated by counting the number of cells that ingested at least 3 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 was calculated as the ratio between orange- stained [dead] and green- stained [alive] bacteria x 100. All of the experiments were performed in duplicate.

2.8 Quantification of cytokines

The cellular supernatant was collected and stored at −80°C prior to analyses. The samples were thawed and cytokines (IL-1β, IL-6, IL-8, IL-10, IL-12, IL-17, TNF-α and IFN-γ) were measured by cytometric bead array [CBA BD Biosciences USA] according to the manufacturer procedures. A flow cytometer was used for these analyses [FACSCalibur BD Biosciences USA]. The data were analyses used the software FCAP Array 1.0 [CBA BD Biosciences USA].

2.9 Statistical analysis

Data were expressed as the mean ± standard deviation (SD). The statistically significant difference was evaluated using the Analysis of variance [ANOVA] and were considered significant when the "p value" was lower than 0.05 ($p < 0.05$).

III. RESULTS

3.1 DLS analysis of the GLY, NP-PEG AND NANO-PEG/GLY

To evaluate the stability of the NANO-PEG/GLY system the samples were submitted to potential Zeta and Zetasize tests and the results are showed in the table 1. The tests were performed at the periods of 0, 15, 30, 45 and 60 days. It was observed that the stability of PEG and NANO-PEG/GLY up to 45 days in relation to arrangement and growth, as well as the average potential of PEG, GLY and NANO-PEG/GLY and after 60 days increased the size.

Biocompatibility and toxicity studies of the GLY, NP-PEG and NANO-PEG/GLY complex were evaluated prior to a potential biomedical use, the cytotoxicity of each stimulus was examined by acridine orange and MTT assays. Cell viability was determined as shown in Fig. 1. The results indicate the high biocompatibility of GLY, NP-PEG and NANO-PEG/GLY. The cell viability index in the presence of Gly, NP-PEG and NANO-PEG/GLY was above 85% (Fig. 1A and Fig.1B).

https://dx.doi.org/10.22161/ijaers *ISSN: 2349-6495(P) | 2456-1908(O)*

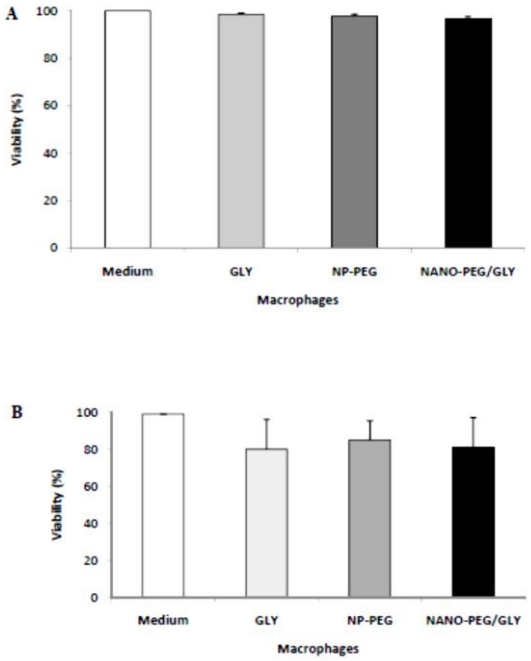

Figure 1. Viability index (mean ± sd; N=5) of colostrum macrophages treated with GLY, NP-PEG and NANO-PEG/GLY in the concentration of 10^{-3} mol/L. Viability index is represented by acridine orange method (A) and MTT method (B).

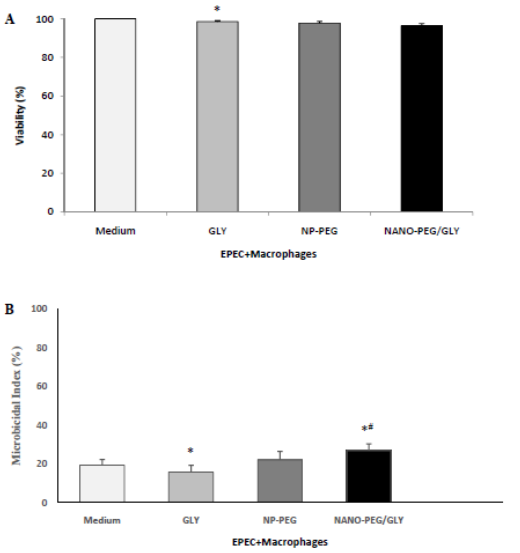

Figure 2. Phagocytosis and microbicidal activity of macrophages from the colostrum treated with glycine (GLY), PEG nanoparticles (NP-PEG) and PEG nanoparticles of glycine (NANO-PEG/GLY).

3.2 Effects of PEG nanoparticles adsorbed to Glycine on phagocytosis and microbicidal by colostrum cells.

Phagocytosis rates in colostrum cells treated with glycine and in the presence of EPEC was higher than in cells from non-treated phagocytes (Fig. 2A). Colostrum macrophages treated with GLY and PEG-NANO-GLY showed higher microbicidal indices in the presence of EPEC than the non-treated phagocytes. The higest microbicidal index were observed in macerophages treated with PEG-NANO-GLY (Fig. 2B)

3.3 Cytokines concentration in supernatant of culture of macrophages in the presence of GLY, NP-PEG and NANO-PEG / GLY.

Table 2 shows cytokine levels in the culture supernatant of colostrum macrophages incubated with the different stimullus. IL-1β and TNF-α levels increased in GLY and NANO-PEG/GLY groups. The levels of IL-12 and IL-17 also increased in the macrophages cultures under the NANO-PEG/GLY treatment. In the supernatant cell culture IL-8 and IFN-γ levels were similar among the treatments.

Table 2- Cytokines concentrations (pg/mL) in culture supernatant of human colostrum macrophages. Cells in untreated, cells treated with Glycine (GLY), PEG nanoparticles (NP-PEG) and PEG nanoparticles adsorbed with glycine (NANO-PEG/GLY). The results are expressed as mean and standard deviation of 6 replicates with human blood cells from different individuals. P>0.05.

Cytokines	Medium	GLY	NP-PEG	NANO-PEG/GLY
IL-1β	13.4±4.4	16.9±1.8*	20.8±1.7*	19.9±2.9*
IL-6	11.8± 4.6	14.2± 2.0	18.5±1.7*	16.5±3.3
IL-8	19.1±1.0	19.3±2.8	19.5±1.7	20.6±3.6
IL-10	4.1 ± 0.2	4.8 ± 0.2	4.5±0.2	5.4±1.2*
IL-12	15.3±6.3	19.9±2.4	22.5±2.9*	21.3±2.7*
IL-17	5.0 ± 1.6	4.7 ±0.8	4.6 ± 0.6	7.1 ± 3.8*
TNF-α	11.2 ± 3.4	17.1±1.9*	17.2 ± 2.5*	17.8 ±1.8*
IFN-γ	3.2 ± 0.1	3.3±0.1	3.4 ± 0.2	3.5±0.5

The results represent the mean and SD of five different individuals. *p<0.05 indicates intergroup differences.

IV. DISCUSSION

The synthesis of functional materials in nanoscale has been increasing interest in the research, due control mechanisms on material in relation the morphology, size, functionalities and properties [24]. Functional materials such as polymers that are produced on nanometer scale as

well as PEG nanoparticles can be used in drug delivery control [25]. Nanoparticles based on polymeric substances can be used in the clinical administration of drugs and / or other bioactive substances due to their incorporation capacity [26,27]. This study it was verified the that nanoparticles of PEG adsorbed glycine, and that this material acts as an immunomodulator on the functional activity of human colostrum cells. The analysis of the NANO-PEG/GLY system was performed by Dynamic Light Scattering (DLS) and Zeta Potential and showed that this material maintained stability for 45 days in relation to arrangement and growth and increased the size after 60 days. Drug delivery is controlled by two main factors: pore size and drug concentration. It is important to understand the physical and chemical properties in the synthesis of neomaterials. It known that PEG nanoscale particle formulations can allow the control of the speed at which the drug is released from the polymer matrix [28]. In this sense the PEG nanoparticles have a high potential in the hormone transport system [19,20]. This material has biocompatible characteristics their degradation products do not present toxicity and are easily metabolized and excreted by natural physiological pathways [29]. Several classes of drugs and bioactive compounds, such as enzymes, cytokines, antibodies and glycine, are significantly improved by the pegylation effect (PEGylation) and can bind to amines [30]. Interactions between amino acids and delivery mechanisms of bioactive compounds have been reported in the literature. Glycine is known for its anti-inflammatory and immunomodulatory properties [7,31]. The effectiveness of association of bioactive substances with the polymer matrix increased the ability to obtain new formulations and activate the immune system [19]. In our experimental model we employed colostrum cells considering that glycine is present in secretion [32] and that can occur easily interactions between this amino acid and this cells reproducing a natural environment. Our results confirm that interaction of NANO-PEG/GLY with colostrum macrophages, independent of method used, did not affect the viability suggesting that this system is non-toxic and can be acts as immunomodulator. The mechanisms of activation of human colostrum phagocytes depend on some signals and stimuli emitted by biologically active molecules that mediates the signals that lead to the production of oxygen free radicals and to the phagocytosis process [33,34]. Phagocytosis and microbicidal activity are important defense mechanisms against several pathological agents such as: virus, bacteria, protozoa infectious and inflammatory processes [7,23, 34, 35]. In this study, the adsorbed glycine PEG nanoparticles were able to modulate the functional activity of human colostrum phagocytes. NANO-PEG/GLY complex increase the microbicidal activity by colostrum macrophages. Studies have shown the immunomodulatory potential of glycine and its cytoprotective capacity to combat infectious and inflammatory processes in various organs and tissues [31]. The NANO-PEG / GLY complex was more efficient at potentiating bacteria death than glycine treatment alone, thus demonstrating the system's immunomodulatory capacity against inflammatory processes. Soluble components present in colostrum interacts with cells and can increase the microbicidal activity [34]. Human colostrum and breast milk are rich in active biological compounds that are essential for pro-oxidative functions. Macrophages also participate in inflammatory response by releasing cytokines and factors that promote cell recruitment to the injured tissue or inflammation site or during the infection [36]. In our work we have showed that macrophages in presence of NANO-PEG/GLY complex increase both cytokines with activity pro-inflammatory such IL-1β, IL-6 and TNF-α and cytokines anti-inflammatory as IL-10 suggeting a balance between these mediators, since it is characteristic of the immunological components of colostrum act together without causing an inflammatory response [37]. Interesting that the macrophages in presence of NANO-PEG/GLY complex increase de IL-17. This cytokines is typical produced by the Th17 T cell subset, but study has demonstrate that macrophages also express Th17 cytokines [38]. In general, the effects of Th17 and the mechanism underlying its action in conditions of systemic inflammation. On the other the presence of IL-17 impairs monocyte/macrophage apoptosis and induces intense differentiation, guaranteeing efficient removal of apoptotic neutrophils and restoration of anti-inflammatory conditions and suggest an unexpected role of IL-17 in the resolution of inflammation [39], that can be important during the phagocitosis and microbicidal process by macrophages. Glycine has therapeutic properties in many models of inflammatory processes [40]. However, cytokines are mediators necessary to conduct the inflammatory response to the sites of infection. The exaggerated production of proinflammatory cytokines from the lesion may manifest systemically with hemodynamic instability or metabolic disorders. Here the microbicidal activity promoted by the presence of NANO-PEG/GLY in phagocytes associated with the balance between pro-inflammatory and anti-inflammatory cytokines may have important clinical implications during the infections.

V. CONCLUSION

The date suggest that NANO-PEG particles produced were able to adsorb the amino acid glycine, and this new bioengineered material is capable of modulating the functional activity of human colostrum macrophages and

represents an alternative route for the treatment of inflammatory deseases.

ACKNOWLEDGEMENTS

This work was supported by the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), Brazil, Fundação de Apoio a Pesquisa de Mato Grosso (FAPEMAT), Brazil.

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