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Modified Caffeine Release System and Its Immunomodulatory Effects on Breast Tumor Cells and Blood Phagocytes

Tatiane Araújo Soares[1,2], André Henrique Furtado Torres[3,4], Letícia Damas Leão Dalcin[1], Luis Carlos Oliveira Gonçalves[1], Anibal Monteiro de Magalhães Neto[1], Adenilda Cristina Honorio-França[1,4], Elton Brito Ribeiro[1], Eduardo Luzía França[1,4]

[1]Postgraduate Program in Imunology and Parasitology, Federal University of Mato Grosso, Barra do Garças, Brazil
[2]Department of Biotechnology, Biotechnology in Regenerative Medicine and Medicinal Chemistry, University of Araraquara (UNIARA), Araraquara, Brazil
[3]Department of Biotechnology, São Paulo State University (Unesp), Institute of Chemistry, Araraquara, Brazil
[4]Postgraduate Program in Materials Science, Federal University of Mato Grosso, Barra do Garças, Brazil

Email: eduardo-franca@ufmt.br

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Abstract

Caffeine is an alkaloid present in a wide variety of plants. Currently the most consumed psychostimulant worldwide, its consumption is associated with several health benefits, including modulation of the innate and adaptive immune response, reduction of oxidative cellular stress, and decreased incidence of some cancers, including breast cancer. Breast cancer is the most common cause of cancer among women and the second leading cause of cancer death in women worldwide. The interaction between biomaterials and drugs has enabled a great advance in science for developing controlled drug delivery systems and has been used to treat numerous pathologies. This work aimed to evaluate the immunomodulatory effects of caffeine associated or not with polyethylene glycol adsorbed in microemulsion (MLP) on MCF-7 cells, phagocytic cells (MN), and coculture. For biological assays, ATCC (American Type Culture Collection, USA) cell lines of breast adenocarcinoma (MCF-7) and phagocytes (MN) obtained from voluntary donors were used. The cells (MN and MCF-7) and coculture were treated with caffeine and MLP and incubated for rheological characterization analyses: flow curve and viscosity, oxidative stress: superoxide anion assay (O_2^-), and activity of the enzyme superoxide dismutase (Cu-Zn-SOD). Caffeine and MLP increased viscosity and blood and MCF-7 cells and affected the immunomodulation of oxidative stress metabolism of MN and MCF-7 cells treated with caffeine and associated caffeine to the MLP. These data suggest that caffeine is associated or not with MLP-induced immunomodulatory effects on MN phagocytes and

MCF-7 cells, demonstrating the antitumor activity via oxidative stress and can be a complementary alternative for treating breast cancer.

Keywords

Breast Cancer, Microemulsion, Polyethyleneglycol, Caffeine, Oxidative Stress

1. Introduction

Breast cancer is the type of cancer that mostly affects women and is the second leading cause of cancer death worldwide [1] [2]. According to the Global Cancer Observatory, in 2018, it was the cancer with the highest incidence, with approximately 2 million cases recorded worldwide, estimated to increase to more than 3 million in 2040 [3]. Breast cancer is rare in women under the age of 25, but with increasing age, the incidence also increases. For example, a woman who lives up to 90 years has a chance in eight to develop breast cancer.

The number of diagnosed cases increased significantly after 1980 due to the introduction of mammography screening [4]. However, a stable screening rate is currently observed in 65% to 75% of women. The main factors contributing to the development of breast cancer are advanced age, genetic factors, hereditary mutations, first-degree relative with breast cancer, life habits, first pregnancy after 30 years of age, early menarche (11 years or less), late menopause (55 years or more), the decline in breastfeeding and obesity [5]. Breast cancer prevention strategies are divided into primary prevention and secondary prevention. Primary prevention applies to moderation in alcohol consumption, regular physical activity, and healthy body weight control [6] in the secondary prevention strategy aims at early detection of breast cancer through screening [7].

The immune system plays an integral and complex role in the biology of breast cancer [8] [9]. The tumor microenvironment comprises cancerous and non-cancerous cells, including fibroblasts, adipocytes, endothelial cells, and immune cells (e.g., macrophages, lymphocytes, and NK cells) that recognize and destroy cancer cells primarily through cytotoxic mechanisms [3]. Doxorubicin [10] and cisplatin [11] are examples of effective chemotherapy for the treatment of breast cancer.

Breast cancer treatment varies and usually involves surgery, radiotherapy, and chemotherapy. In recent years, molecular target therapy, immunotherapy, and modified release system are gaining strength. Chemotherapy is the main approach for the treatment of metastatic tumors. However, it is associated with serious side effects such as bone marrow suppression, gastrointestinal reaction, and liver and kidney damage, as well as low specificity and toxicity to normal healthy cells [12] [13] [14].

Modified release systems are a promising therapeutic alternative, enabling the administration of biologically active molecules at a specific location with reduced therapeutic levels and decreased side effects. In this context, microemul-

sions (ME) and Polyethylene Glycol-400 (PEG-400) stand out as interesting pharmaceutical forms for the dissemination of molecules with therapeutic activity [15] [16].

Microemulsions are water, oil, optically isotropic, thermodynamically stable [17] [18] [19], and low viscosity systems with a diameter of 10 to 100 nm, which form after mixing oil, water, and tensoactives [20] [21]. The tensoactive can be pure, mixed, or combined with other components, whose main function is reducing interfacial tension [22]. These mycelial systems may better solubilize drugs or unsolvable materials besides improving their bio-pharmaceutical and pharmacokinetic properties [23]. PEG-400 is a polymer that forms microparticles with various pharmacological properties in modulating and prolonging drug action [24]. PEG-400 is a polymer that forms microparticles with various pharmacological properties in modulating and prolonging the action of drugs. They are promising because they prevent the degradation of adsorbed substances. The administration of PEG-adsorbed drugs has been an alternative treatment for some diseases, including breast cancer [25] [26].

Caffeine is an alkaloid present in more than sixty plant species. The main sources are beans, coffee beans and leaves, cola nuts, cocoa beans, yerba mate, and guarana [27] [28]. It can influence cognitive performance by increasing alertness and wakefulness and can also improve performance in memory tasks [29] [30] [31].

Caffeine consumption is associated with some health benefits, including reduced risk of cardiovascular disease, lower incidence of diabetes mellitus and Alzheimer's disease, decreased mortality from inflammatory diseases [32] [33], and decreased incidence of some types of cancers, including colorectal, colon, endometrial, prostate and breast cancer [34] [35] [36].

Caffeine can modulate both the innate and adaptive immune response [37]. It affects the cell cycle [38] and acts on protein kinases that play important roles in repairing DNA damage, which induces strain of the cell cycle in phase G1 and signaling apoptosis [39]. It also can increase antioxidant defenses [38], and chronic intake improves oxidative stress [40]. Strategies to eliminate tumor cells include modulation of oxidative metabolism and strengthening of antitumor effects of drugs associated with a modified release system [25] [41]. Since caffeine contains antioxidant properties [42] [43] that probably modulate the oxidative stress of tumor cells, the present study produced a controlled release system + PEG-400 adsorbed with caffeine and tested its effects on breast adenocarcinoma cells (MCF-7), mononuclear cells (MN) and in coculture with blood MN cells.

2. Material and Methods

2.1. Composition of the Microemulsified System Containing PEG 400

Polyethylene glycol adsorbed in microemulsion (MLP) was formulated from an aqueous phase (distilled water), oily phase (isopropyl myristate-IPM®-EHL 11.5 (Bianquimica®, São Paulo, Brazil)), tensoactive (polysorbate 80-Tween 80® (TW)-

EHL = 15.0 (Dynamics®, São Paulo, Brazil)) and polyethylene glycol 400-PEG 400@EHL = 8.5 (Dynamics®, São Paulo, Brazil) modified according to the method of Torres *et al.* (2021) where each component ranged from 10% to 75%. Being used for formulation, 10% of the oily phase, 15% of the phase aqueous, and 75% of tensoactive [44].

2.2. Caffeine Preparation

Caffeine was weighed on an analytical scale, diluted in Buffered Saline Solution (PBS), and adjusted at a 100 ng/mL concentration. Diluted caffeine was associated with the microemulsified system containing PEG 400 in the equivalent amount of the aqueous phase of the formulation, where diluted caffeine was homogenized with PEG 400, plus the oily phase, and then the tensoactive was added. Caffeine concentration was determined based on a previous study in which cellular modulation was observed [26].

2.3. Obtaining and Separating Mononuclear Cells (MN) from Human Peripheral Blood

A 10 mL of the peripheral blood sample from 18 clinically healthy donors aged 18 to 35 was collected in Vacutainer tubes with EDTA (Beckton Dickinson, Franklin Lakes, NJ, USA®) to obtain MN cells. Then, the cell populations were separated by density gradient with Ficoll-Paque (Pharmacia, Upsala, Sweden) by centrifugation for 40 minutes at 160 ×g at laboratory temperature (25˚C). Next, the mononuclear phagocyte ring (MN) was removed with a Pasteur pipette and centrifuged twice at 160 ×g for 10 minutes with buffered saline solution (PBS) for cell washing. Finally, the supernatant was discarded, and the pellet was added 1 ml of PBS. The cells were then counted in the Neubauer chamber, and the cell concentration was adjusted to 2×10^6 cells/mL according to Honorio-França [45].

2.4. MCF-7 Cell Culture

ATCC (American Type Culture Collection, USA) cell lines of breast adenocarcinoma (MCF-7) were used for the biological assays with the immunomodulator. The cells were cultivated in RPMI medium (Sigma), plus 10% Bovine fetal serum (FBS, Cultilab), 1.0 ml pyruvate, 1.0 ml Hepes, 4.5 ml glucose, 100 µl gentamicin, the final volume of 100 ml of the medium. The cells were grown in bottles and maintained at 5% CO_2 and 37˚C until the formation of a cell monolayer. Every 96 hours, the cells were removed from the CO_2 oven, added in a 15 ml falcon tube, and 1 ml of trypsin was placed in each bottle for 5 minutes. Trypsin was removed from the bottles and added to the corresponding falcon tubes. The bottles with RPMI medium were washed and added to the corresponding falcon tubes to avoid cell loss. The cells in the falcon tube were centrifugated for 10 minutes at 160 g. The Avernum was discarded, and the pellet was suspended in a 2 ml RPMI medium. Next, 1 ml of MCF-7 cells were added in two new bottles; each bottle was completed with 4 ml of RPMI medium and taken to a CO_2 kiln repeating the cycle until the number of cells suitable for the experiment was ob-

tained.

2.5. Superoxide Anion (O_2^-)

The MN and MCF-7 cells were incubated with caffeine associated or not with polyethylene glycol adsorbed in microemulsion to verify the release of superoxide anion, using the chromogen Ferricitochrome C, according to the Pick and Mizel method [46] and adapted by Honório-França *et al.* [47]. They were analyzed by spectrophotometer at 550 nm absorbance. For the assays, the samples were divided into groups containing 500 µL of MN, MCF-7 cells, and cocultures incubated with 50 µL of caffeine associated or not with polyethylene glycol adsorbed in microemulsion and without stimuli used as control.

The samples were incubated for 24 hours at 37˚C in a greenhouse with 5% CO_2.

At the and of this time, the samples were centrifuged at 1600 g for 10 min. The supernatant was collected and reserved for analysis of the SOD enzyme, while the pellets were resuspended in 150 µL of ferricytochrome C solution (2 mg/mL of glucose PBS). Subsequently, the samples were transferred to a 96-well plate and accommodated in an oven at 37˚C for 1 hour without light. Finally, the reading was performed in a spectrophotometer (Thermo Plate TP-Reader) with a 540 nm filter.

The concentration of the superoxide anion was calculated according to the method adapted from Pick and Mizel [46] by the equation:

$$\text{Concentration } O_2^- = (\text{DO}/6.3) \times 100$$

where: DO: Optical density.

2.6. Superoxide Dismutase Enzyme (Cu-Zn-SOD)

The spectrophotometer performed the enzymatic activity of superoxide dismutase (Cu-Zn-SOD) at 560 nm absorbance. For the dismutation assays, 500 µL of the cell suspension overeating was pipetted in different tubes, and the stimulus was removed from the O_2^- dosage. Then, in each tube, in the following order were added: 500 µL of the chloroform-ethanol mixture (1:1), 500 µL of the reactive mixture of Nitro Bluetetrazolium (NBT) and tetraacetic ethylenediamine acid (EDTA) (1:1.5) and 2 mL of carbonate and hydroxylamine buffer solution. For calibration of the device, from the reactive mixture of NBT and EDTA. The standard absorbance was measured from the following solutions: 500 µL of the hydroalcoholic mixture (1:4), 500 µL of the chloroform-ethanol mixture (1:1), 500 µL of NBT and EDTA reactive mixture, and 2 mL of carbonate buffer and hydroxylamine.

SOD values were expressed in SOD g^{-1} units, *i.e.*, in terms of enzyme activity, a SOD unit is defined as the amount of enzyme required to inhibit 50% of the NBT reduction. The equation gives the calculation of % inhibition:

$$\%\ \text{reduction of NBT} = (\text{Abs. standard} - \text{Abs. sample})/\text{Abs. standard} \times 100$$

where:

Abs. standard: Absorbance of standard sample;

Abs. Sample: Absorbance of each sample at 560 nm.

2.7. Rheological Characterization

Rheological parameters were determined in Modular Compact Rheometer—MCR 102 (Anton Paar®, GmbH, Ostfildern, Germany). For the hemorheological analyses, 600 µL of blood and 60 µL of MLP stimulus and caffeine associated or not with polyethylene glycol adsorbed in microemulsion were added to the surface of the reading plate, and the excess sample was removed. The readings were performed with permanent control of the measurement gap with TruGap™ support at 0.099 mm, unit measuring cell Toolmaster™ CP 50 (angle 1˚), and precise temperature control with T-Ready feature™, using Software Rheoplus V3.61. The rheological charts were all treated with Rheoplus Software. For the flow and viscosity curves, the established parameters were based on the control of shear stress (τ), and the last 5 points of the ascending curve and the first 5 points of the descending curve for statistical analysis were used, modified according to the method of France *et al.* [48]. The tests were carried out under isothermal conditions at 37˚C.

2.8. Statistical Analysis

Statistical analyses were performed in the BioEstat 5.3 program through a variance analysis (ANOVA), followed by the Tukey test for the analyses that presented $p < 0.05$. Spearman's test performed the correlation between the values of the viscosity and apoptosis assays.

3. Results and Discussion

3.1. Hemorreological Evaluations of Blood Treated with Caffeine Associated or Not with Polietylene Glycol Adsorbed in Microemulsion

The rheological evaluations of the blood with caffeine associated or not with polyethylene glycol adsorbed in microemulsion (MLP) were able to alter the rheological behavior, increasing viscosity (**Figure 1**) and altering the behavior of the blood (**Figure 2**). A recent study with melatonin associated with microemulsion and polyethylene glycol 400 (MLMP) also showed that there was a rheological change, where it was observed that the viscosity of the blood treated with MLMP increased [44].

A similar result was observed in a study conducted with Dillenia, indicating a change in the viscosity of peripheral blood in the presence of Dillenia indica extract at the concentration of (100 ng/ml) [49]. The viscoelastic properties of cells are important biomarkers of disease status and progression. A simpler approach to defining cells' viscoelastic properties examines two parameters: stiffness and viscosity [50].

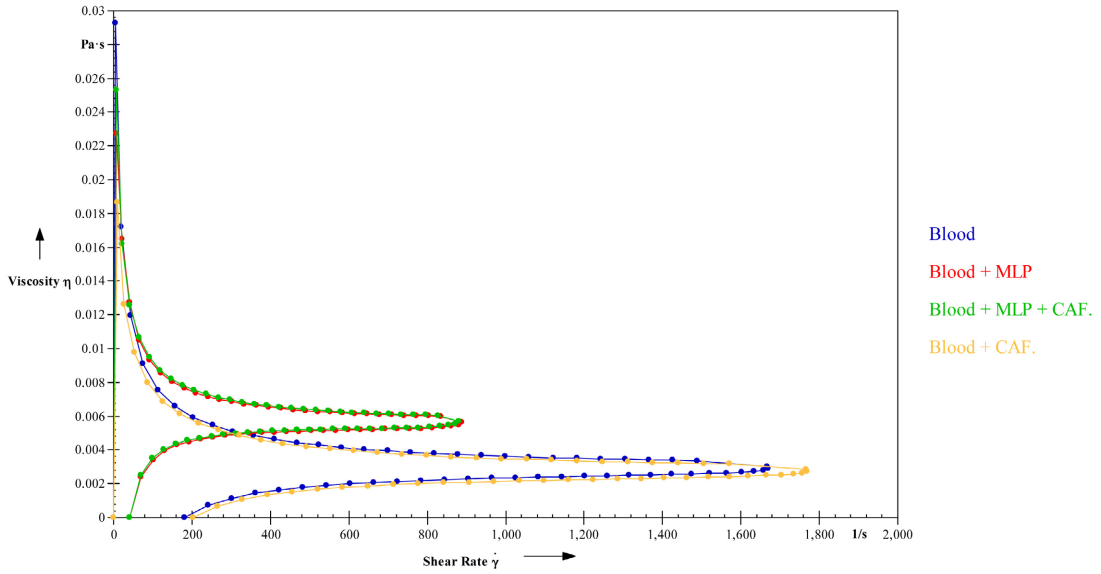

Figure 1. The viscosity of hemorheological parameters in the presence of stimuli Caffeine, MLP, MLP with Caffeine. *p < 0.05. Differences between blood and Blood + MLP + CAF or blood and Blood + CAF; MLP: Polyethylene glycol adsorbed in microemulsion. Blue line: blood, red line: blood treated with MLP, green line: blood treated with caffeine associated with MLP, yellow line: blood treated with caffeine.

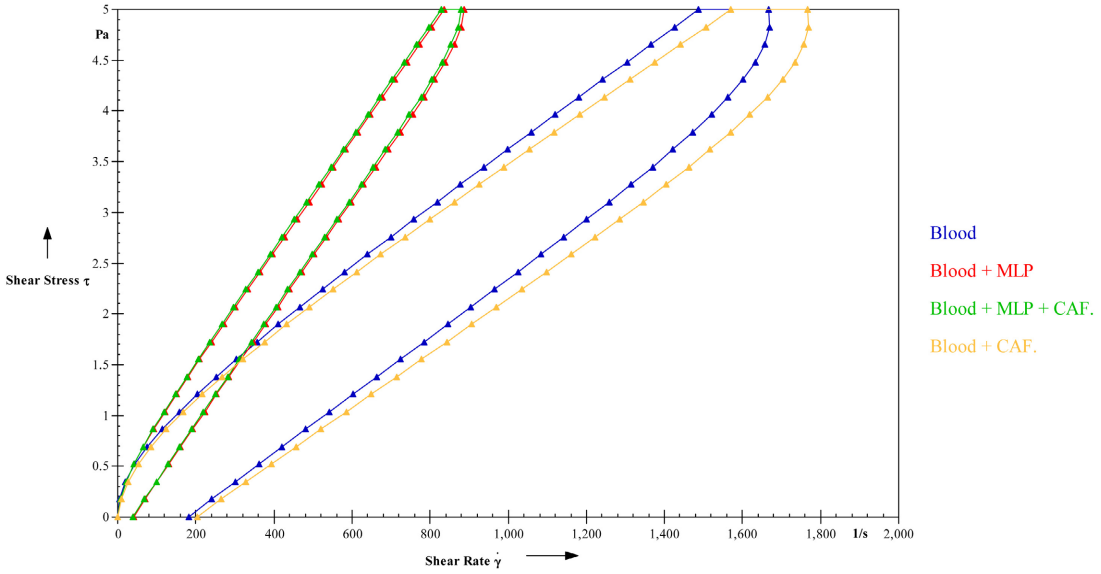

Figure 2. Flow curve of hemorheological parameters in the presence of stimuli Caffeine, MLP, MLP with Caffeine. *p < 0.05. Differences between blood and Blood + MLP + CAF; MLP: Polyethylene glycol adsorbed in microemulsion. Blue line: blood, red line: blood treated with MLP, green line: blood treated with caffeine associated with MLP, yellow line: blood treated with caffeine.

3.2. Rheological Evaluations of MCF-7 Cells Treated with Caffeine Associated Not with Polyethylene Glycol Adsorbed in Microemulsion

The rheological evaluation of MCF-7 cells with caffeine associated or not with polyethylene glycol adsorbed in a microemulsion is presented in **Figure 3** and **Figure 4**. An increase in viscosity was obtained (**Figure 4**) in MCF-7 cells treated

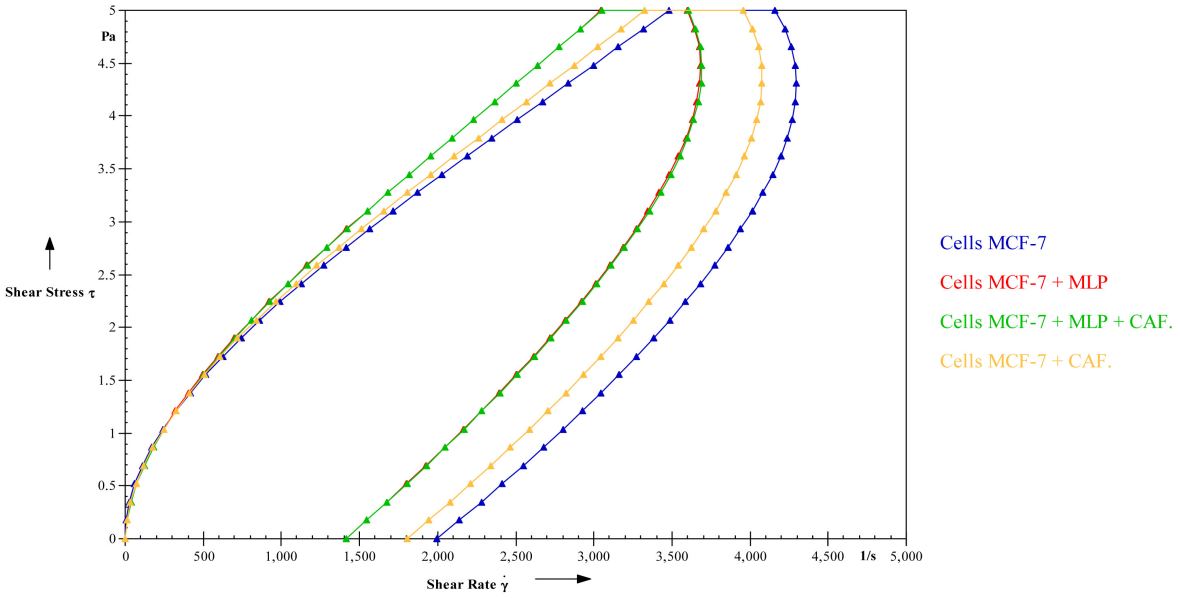

Figure 3. Rheological parameters of flow curve of MCF-7 cells treated with caffeine, MLP, MLP with Caffeine. *p < 0.05. Differences between MCF-7 and MCF-7 + MLP + CAF; MLP: Polyethylene glycol adsorbed in microemulsion. Blue line: MCF-7 cells, red line: MCF-7 cells treated with MLP, green line: MCF-7 cells treated with caffeine associated with MLP, yellow line: MCF-7 cells treated with caffeine.

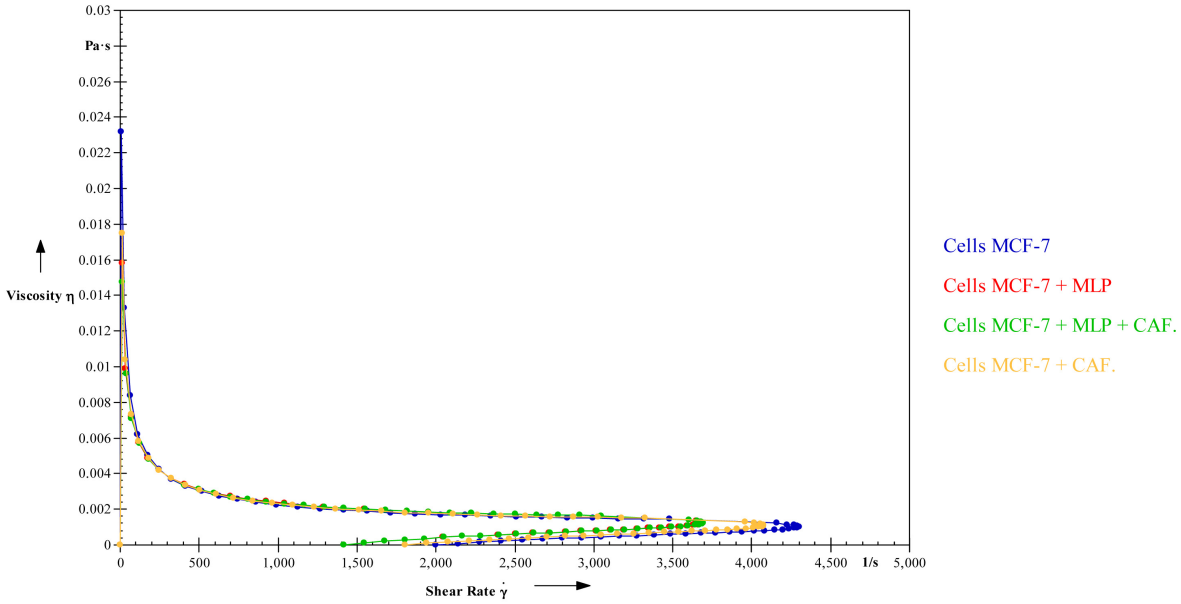

Figure 4. Rheological viscosity parameters of MCF-7 cells treated with Caffeine, MLP, and MLP with Caffeine. *p < 0.05. Differences between MCF-7 and MCF-7 + MLP + CAF; MLP: Polyethylene glycol adsorbed in microemulsion. Blue line: MCF-7 cells, red line: MCF-7 cells treated with MLP, green line: MCF-7 cells treated with caffeine associated with MLP, yellow line: MCF-7 cells treated with caffeine.

with MLP and caffeine associated with polyethylene glycol adsorbed in micro-emulsion. It is observed that there was also a change in the rheological behavior of MCF-7 cells (**Figure 3**).

Research conducted with cancerous liver cells of the HepG2 line treated with

dexamethasone also observed an increase in cellular viscosity, corroborating our study [51].

A study using mononuclear phagocytes (MN), MCF-7 cells, and coculture treated with Parrychloride (BaCl2) adsorbed to PEG microspheres and a study using Bryophytes spp. and MCF-7 cells observed that blood flow and viscosity curves were not affected [41] [52].

3.3. Release of Superoxide Anion from MN Cells, MCF-7 and Co-Culture

The release of O_2^- was higher in MCF-7 cells treated with caffeine adsorbed in MLP (**Figure 6**), suggesting the stimulating effect of MLP in these cells. A study previously carried out by the group also observed an increase in the release of superoxide in MN phagocytes co-cultivated with MCF-7 cells treated with mangaba fruit extract (Hancornia speciosa), adsorbed or not in PEG microspheres [26].

A similar result was observed in the combined treatment of menadione (vitamin K) and calcitriol (vitamin D) in MCF-7 cells. In addition, it was observed that many trials performed with menadione and calcitriol increased O_2^-, but these values intensified when both drugs were administered simultaneously [53].

In general, when compared to normal cells, tumor cells produce more ROS due to metabolic and signaling alterations [54]. This may explain why MN phagocytes treated with caffeine associated with MLP did not demonstrate a significant increase in the release of O_2^- (**Figure 5**) compared to MCF-7 cells (**Figure 6**).

Caffeine showed modulating activity in MCF-7 cells, decreasing O_2^- (**Figure 7**). The same was observed in MN and MCF-7 cells in coculture, probably due to caffeine's antioxidant properties. Several studies have shown that coffee reduces oxidative stress [40] [42] [43]. A previous study with pomiferin will identify the significant regulation of two important genes, the MN-SOD and ACOX1, of antioxidant enzymes that help remove excess superoxide anions in MCF-7 cells [55].

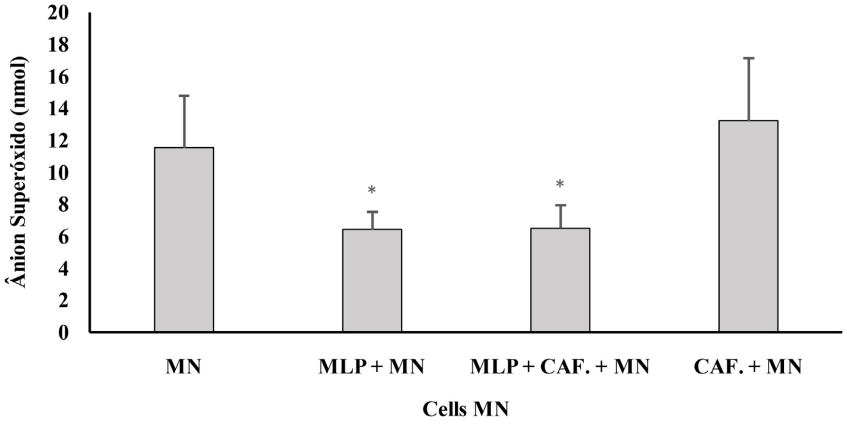

Figure 5. Release of superoxide anion (O_2^-) by MN cells treated with Caffeine, MLP, MLP with Caffeine. *$p < 0.05$. Differences between MN and MLP+MN or MN and MLP + CAF + MN.

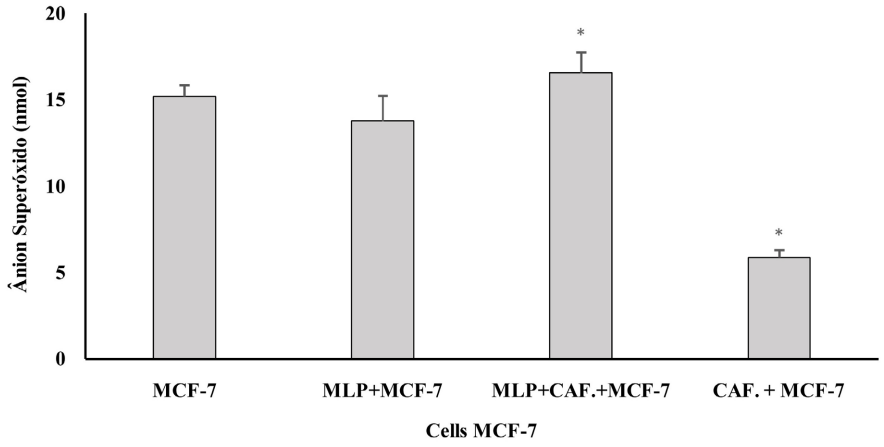

Figure 6. Release of superoxide anion (O_2^-) by MCF-7 cells treated with Caffeine, MLP, MLP with Caffeine. *p < 0.05. Differences between MCF-7 and MLP + CAF + MCF-7 or MCF-7 and CAF-MCF-7.

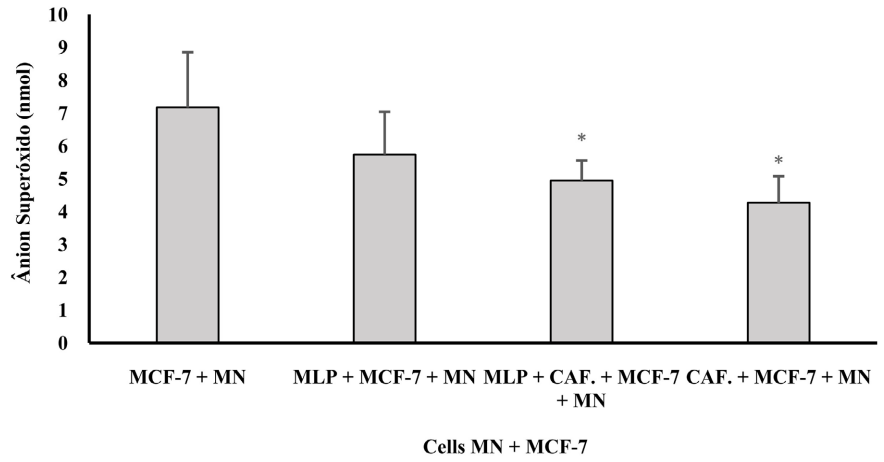

Figure 7. Release of superoxide anion (O_2^-) by MN and MCF-7 cells treated with Caffeine, MLP, and MLP with Caffeine. *p < 0.05. Differences between MCF-7 + MN and MLP + CAF + MCF-7 + MN or MCF-7 and CAF-MCF-7 + MN.

The regulation of oxidative stress is important in tumor development and responses to anticancer therapies. Cell survival and death can be influenced by oxidative stress. Although high levels of ROS cause cell death, low levels of free radicals can directly modulate transcription factors that regulate apoptosis [56]. Because of this, scientists are looking for potential modulators of oxidative stress as anticancer strategies [26] [44].

3.4. The Activity of the Enzyme Superoxide Dismutase (SOD) Cellular MN and MCF-7 and Coculture

In metabolic reactions, they also have antioxidant enzymes that control THE concentration. Superoxide dismutase (SOD) is a very important enzyme that functions in the dismutation of O_2^- in H_2O and O_2 [42]. The antioxidant action of caffeine can aid in eliminating ROS by avoiding cellular damage. Many stu-

dies have shown that caffeine can eliminate free radicals and increase the activity of superoxide dismutase (SOD) in vivo [57] [58]. SOD activity was increased in MN phagocytes treated with MLP and caffeine associated with MLP (**Figure 8**). The result suggests that the antioxidant system of MN phagocytes was maintained, where we observed increased SOD activity and O_2-reduction. A similar result performed with melon-são-Caetano (Momordica charantia) altered the SOD production of mononuclear cells, increasing their antioxidant effects [59].

There was a reduction in the sod activity of MCF-7 cells when treated with caffeine (**Figure 9**) this is a good factor, as it can prevent the antioxidant escape mechanism used by the pre-established tumor. Tumor cells increase their antioxidant defense mechanisms to prevent the high concentration of ROS in the tumor microenvironment from inducing cell death [60].

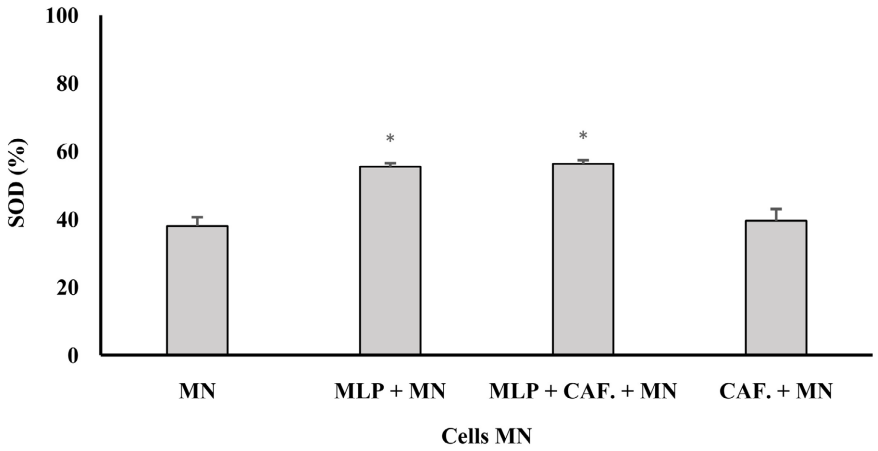

Figure 8. Percentage of NBT inhibition by the enzyme superoxide dismutase (Cu-Zn-SOD) in MN cells treated withCaffeine, MLP, MLP with Caffeine. *p < 0.05. Differences between MN and MLP + MN or MN and MLP + CAF + MN.

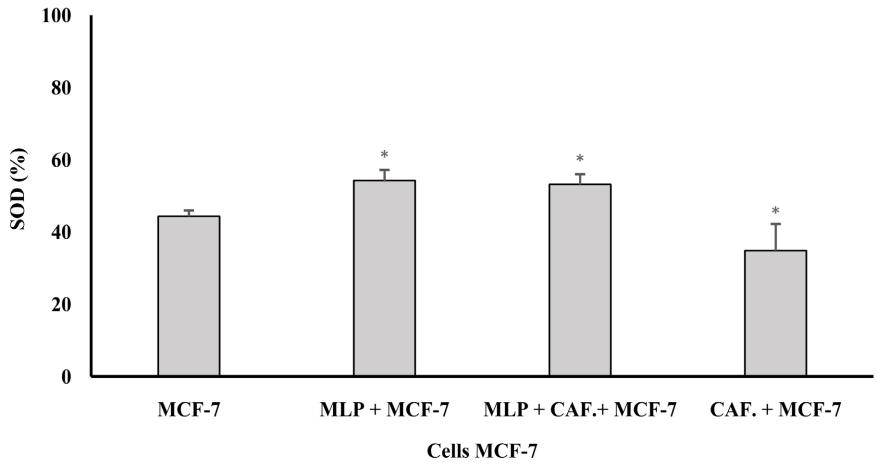

Figure 9. Percentage of NBT inhibition by the enzyme superoxide dismutase (Cu-Zn-SOD) in MCF-7 cells treated with Caffeine, MLP, MLP with Caffeine. *p < 0.05. Differences between control group (MCF-7) and the groups treated (MLP + MCF-7; MLP + CAF + MCF-7; CAF + MCF-7).

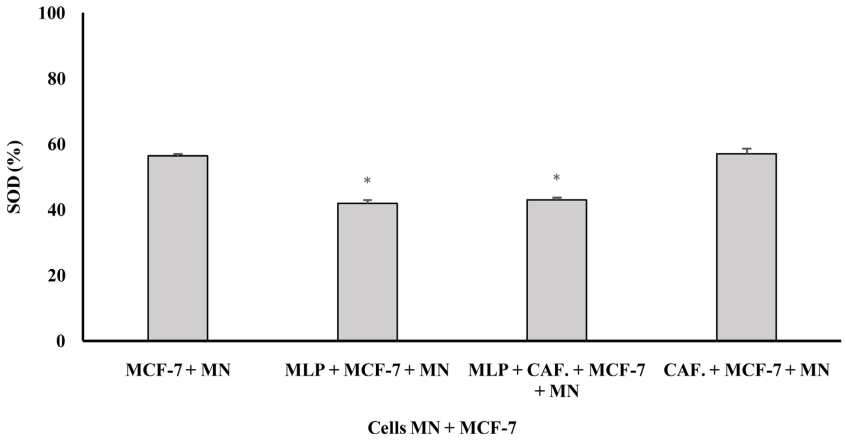

Figure 10. Percentage of NBT inhibition by the enzyme superoxide dismutase (Cu-Zn-SOD) in MN and MCF-7 cells treated with Caffeine, MLP, and MLP with Caffeine. *$p < 0.05$. Differences between MCF-7 + MN and MLP + MCF-7 + MN or MCF-7 + MN and MLP + CAF + MCF-7 + MN.

In the present work, modulation was observed MCF-7 cells treated with MLP and caffeine associated or not with polyethylene glycol adsorbed in microemulsion, in which the MLP and caffeine associated with MLP significantly increased the activity of sod of MCF-7 cells (**Figure 9**). A study conducted with *Hancornia speciosa* adsorbed in PEG microsphere in MCF-7 breast cancer cells and cocultured with blood cells also shows high levels of SOD in these cells when treated with *H. speciosa* ethanol extract (HSEE) [26].

Caffeine can potentially increase the antioxidant defenses of breast tumor cells. The same was observed in MCF-7 and MDA-MB-231 cells treated with caffeine [38].

Phagocytes MN and MCF-7 in coculture treated with MLP and caffeine associated with MLP had the reduction of SOD (**Figure 10**). A similar result was observed in a study conducted with MN phagocytes with enteropathogenic Escherichia coli (EPEC) bacteria treated with Psidium guajava leaf extract. It was observed that at concentrations of 200 ng/mL and 200 pg/mL, the extract significantly reduced the activity of the SOD enzyme [61]. A study conducted with cells treated with guarana and their combinations with caffeine and/or taurine showed an exponential decrease in SOD activity in human neuronal cells (SH-SY5Y) [62].

4. Conclusion

These results suggest that caffeine associated with MLP modulated the superoxide anion and the superoxide dismutase enzyme in mcf-7 cells, MN phagocytes, and coculture. It may be an important mechanism of antitumor response via oxidative stress in breast cancer treatment.

Acknowledgements

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Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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