Intracellular calcium is a target of modulation of apoptosis in MCF-7 cells in the presence of IgA adsorbed to polyethylene glycol

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Purpose: Clinical and epidemiological studies have indicated that breastfeeding has a protective effect on breast cancer risk. Protein-based drugs, including antibodies, are being developed to attain better forms of cancer therapy. Secretory IgA (SIgA) is the antibody class in human breast milk, and its activity can be linked to the protective effect of breastfeeding. The aim of this study was to investigate the effect of polyethylene glycol (PEG) microspheres with adsorbed SIgA on MCF-7 human breast cancer cells.

Methods: The PEG microspheres were characterized by flow cytometry and fluorescence microscopy. The MCF-7 cells were obtained from American Type Culture Collection. MCF-7 cells were pre-incubated for 24 hours with or without SIgA (100 ng/mL), PEG microspheres or SIgA adsorbed in PEG microspheres (100 ng/mL). Viability, intracellular calcium release, and apoptosis in MCF-7 cells were determined by flow cytometry.

Results: Fluorescence microscopy and flow cytometry analyses revealed that SIgA was able to adsorb to the PEG microspheres. The MCF-7 cells that were incubated with PEG microspheres with adsorbed SIgA showed decreased viability. MCF-7 cells that were incubated with SIgA or PEG microspheres with adsorbed SIgA had increased intracellular Ca²⁺ levels. In the presence of SIgA, an increase in the percentage of apoptotic cells was observed. The highest apoptosis index was observed when the cells were treated with PEG microspheres with adsorbed SIgA.

Conclusion: These data suggest that colostral SIgA adsorbed to PEG microspheres has antitumor effects on human MCF-7 breast cancer cells and that the presence of large amounts of this protein in secreted breast milk may provide protection against breast tumors in women who breastfed.

Keywords: MCF-7 cells, colostrum, SIgA, apoptosis, PEG microsphere

Introduction
Clinical and epidemiological studies have shown that breastfeeding has a protective effect on breast cancer risk.¹⁻³ Accumulating evidence has suggested that human breast milk may confer long-term benefits, such as reduced risks of certain autoimmune diseases, inflammatory bowel disease, and certain malignancies. Some studies have also indicated that a reduced incidence of breast cancer is the most well-documented long-term effect of breastfeeding on mothers;⁴ however, the degree to which some of these health benefits may be realized depends on the breastfeeding duration, breastfeeding frequency, breastfeeding exclusivity, and other personal factors.⁵

Efforts have been directed to identify the various immunoreactive substances in human breast milk that account for the protective effects of breastfeeding against diseases.⁶⁻⁷ However, additional studies must be conducted to elucidate the mechanisms...
involved in the protective effects of breastfeeding on reducing the risk of breast cancer.²

Human breast milk is particularly rich in secretory IgA (SIgA) antibodies,³–¹⁴ which play a protective role against several diseases. These antibodies neutralize pathogens and simultaneously limit the damaging effects of tissue inflammation caused by other antibody types.¹⁵ The biological activity of SIgA is important because this protein is the primary antibody class in human breast milk¹²,¹⁶ and because its activity can most likely be linked to the effect of breastfeeding on the reduced incidence of breast cancer.

Breast cancer therapy includes radiotherapy, chemotherapy, endocrine therapy, and biological agents, and treatments are increasingly being tailored to the individual tumor and patient to provide the maximum survival benefit with minor toxicity.¹⁷

However, protein formulation development and protein integration into modified drug release systems are critical steps in developing proteins as therapeutic products. Protein-based drugs, including antibodies, are being developed to attain better forms of therapy. The best formulation should be biocompatible, have minor toxicity, a good degree of tissue-specific action, and be easily prepared and administered and should not be cost-prohibitive.¹⁸,¹⁹

Polyethylene glycol (PEG) microspheres are polymeric particles that have the capacity to adsorb organic compounds and are considered a major drug carrier.²⁰ The adsorption capacity of microspheres for organic compounds can be modified to improve their biological function.²¹ In a previous study, we showed that PEG microspheres are a promising agent for the delivery of amino acids, hormones, and medicinal plants because these microspheres can prevent the degradation of and increase the bioavailability of these substances within the organism, thus improving the immune function.²²–²⁸

Nevertheless, no studies have linked SIgA adsorbed to PEG microspheres to the mechanisms of tumor cell growth; however, SIgA adsorbed to PEG microspheres may have an effect on breast tumor cells. The aim of this study was to investigate the effect of PEG microspheres with adsorbed SIgA on MCF-7 human breast cancer cells.

Materials and methods

Ethics

This study was approved by the Institutional Research Ethics Committee of the Campus of Araguaia of Federal University of Mato Grosso, Brazil (Protocol Number CAAE: 45102815.3.0000.5587) and all the subjects gave informed written consent for the use of their colostrum.

Cell culture

MCF-7 human breast cancer cells were obtained from American Type Culture Collection (ATCC, Manassas, VA, USA). The cells were maintained as monolayer cultures in 75 cm² plastic culture flasks in Roswell Park Memorial Institute (RPMI) 1640 medium supplemented with 10% fetal bovine serum (Sigma-Aldrich Co., St Louis, MO, USA), penicillin (20 U/mL) and streptomycin (20 µg/mL) (Sigma-Aldrich Co.) at 37°C in a humid atmosphere containing 5% CO₂. The cells were sub-cultured every 5±2 days.

Colostral purified SIgA

Human colostrum SIgA was purified from a defatted colostrum pool by affinity chromatography on Cyanogen bromide-activated Sepharose-4B (CNBr-Sepharose-4B; Sigma-Aldrich Co.) bound with sheep anti-human α chain as proposed by March et al.²⁹ To ensure SIgA depletion, fractions eluted from the affinity chromatography column were pooled and passed five times through the same column. Bound SIgA was eluted from the column with 6N glycine-HCl buffer, pH 2.8. The purified preparations were restored to the initial volume. The concentration of SIgA was determined by simple radial immunodiffusion with a sheep anti-human α chain serum on agarose plates.¹⁶ Total protein concentration was determined by Lowry Method. The purified SIgA preparation was also tested by immunoelectrophoresis with goat anti-human γ and μ chain antisera. Both IgG and IgM were undetectable in the preparation. The purified SIgA was 4.0 g/L adjusted to a concentration of 100 ng/mL. The aliquots were stored at −80°C and subsequently used for experiments.

PEG microsphere preparation

The microspheres were obtained from PEG 6000 using a modification²²,²³ of a previously described protocol.²¹ 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 minutes. After incubation, the PEG microspheres were diluted 3:1 in PBS and washed twice in PBS (centrifuged for 500× g, 5 minutes). The PEG microspheres were resuspended in PBS. The formation of the microspheres was thermally induced by subjecting the solution to 95°C for 5 minutes. For adsorption, the suspensions of PEG microspheres in PBS were incubated with SIgA (Sigma-Aldrich Co.; concentration 100 ng mL⁻¹) at 37°C for 30 minutes. After this period the PEG microspheres with adsorbed SIgA were washed twice in PBS (centrifuged for 500× g, 5 minutes). For verifying the loading efficiency of adsorption, the PEG microspheres with or without adsorbed
SLgA were fluorescently labeled overnight at room temperature with a solution of Dylight-488 (Pierce Biotechnology, Rockford, IL, USA; 10 µg mL⁻¹) in dimethylformamide at a 100:1 molar ratio of PEG:Dylight. The samples were then analyzed by fluorescence microscopy.

Characterization of the PEG microspheres by flow cytometry
Immunofluorescence staining was performed with phycoerythrin (PE; Sigma-Aldrich Co.) to compare the abilities of the PEG and polymethylmethacrylate microspheres (CaliBRITE; BD Biosciences, San Jose, CA, USA) to bind fluorescent markers. The PEG microspheres were incubated with 5 µL of PE (0.1 mg mL⁻¹) for 30 minutes at 37°C. After the incubation, the PEG microspheres were washed twice in PBS containing BSA (5 mg mL⁻¹; centrifuged for 500×g, 10 minutes, 4°C). In all of the experiments, the PEG microspheres were analyzed by flow cytometry. The study was performed on a FACSCalibur (BD Biosciences). The PEG microspheres sizes were compared to those of the BD microspheres (6 µm CaliBRITE 3 Beads, BD Biosciences catalog number 340486) bound or unbound to phycoerythrin (PE). The fluorescence intensity of the PEG microspheres was expressed as the geometric mean fluorescence intensity, and the size was calculated according to the geometric mean of the forward scatter.

Immunofluorescence of adsorbed SLgA
The PEG microspheres with or without adsorbed SLgA were fluorescently labeled with a F(ab')2 fragment of anti-human IgA conjugated to fluorescein isothiocyanate (FITC) (Sigma-Aldrich Co.) for 30 minutes at 4°C. The microspheres were analyzed by flow cytometry in all experiments (FACSCalibur; BD Biosciences).

Release of IgA adsorbed in PEG (PEG-IgA) microspheres
After adsorption of the IgA (100 ng/mL) onto the PEG microspheres the preparation was resuspended in RPMI culture medium to verify the IgA release. The PEG-IgA microspheres were incubated for 2, 12, and 24 hours at 37°C with 5% CO₂. After this period, the suspensions of PEG-IgA were centrifuged for 10 minutes at 160×g. The IgA release in supernatant was determined using the IgA human enzyme-linked immunosorbent assay (Abcam, Cambridge, UK). Reaction rates were measured by absorbance plate-reading spectrophotometer with a 450 nm filter. The results were calculated according to the standard curve.

Cell treatment
To explore cell viability (propidium iodide [PI] permeability test), apoptosis induction (annexin V staining), and intracellular calcium release (fluor3-acetoxymethyl staining), sub-confluent (80%) monolayers of MCF-7 cells were treated with a concentration of trypsin (Sigma-Aldrich Co.) adjusted for 5×10⁶ cells/mL. Then, the trypsinized cells were pre-incubated for 24 hours with or without 50 µL of SLgA (100 ng/mL final concentration), 50 µL of PEG microspheres or 50 µL of SLgA adsorbed in PEG (PEG-IgA) microspheres (100 ng/mL final concentration). Next, the cells were diluted in RPMI 1640 medium supplemented with 10% fetal bovine serum (Sigma-Aldrich Co.), penicillin (20 U/mL), and streptomycin (20 µg/mL) (Sigma-Aldrich Co.) at 37°C for 24 hours in a humid atmosphere containing 5% CO₂.

Cellular viability
After 24 hours of treatment, the culture medium was removed, and the cells were washed twice with PBS. The viability test was performed using PI fluorescence assay. Then, the cells were stained with 10 µL of PI (1 mg/mL), Triton X-100 (5.5%), and ethylenediamine tetraacetic acid (110 mM) and incubated for 10 minutes at room temperature. Unstained cells were used as controls. The fluorescence of the cells was analyzed by flow cytometry (FACSCalibur system). The percentage of viable cells was conversely proportional to the geometric mean fluorescence intensity of PI.

Intracellular Ca²⁺ release determination
We analyzed fluorescence staining using a FACSCalibur system to assess intracellular Ca²⁺ release in MCF-7 cells. Cells were loaded with the fluorescent radiometric calcium indicator fluo3-acetoxymethyl (Fluo3-AM; Sigma-Aldrich Co.). Cell suspensions were incubated with 5 µL of fluo3-acetoxymethyl (1 µg/mL) for 30 minutes at 37°C. After incubation, the MCF-7 cells were washed twice in PBS containing BSA (5 mg/mL; centrifuged for 160×g, 10 minutes, 4°C) and then analyzed by flow cytometry (FACSCalibur system). Fluo-3 was detected using a 530/30 nm filter for intracellular calcium release (fluo3-acetoxymethyl stain). The rate of intracellular Ca²⁺ release was expressed as the geometric mean fluorescence intensity of Fluo-3.

Apoptosis assay
Annexin V staining was used to assess apoptosis. Untreated cells were used as negative controls, and cells treated with staurosporine (Sigma-Aldrich Co.), which was used to induce apoptosis, were used as positive controls according to Pundt.
et al. The cells were resuspended in 500 µL of binding buffer containing 5 µL of annexin V-FITC (Annexin V-FITC Apoptosis Detection Kit, Alexis™, San Diego, CA, USA) and 5 µL of PI and then incubated for 10 minutes at room temperature. The fluorescence of the cells was analyzed by flow cytometry (FACSCalibur system). The obtained data were analyzed using CellQuest software. The cells were classified as follows: viable cells (annexin−/PI−), early apoptotic cells (annexin+/PI−), late apoptotic cells (annexin+/PI+), and necrotic cells (annexin+/PI+).

**Separation of colostral cells**

To verify whether PEG or PEG-IgA induces apoptosis in normal cells, ~8 mL samples of colostrum from five women were collected in sterile plastic tubes between 48 and 72 hours postpartum. The samples were centrifuged (160 × g, 4°C) for 10 minutes, which separated the colostrum into three different phases: cell pellet, an intermediate aqueous phase, and a lipid-containing supernatant. The upper fat layer and the aqueous supernatant were discarded. Cells were separated by a Ficoll-Paque gradient (Pharmacia, Uppsala, Sweden), producing preparations with 98% pure mononuclear (MN) cells, analyzed by light microscopy. Purified MN cells were resuspended independently in serum-free medium 199 at a final concentration of 2×10⁶ cells/mL. The cells were used for apoptosis assays.

**Statistical analysis**

An analysis of variance (ANOVA) was used to evaluate cellular proliferation, culture supernatant calcium levels, and the intracellular calcium and apoptosis index of MCF-7 cells in the presence or absence of PEG microspheres with adsorbed SIgA. Statistical significance was considered when P<0.05.

**Results**

Figure 1A shows a fluorescence microscopy image of PEG microspheres produced in PBS. This result confirmed that our method produces microspheres that are easily separated in the suspension. The produced microspheres retained their spherical structure without deformation (Figure 1A). Fluorescence microscopy showed that PEG microspheres are able to adsorb SIgA, which is distributed throughout the PEG microsphere surface (Figure 1B). To confirm the binding of SIgA to the microspheres, we used an F(ab′)2 fragment of anti-human IgA conjugated to FITC. Figure 2 shows the positive binding of SIgA on the PEG microsphere surface.

Figure 3A compares the PEG microspheres, PEG microspheres with adsorbed SIgA, and BD microspheres (standard) in terms of fluorescence intensity. The PEG microspheres showed a lower geometric mean fluorescence intensity when compared with the BD microspheres. The PEG microspheres with adsorbed SIgA showed geometric mean fluorescence intensity values similar to those of the BD microspheres. The PEG microspheres with adsorbed SIgA were also similar in size to the standard microspheres (Figure 3B and C).

The release of SIgA adsorbed in PEG microspheres during 24 hours is illustrated in Figure 4. The SIgA release increased over time; after 24 hours, more than 90% (92.4 ng/mL) of the antibody had been released.

The viability of MCF-7 cells is shown in Figure 4. The MCF-7 cells which were not stimulated had higher viability. The PEG microspheres did not alter cell viability. When the MCF-7 cells were incubated with SIgA, cell viability decreased. MCF-7 cells had a lower viability index when incubated with PEG microspheres with adsorbed SIgA (Figure 5).

Figure 6 shows the rate of intracellular Ca²⁺ release in MCF-7 cells as measured by Fluo-3 fluorescence intensity. MCF-7 cells had low spontaneous intracellular Ca²⁺ release. When these cells were incubated with SIgA or PEG microspheres with adsorbed SIgA, they had increased intracellular Ca²⁺ levels (Table 1). Intracellular Ca²⁺ release was higher in MCF-7 cells treated with PEG microspheres with adsorbed SIgA compared with cells treated with PEG microspheres.

To evaluate apoptosis induction in MCF-7 cells, cells were stained with annexin V and analyzed by flow cytometry (Figure 7). The control MCF-7 cells showed low levels of apoptosis. When these cells were incubated with PEG, we observed an increase in necrotic cells. In the presence of SIgA, the percentage of apoptotic cells increased. The highest apoptosis index was observed when the cells were treated with PEG microspheres with adsorbed SIgA (Table 2).
The apoptosis index of colostral MN cells was also evaluated. The rates of apoptosis (%) were 6.75±2.78 in cells incubated with PBS, 14.35±3.55 in cells treated with PEG microspheres, 11.42±1.50 in cells incubated with SlgA, and 17.84±2.35 in cells treated with PEG microspheres with adsorbed SlgA.

**Discussion**

The protective effects of breastfeeding on breast cancer risk have been difficult to study due to the high correlation with parity. Reproductive factors may induce permanent changes in the mammary gland epithelium or in the surrounding tissue. This study suggests that breastfeeding may provide protection against breast cancer by inducing apoptosis in breast cancer cells.
stromal tissue. These tissue changes most likely make the breast more or less susceptible to carcinogenic factors. Secretory immunity is of great importance as a natural barrier because S IgA forms the first line of defense against pathogens and other potentially harmful agents. In this study, we produced PEG microspheres with adsorbed colostral S IgA and used an in vitro model to demonstrate that this biocomposite showed antitumor activity against MCF-7 human breast cancer cells, as evidenced by the decreases in cell viability, intracellular calcium release, and apoptosis.

Microsphere-based polymeric substances can be employed as release delivery systems for drugs and therapeutic proteins. In this study, fluorescence microscopy and flow cytometry showed that the PEG microspheres had ellipsoid shapes and were easily separated from the suspension. Flow cytometric analysis indicated that the PEG microspheres had a size of approximately 5.1 µm. The adsorption of colostral S IgA enhanced the microsphere size to approximately 5.7 µm, suggesting that S IgA may bind at the same site as the marker. The adsorption of S IgA to PEG microspheres was confirmed using monoclonal anti-IgA antibody conjugated to FITC. These data are consistent with previous studies, which found that PEG microspheres changed in size or in their ability to bind to fluorescent substances after the adsorption of bioactive molecules.

When a drug or protein is administered using a carrier, the drug/protein clearance decreases (half-life increases), volume of distribution decreases, and the area under the time versus concentration curve increases. The carrier also maintains the drug/protein for an adequate period until the drug or protein reaches its required site of action and then releases it in a controlled fashion.

### Table 1: Release of Intracellular Ca²⁺ by MCF-7 Cells in the Presence of S IgA Adsorbed to PEG Microspheres

| Medium       | Fluorescence intensity (Mean ± SD) |
|--------------|-----------------------------------|
| PBS          | 5.61 ± 1.61                       |
| PEG          | 6.70 ± 1.59                       |
| S IgA        | 14.91 ± 1.09*                     |
| PEG + S IgA  | 15.05 ± 2.10**                    |

**Notes:** Intracellular Ca²⁺ release is represented by mean fluorescent intensity as determined by flow cytometry. The results represent the mean ± standard deviation (SD) of five experiments. *P < 0.05: treated cells compared with non-treated cells (PBS); **P < 0.05: comparing the different treatments (S IgA compared with PEG-S IgA).

**Abbreviations:** PBS, phosphate-buffered saline; PEG, polyethylene glycol; S IgA, secretory immunoglobulin A; PEG-S IgA, S IgA adsorbed to PEG microspheres.
action. In this study we verified that PEG-IgA microspheres had a release profile that was satisfactory in terms of concentration and time.

The functions of SIgA in antigen-specific immunity have been shown to contribute in novel ways to the regulation of innate immunity. SIgA protects against a number of microorganisms by acting as an opsonin, which blocks bacterial adherence to epithelial cells, neutralizes toxins, and prevents viral infections, and by simultaneously limiting the damaging effects of tissue inflammation caused by other antibody types.

Here, the PI fluorescence assay was used to verify that colostral SIgA decreased the growth of human MCF-7 cells; this inhibition of cellular growth was more evident when the cells were treated with SIgA adsorbed to PEG microspheres. These data show that the PEG microspheres are able to modify the release of SIgA and suggest that colostral SIgA presents anti-tumor properties and that SIgA is an important component of human breast milk that may decrease the risk of breast cancer.

Table 2 Apoptosis (%) and necrosis (%) of MCF-7 human breast cancer cells in the presence of SIgA adsorbed to PEG microspheres

| Viable | Apoptosis (Q2 + Q3) | Necrosis | Q4 |
|--------|---------------------|----------|-----|
| MCF-7  | 93.1 ± 0.8          | 3.1 ± 0.6 | 3.8 ± 1.8 |
| MCF-7 + Peg | 80.2 ± 0.6*       | 4.9 ± 0.5 | 14.5 ± 0.7* |
| MCF-7 + SIgA | 49.7 ± 8.3*       | 41.2 ± 10.3*       | 9.1 ± 0.9* |
| MCF-7 + Peg-SIgA | 31.3 ± 4.5*      | 55.8 ± 7.1*      | 12.9 ± 1.7* |

Notes: The results represent the mean ± standard deviation of five experiments. Q1: viable cells (annexin-V/Pi-); Q2: (annexin-V/Pi-); Q3: (annexin-V/Pi+); total apoptotic cells; Q4: necrotic cells (annexin-V/Pi+). *P<0.05: treated cells compared with non-treated cells; **P<0.05: comparing the different treatments (SIgA and Peg microspheres).

Abbreviations: Peg, polyethylene glycol; SIgA, secretory immunoglobulin A; Pi, propidium iodide; Peg-IgA, SIgA adsorbed to Peg microspheres.

Figure 7 PEG microspheres with adsorbed SIgA induce apoptosis in MCF-7 human breast cancer cells.

Notes: Modes of cell death were established using flow cytometry with annexin V/propidium iodide (PI) staining. (A) MCF-7 cells incubated with RPMI 1640 medium; (B) MCF-7 cells incubated with PEG microspheres; (C) MCF-7 cells incubated with SIgA; (D) MCF-7 cells incubated with SIgA adsorbed to PEG microspheres. The summation of the upper-right (Q3) and lower-right (Q2) quadrants is presented as the percentage of total apoptosis. The upper-left (Q4) quadrant is the percentage of necrosis, and the lower-left (Q1) quadrant corresponds to viable cells. Data are representative of an experiment with the different treatments.

Abbreviations: SIgA, secretory immunoglobulin A; Peg, polyethylene glycol; RPMI, Roswell Park Memorial Institute; Pi, propidium iodide.
In conclusion, these data suggest that colostral SIgA adsorbed to PEG microspheres has antitumor effects on human MCF-7 breast cancer cells and that the presence of large amounts of this protein in secreted human breast milk may provide protection against breast tumors in women who breastfed. In addition, the modified delivery of SIgA may represent a possible alternative therapy for breast cancer treatment.

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Author contributions
AC Honorio-França and EL França conceived the study, carried out the assays, and participated in its design and co-ordination, and helped to draft the manuscript.

GT Nunes, DLG Fagundes, PGF de Marchi, RTS Fernandes, and JL França participated in the collection of samples, carried out the assays, and helped to draft the manuscript.

AC França-Botelho, LCA Moraes, and FP Varotti carried out the assays, participated in the sequence alignment, and helped to draft the manuscript.

All authors read and approved the final version of the manuscript.

Disclosure
The authors declare no conflicts of interest in this work.

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