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# Anti-Inflammatory Effects of Nanoparticles Containing Alpha-Humulene in a Model of Alzheimer's Disease

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***Authors' contributions***

*This work was carried out in collaboration among all authors. All the authors contributed to the study conception and design. Author's IIK, ACDB, CSM and RMM designed the experiments. Author's SAW, JRN, LFD, JAS and LHGM carried out the experiments. Author's IIK, ACDB, CSM, RMM and SAW did data collection and performed the analysis. Author's IIK, RMM, CSM and SAW wrote the first draft of the manuscript. All the authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.*

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## ABSTRACT

Alzheimer's disease (AD) is a neurodegenerative disorder that causes impairment in daily living activities. Although there is no consensus on the pathophysiology of this disease, neuroinflammation is known to be associated with this disease. Objective: To evaluate the anti-inflammatory effects of nanoparticles containing alpha-humulene (HUM) in an experimental model of AD. Methodology: Thirty-three animals were included in the sample, 3 of which were in the negative control group. The other 30 patients received the amyloid-beta peptide in the CA1 region of the hippocampus for neuroinflammation. The cells were allowed to rest for 30 days for the inflammatory process to occur. The HUM group was treated with α-humulene particles, the NHUM group was treated with α-humulene nanoparticles for 15 days, and the CP group was not treated. Results: There were significant differences in IFN concentrations between the CP and CN (p=0.0001), HUM and CN (p=0.0003), CP and NHUM (p=0.0006), and HUM and NHUM (p=0.0495) groups. There was no difference in TNF-α levels between the groups. IL-6 levels were significantly different between the CP and NHUM groups (p=0.0078) and between the CP and CN groups (p=0.0009). IL-12 levels were significantly different between the CP and CN (p=0.0001) and between the NHUM and CN (p=0.0160). Overall, the highest concentration was in the CP group. Concerning IL-10, there was a difference between the CP and NHUM (p=0.0003) and between the NHUM and CN (p=0.0005), with the NHUM having the highest concentration. Immunohistochemistry analysis of the anti-Tau and anti-GAPF antibodies revealed strong positivity in the CP group, positivity in the HUM group, and weak positivity in the NHUM group. Conclusion: Treatments for Alzheimer's disease and HUM and NHUM were effective.

*Keywords: Neuroinflammation; Alzheimer's; Alpha-Humulene; nanoparticles.*

## 1. INTRODUCTION

According to the Therapeutic Guidelines for Alzheimer's Disease (AD), prepared by the Brazilian Ministry of Health [1], the pathology is a neurodegenerative disorder that generates progressive brain impairment affecting activities of daily living. Individuals with AD exhibit a variety of neuropsychiatric symptoms and behavioral changes due to cognitive and memory deterioration.

AD is defined as a multifactorial disease in which genetic, environmental, behavioral, and mental development components critically influence its pathogenesis, with age being the most important risk factor [2].

The etiology of AD has not been fully elucidated, although considerable progress has been made in understanding its biochemical and genetic mechanisms. The diagnosis is usually clinical, by exclusion, based on signs, symptoms, and brain imaging. The measurement of biomarkers such as tau and Aβ-42 (amyloid-beta peptide) in the cerebrospinal fluid (CSF) is also well established in the differential diagnosis of AD [3].

Furthermore, the hypothesis linking neuroinflammation to the pathogenesis of AD has gained strength in recent years, even suggesting that its onset occurs long before memory impairment becomes clinically evident. Neuroinflammation is a physiological response of the immune system that is regulated by the central nervous system (CNS). Additionally, autophagy is a defense mechanism against molecular changes in brain tissue [2].

Increasing evidence suggests that the pathogenesis of AD is not restricted to neurons but also involves immunity in the brain. Thus, the activation of astrocytes and microglia, resident immune cells of the CNS, is a feature of neuroinflammation that is observed in most neurodegenerative conditions, including AD. In general, glial cell activation induces many biochemical and cytological changes, such as the production and secretion of proinflammatory cytokines [4].

Therefore, the objective of this research was to understand the mechanism of neuroinflammation in an experimental model of AD through the treatment of neuroinflammation with a molecule that has few side effects. Additionally, this study aimed to analyze the effectiveness of nanoparticles in crossing the blood-brain barrier (BBB). If an effective treatment is found, people with AD will have a better quality of life, and the cost of the disease, as well as mortality, can decline dramatically.

Based on the above considerations, the overall objective of this study was to evaluate the anti-inflammatory effects of nanoparticles containing alpha-humulene in an experimental model of AD after induction by Aβ 1-42 toxin.

## 2. METHODOLOGY

The central limit theorem was used to determine the sample size to be surveyed. Based on a population of 2000 individuals with Alzheimer's disease in the municipality of Guarapuava, a margin of error of 10%, a degree of reliability of 95%, and a homogeneous distribution of 99%, the sample size was 40 animals.

According to the equation used: $n = p(1-p)Z^2/e^2$

where *n* = sample size, *p* = population proportion, *Z* = normal distribution value for a given confidence level, and *e* = margin of error.

The initial sample consisted of 33 Wistar rats (*Rattus norvegicus*) weighing 300-350 grams were purchased from the animal facility of the State University of Maringá after approval by the ethics committee for using the animals (protocol number 009/2021). The animals were divided into four groups. The negative control (NC) group consisted of 3 animals for testing the predominance of normal tissues; the materials were collected, and the animals were euthanized in addition to the other groups. The positive control (PC) group consisted of 10 animals with lesions in the CA1 region that were not treated; the animals were collected and euthanized on the 45th day after amyloid-beta 1-42 induction. Group treated with α-humulene (HUM): composed of 10 animals that were treated 30 days after the induction of neuroinflammation by amyloid-beta 1-42; the treatment was performed with 6.5 µg of α-humulene dissolved in 0.9% saline by gavage for 14 days, with material collection and euthanasia on the 45th day. The group treated with α-humulene nanoparticles **(**NHUMs) was composed of 10 animals that were treated 30 days after the induction of neuroinflammation by amyloid-beta 1-42; the treatment was performed with polymeric nanoparticles and 6.5 µg of α-humulene for 14 days, after which the materials were collected and euthanized on the 45th day.

The nanoparticles were produced by the Nanotechnology Laboratory at Midwestern State University (UNICENTRO). These data were obtained by the antisolvent precipitation method.

The average diameter and polydispersity index (PDI) of the nanoparticles were determined via dynamic light scattering (DLS) (BIC 90 Plus, Brookhaven Instruments Corp., Holtsville, NY).

The zeta potential was determined from the electrophoretic mobility of the suspended nanoparticles. To determine the amount of α-humulene encapsulated in the nanoparticles, indirect analysis was applied. An aliquot of the supernatant obtained after ultracentrifugation of the nanoparticles was diluted in the mobile phase, filtered through a 0.22 µm membrane and analysed via high-performance liquid chromatography (HPLC) (Waters 2695-Alliance, Milford, USA). The analysis was performed in triplicate. The mobile phase consisted of a mixture of methanol, pH 6.8 phosphate buffer, and acetonitrile (63:30:7, v/v/v) at a flow rate of 0.9 ml/min. The Photodiode Array (PDA) detector was set to 306 nm. The percentage of EE (encapsulation efficiency) was determined in at least three repetitions, according to Equation (1). The results are expressed as the mean ± standard deviation.

Equation 1: % EE = ((initial amount of drug - recovered amount of drug)/initial amount of drug)*100

The animals were anesthetized. Then, the fish were taken to a stereotaxic device (David Kopf, USA), where their heads were fixed by the petrous temporal bone and upper incisors, under coordinates AP = -3.0 mm, ML = 1.6 mm, -1.6 mm, and DV = 3,00 mm, taking as a reference the bregma point. In addition, 4 µl of amyloid-beta peptide toxin was administered through a Hamilton syringe in the hippocampal region of CA1 to induce the formation of senile plaques [5,6].

For postsurgical analgesia, tramadol hydrochloride was used at a dose of 10 mg/kg every 12 hours orally for 7 days.

After the induction of neuroinflammation, the animals were allowed to rest for a period of 30 days for the inflammatory processes in the hippocampal neurons to occur. The animals received 6.5 µg of α-humulene orally (via gavage), and the same concentration of polymeric nanoparticles containing the substance was used for a period of 14 days.

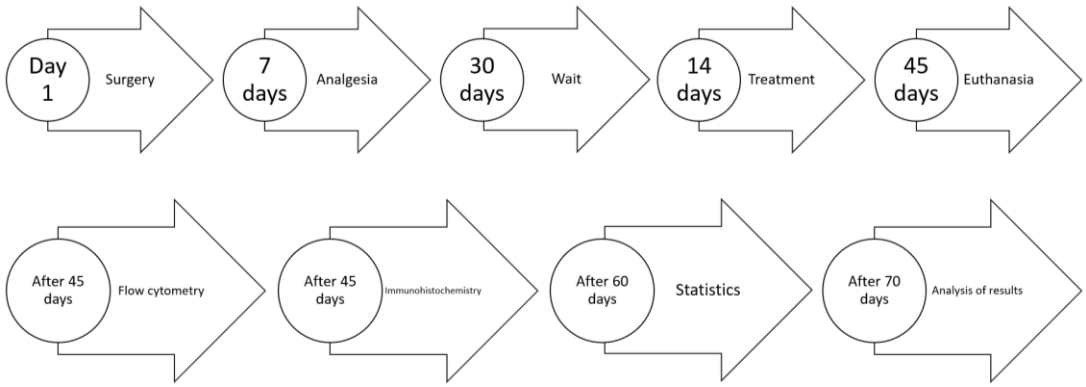


**Fig. 1. Timeline scheme representing the methodology used in the research**

Then, one animal per group was euthanized to verify the presence of plaques and neurofibrils in the hippocampus. Only after this verification were the animals in the group treated with α-humulene particles and α-humulene nanoparticles.

This experimental model of AD has been used by UNICENTRO's Neuroanatomy Laboratory for several years [6].

After treatment, two ml of blood was taken for analysis. The BD™ Cytometric Bead Array Mouse Inflammation Cytokine Kit (Becton Dickinson, USA) was used for the analysis. The cytokines analyzed were tumor necrosis factor (TNF), interferon (IFN), interleukin-6 (IL-6), interleukin-10 (IL-10), monocyte chemoattractant protein-1 (MCP-1), and interleukin-12p70 (IL-12p70).

The reading in the cytometer was performed manually by acquiring 10,000 events from each sample. Flow cytometry data were analyzed using FCap 3.0 Array Software (Becton Dickinson, USA), and the results were plotted as graphs of means and standard deviations.

Immunohistochemistry (IHC) was performed by Guarapuava's Pathology Support Center. The vials were lyophilized with 0.05 or 0.2 ml of deionized water. Antibody dilutions were made in a buffer containing 1-3% bovine serum albumin (BSA). Anti-Tau and anti-GAPF antibodies were also used. Furthermore, IHC analysis was performed as recommended by the manufacturers: Sigma-Aldrich and Biogen.

For statistical analysis, the data obtained were arranged in spreadsheets and analyzed using Prisma software. For Gaussian analysis, the Shapiro–Wilk test was used. For nonparametric samples, one-way analysis of variance (ANOVA) with Tukey's post hoc test was used.

Fig 1 shows all the phases of the methodology used in this research, including schematic, simplified and easy-to-interpret methods.

## 3. RESULTS AND DISCUSSION

By euthanizing one rat from each group after surgery for the induction of amyloid-beta 1-42 by electron microscopy, one can observe that there was deposition of amyloid-beta 1-42 in the hippocampal CA1 region (Fig 2).

The average diameter, polydispersity index, zeta potential, and encapsulation efficiency were used to evaluate the nanoparticles. The results of the preparation of these nanoparticles are shown in Table 1.

In this study, the nanoparticles had the expected diameter. The results obtained for the encapsulation efficiency, as well as the polydispersity index, were satisfactory.

According to Surender and Deepika [7], the zeta potential provides an index of the magnitude of electrostatic repulsion of particles. The electrical charge on the surface of a particle is a parameter used for determining the physical stability of colloidal systems. In this work, the potential was 45.0 mV.

Reactions with anti-TAU and anti-GAPF antibodies were performed on the samples (for a total of 33 samples). The selection of the samples was based on morphological evaluation,

giving preference to those that had characteristic findings for each IHC. Its evaluation was performed following positivity standards. Negative controls were prepared in serial sections.

The intensity and localization of immunoreactivity with all primary antibodies used were examined throughout the slide using an optical microscope. As a negative control, the primary antibody was omitted. For the imaging analysis, color photomicrographs of representative areas (400x magnification) were digitally acquired.

For the semiquantitative score, a total of 10 images for each section of each animal were evaluated using the color deconvolution tool of ImageJ software (NIH, USA). Pixels were categorized as described previously by Chatterjee et al. [8] as strongly positive (3+), positive (2+), weakly positive (1+), or negative (0).

According to the results of the semiquantitative evaluation, group C exhibited weak positive staining, group PC exhibited strong positive staining, group HUM positive, and group NHUM showed weak negative staining due to the coloration present in each group, where brown indicates the deposition of TAU (Fig 3).

According to Laurent, Buée, and Blum [9], in tauopathies, some hyperphosphorylated tau proteins and some NFTs are sufficient to induce morphological changes in astrocytes, impacting their function. These cells switch to an inflammatory profile, indicating positive regulation of glial fibrillary acidic protein (GFAP) and secretion of proinflammatory factors.

Evidence from Naseri et al. [10] demonstrated the association between glia and tauopathies. Activated glial cells were observed near neurons with hyperphosphorylated tau in postmortem brains with tauopathies. Observations suggest that pathological tau may contribute to the activation of glial cells and the development of neuroinflammation in AD. However, the way in which pathogenic tau activates microglia at the cellular and molecular levels is unclear. There is evidence that the modulation of tauopathies by microglia is due to genetic factors.

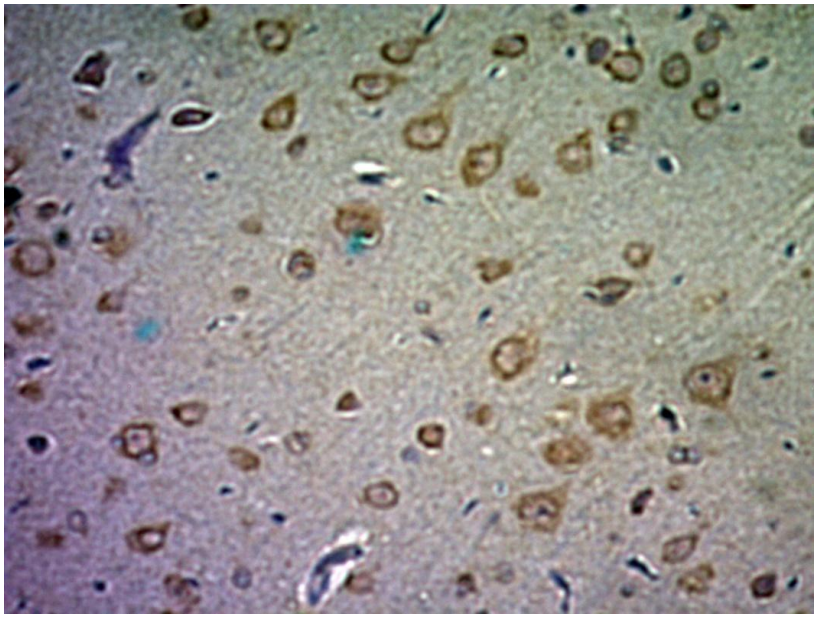

**Fig. 2. Histological section to verify the deposition of amyloid-beta 1-42 in the hippocampal CA1 region; electron microscopy images at 400x magnification**

**Table 1. Mean diameter, polydispersity index, zeta potential and encapsulation efficiency of the humulene nanoparticles**

| Average diameter (nm) | IP | Zeta Potential (mV) | Encapsulation efficiency (%) |
|---|---|---|---|
| 210.1 ± 3.1 | 0,090 ± 0,037 | +45,0 ± 1,60 | +64,0 ± 1,93 |

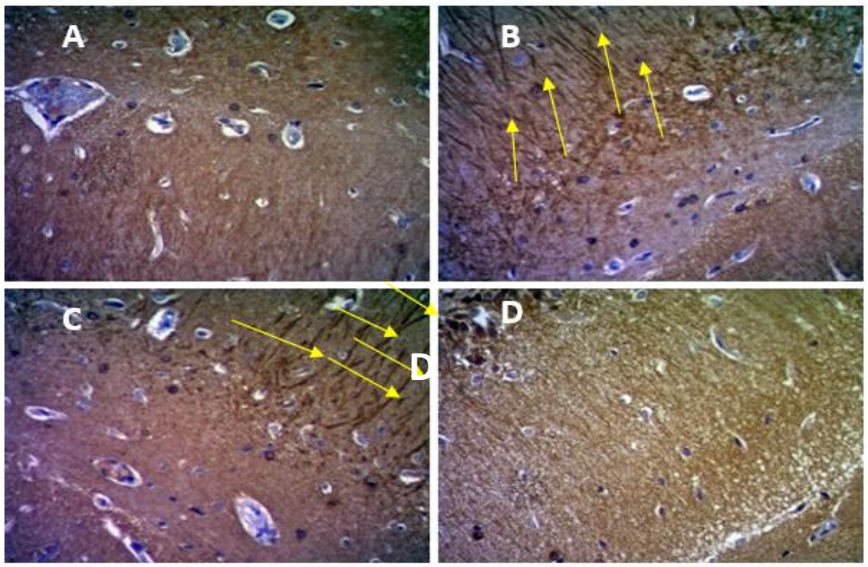


**Fig. 3. Immunohistochemical staining for anti-TAU in A (control), B (positive control), C (HUM) and D (NHUM) at 400x magnification**

According to Liddelow et al. [11], in addition to amyloid and tau pathologies, another characteristic of AD is the accumulation of reactive astrocytes and microglia in the vicinity of NFT, commonly referred to as the neuroinflammatory response. In the healthy brain, astrocytes provide a neuronal energy supply, but other cytokines are triggered by activated microglia, favoring the formation of a neurotoxic subset of reactive astrocytes called A1. A1 astrocytes lose their normal functions and ability to promote synapse formation and, through the secretion of harmful factors, contribute to neuronal death. A greater proportion of A1 astrocytes was observed in the brains of patients with AD.

A semiquantitative evaluation of GFAP revealed that, in the C group, the cells were positive, strongly positive in the PC, HUM positive, and weakly negative in the NHUM, which can be observed by the brown color (Fig 4).

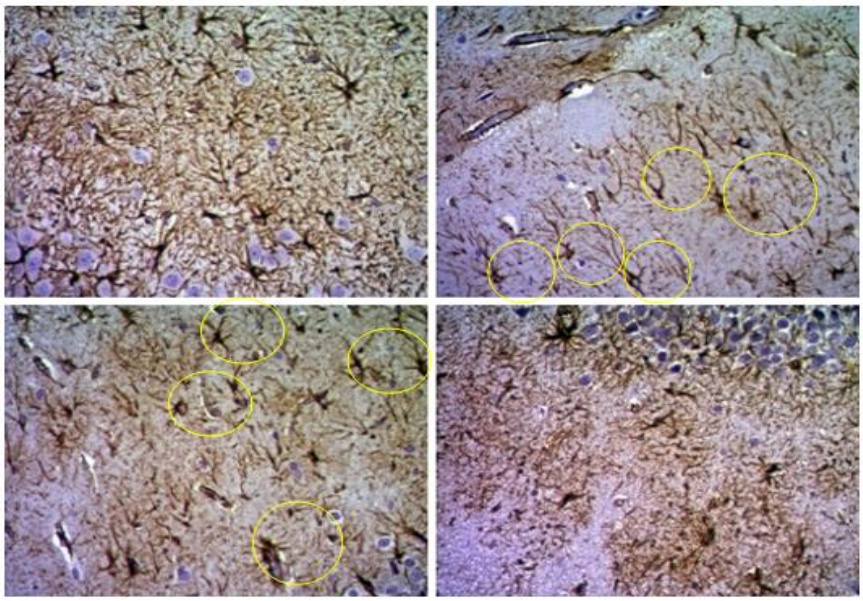

**Fig. 4. Immunohistochemical staining for anti-GAPF in A (control), B (positive control), C (HUM), and D (NHUM) at 400x magnification**

One study verified that in the C group, astrocytes presented fine extensions, while in the CP, astrocytes presented thicker extensions, characterizing fibrous astrocytes. In the HUM group, the same characteristics were observed as in the PC group, and in the NHUM group, astrocytes with thin filaments were observed.

According to Oeckl et al. [3], GFAP is a marker of astrogliosis and is increased in brains of patients with AD, which is why it can be considered a marker of this pathology.

Laurent, Buée, and Blum [9] reported that activated microglia are also prone to secrete proinflammatory cytokines, including TNF.

The mean and standard deviation values for TNF-α are illustrated in Fig 5, where it is possible to observe that the mean for group C was 5.001 pg/mL with a standard deviation of 1.866 pg/mL, the mean for PC was 7.847 pg/mL with a standard deviation of 1.646 pg/mL, the mean for the NHUM group was 6.152 pg/mL with a standard deviation of 2.510 pg/mL, and the mean for the HUM group was 6.755 pg/mL with a standard deviation of 2.948 pg/ml. The TNF concentration was greater in the PC group than in the other groups. Moreover, there was no statistically significant difference between the groups analyzed.

In their study, Fischer, Kontermann, and Pfizenmaier [12] suggested that TNF-α is a key regulatory component of the immune system that regulates innate and adaptive immunity and contributes to the onset and maintenance of inflammation.

The main cellular sources of TNF-α are macrophages and immune cells that are activated in response to infections or tissue damage. Therefore, the regulated expression of TNF-α is essential for promoting tissue homeostasis and combating infections. In contrast, dysregulated expression of TNF-α and signaling can induce pathology, resulting in inflammation and tissue damage. Indeed, increased levels of TNF-α have been detected in patients with autoimmune and degenerative diseases.

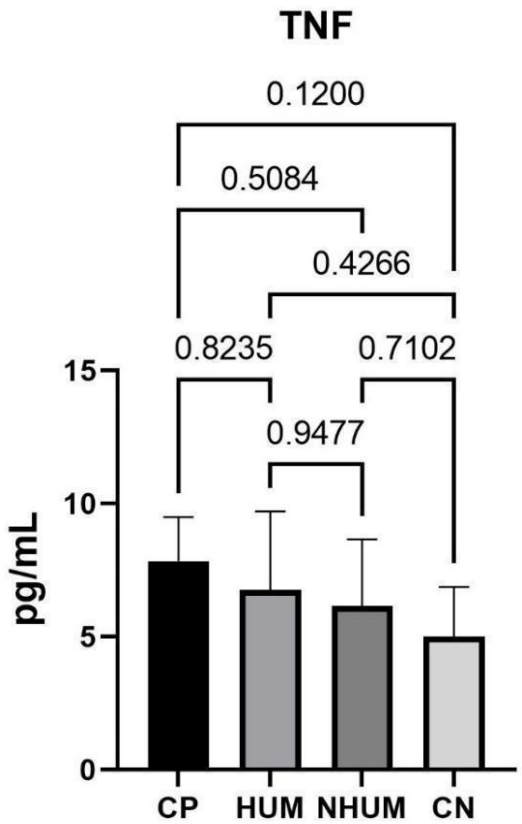


**Fig. 5. Quantitative analysis of TNF-α levels using flow cytometry**

Consistent with the flow cytometry results of this study, the PC group had high concentrations of TNF-α, indicating an active inflammatory process, with lower concentrations in the HUM, NHUM, and C groups.

Laurent, Buée, and Blum [9] state that TNF is known to favor tauopathies. Studies suggest that reducing the amount of secreted proinflammatory mediators can directly impact tau protein phosphorylation in mice.

In this study, a high concentration of TNF-α was detected in the PC group, and according to the immunohistochemistry results, there was a strong positive expression when using anti-tau; in the HUM and NHUM groups, a positive and a weak positive expression, respectively, were noted. A decrease in the TNF concentration was noted in the HUM group compared with the NHUM group.

The mean and standard deviation values for MCP-1 are shown in Fig 6. The mean for the C group was 4.745 pg/ml, with a standard deviation of 1.988 pg/ml; the mean for the CP group was 6.288 pg/ml, with a standard deviation of 2.648 pg/ml; the mean for the NHUM group was 7.835 pg/ml, with a standard deviation of 2.594 pg/ml; and the mean for the HUM group was 7.225 pg/ml, with a standard deviation of 1.555 pg/ml. The concentration of the monocyte chemoattractant protein-1 was greater in the NHUM group than in the other groups. Furthermore, a statistically significant difference in the concentration between the NHUM and C groups (p=0.0315) was observed.

Lee et al. [13] reported that the main function of MCP-1 is to regulate the migration and infiltration of monocytes, memory T lymphocytes, and natural killer cells and that monocytes/macrophages are the main source of MCP-1. Furthermore, recent evidence suggests that in AD, the inflammatory process related to MCP-1 is associated with the accumulation of Aβ.

According to Shen et al. [14], this chemokine plays an important role in neuroinflammation and neuronal degeneration in AD. MCP-1 is a member of the beta-chemokine subfamily. Studies suggest that MCP-1 levels in cerebrospinal fluid (CSF) are elevated in patients with AD and are promising for the diagnosis of AD.

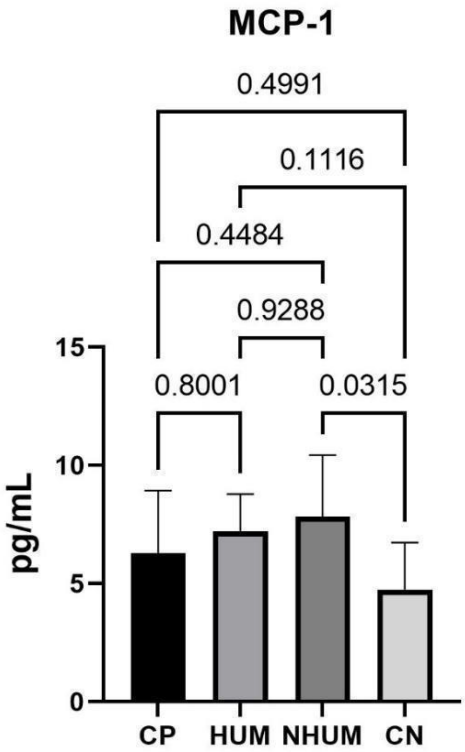


**Fig. 6. Quantitative analysis of MCP-1 expression by flow cytometry**

Xu et al. [15] reported that MCP-1 is a potent chemotactic factor for monocytes that regulates the activation of inflammation. It has been found that MCP-1 levels increase with age in the blood. CSF and plasma MCP-1 levels are greater in patients with AD and are associated with disease severity and cognitive decline. The same authors also claim that changes in MCP-1 metabolism are involved in the development of AD.

In the present study, MCP-1 was one of the cytokines that was expressed at a higher concentration in the CP group than in the NHUM group. Furthermore, a statistically significant difference was detected between the NHUM and C groups (p=0.0315), indicating an active inflammatory process.

On the other hand, the chemokine MCP-1 acts on monocytes through its CCR2 receptor, suppressing IL-12 production by activated monocytes, which was observed in the present study [16].

The mean values and standard deviations for IL-12 are shown in Fig 7. The mean for the C group was 1.648 pg/ml, with a standard deviation of 0.4555 pg/ml; the mean for the PC group was 3.510 pg/ml, with a standard deviation of 0.8944 pg/ml; the mean for the NHUM group was 2.827 pg/ml, with a standard deviation of 0.8779 pg/ml; and the mean for the HUM group was 2.607 pg/m, with a standard deviation of 0.8278 pg/ml. The concentration of IL-12p70 was greater in the PC group than in the NHUM, HUM, and C groups. There was a statistically significant difference in concentration between the PC and C groups (p=0.0001) and between the NHUM and NC groups (p=0.0160).

According to Riphagen et al. [17], biomarkers such as tau protein, Aβ42, IL-1β, IL-6, and IL-12 are associated with chronic neuroinflammation. These parameters increase in AD patients and may also cause a risk of vascular disease. In addition, the cytokine IL-12 induces IFN-γ.

This study showed a difference between the PC and NC groups (p=0.0001), indicating that inflammation occurred in the groups that underwent surgery. Furthermore, there was a statistically significant difference between the NHUM and C groups (p=0.0160). However, among the analyzed cytokines, IL-12 had the lowest concentration.

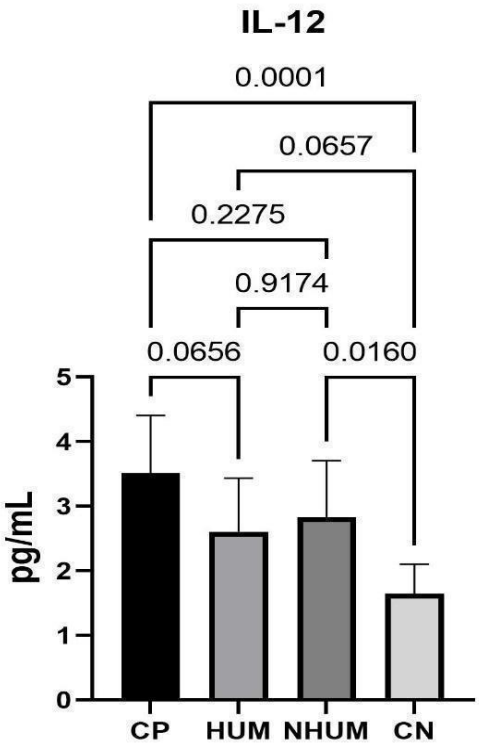


**Fig. 7. Quantitative analysis using IL-I2 flow cytometry**

The mean and standard deviation values for IFN are shown in Fig 8. The mean value in the C group was 1.881 pg/ml, with a standard deviation of 1.239 pg/ml; the mean value in the PC group was 5.042 pg/ml, with a standard deviation of 1.351 pg/ml; the mean value in the NHUM group was 2.786 pg/ml, with a standard deviation of 0.5929 pg/ml; and the mean value in the HUM group was 4.259 pg/ml, with a standard deviation of 0.4341 pg/ml. The concentration of IFN-γ was greater in the PC group than in the other groups. There were significant differences in concentrations between the NC and C groups (p=0.0001), between the HUM and C groups (p=0.0003), between the PC and NHUM groups (p=0.0006), and between the HUM and NHUM groups (p=0.0495).

Moore et al. [18] reported that, similar to microglia, the type I interferon (IFN) family of cytokines is also critical for neuroinflammation. Type I IFNs are indeed able to regulate the levels of IL-1β, IL-6, and TNFα, which are consistently positively regulated in AD. We postulate that microglial type-I IFN signaling contributes to neuroinflammation in AD. The authors have demonstrated for the first time that type-I IFNs participate in phagocytosis and alter the microglial response to $A\beta_{1-42}$.

In the present study, IFN showed the greatest difference between the concentrations in the PC and C groups (p=0.0001). In addition, there was also a difference between PC and NHUM (p=0.0006), thus demonstrating that HUM carried by nanoparticles was effective at reducing IFN. Moreover, there was no significant difference between the PC and HUM groups (p=0.3880), but there was a difference between the HUM and NHUM groups (p=0.0495). Therefore, the HUM compound needs to be carried by nanoparticles to further reduce IFN levels.

Fig 9 shows the mean and standard deviation for IL-6; the mean value in the C group was 2.170 pg/ml, with a standard deviation of 0.5325 pg/ml; the mean value in the PC group was 4.454 pg/ml, with a standard deviation of 0.3004 pg/ml; the mean value in the NHUM group was 2.735 pg/ml, with a standard deviation of 1.047 pg/ml; and the HUM group mean was 3.155 pg/ml, with a standard deviation of 0.8406 pg/ml. The interleukin-6 concentration was greater in the PC group than in the other groups. PC concentrations were significantly different between the NHUM (p=0.0078) and PC and C (p=0.0009) groups.

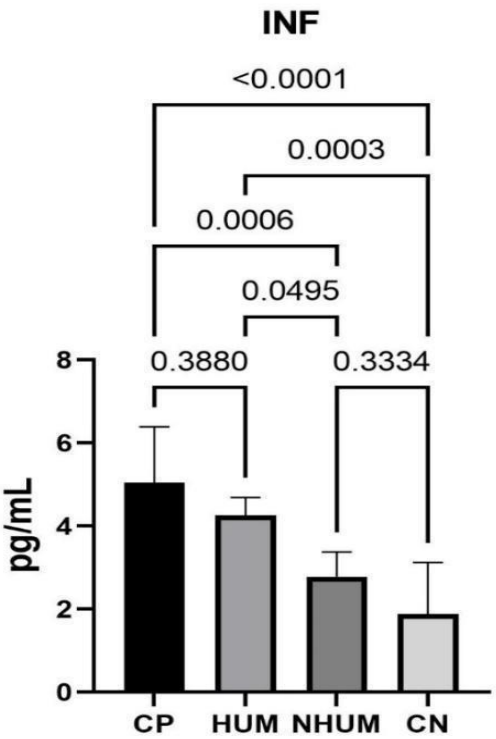


**Fig. 8. Quantitative analysis using IFN flow cytometry**

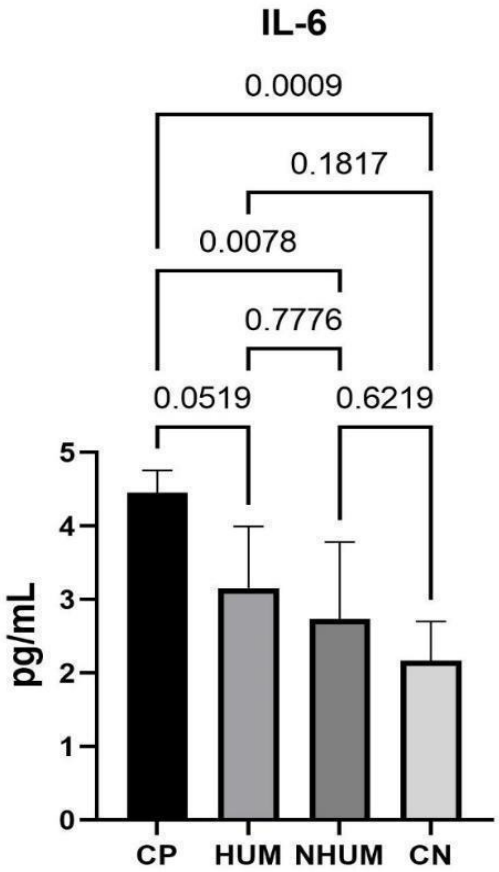


**Fig. 9. Quantitative analysis using IL-6 flow cytometry**

According to Ng et al. [19], IL-6 is produced by macrophages, T lymphocytes, endothelial cells, smooth muscle cells, and adipocytes. Peripheral IL-6 levels precede the onset of AD but are not elevated during the course of AD.

Kaur et al. [20] reported that IFN-γ acts in the activation of microglia, which acts as a defense mechanism against pathogenesis and tumor growth; thus, proinflammatory cytokines such as IL-6 and free radicals (reactive oxygen species) are produced, accompanied by neuronal loss. This process is followed by the activation and release of several anti-inflammatory cytokines, such as IL-10, which accelerate tissue remodeling, repair, and angiogenesis.

These data corroborate the findings in this study, in which there was an increase in the concentration of IL-6 in the PC group compared to that in the C group. However, the NHUM group had lower concentrations of this cytokine and higher concentrations of IL-10.

As shown in Fig 10, the mean IL-10 concentration in the C group was 4.890 pg/ml, with a standard deviation of 1.871 pg/ml; the mean IL-10 concentration in the CP group was 4.806 pg/ml, with a standard deviation of 1.444 pg/ml; the mean IL-10 concentration in the NHUM group was 7.642 pg/ml, with a standard deviation of 1.439 pg/ml; and the mean IL-10 concentration in the HUM group was 6.440 pg/ml, with a standard deviation of 1.472 pg/ml. The concentration of interleukin-10 was greater in the NHUM group than in the other groups. The concentrations of PC were significantly different from those of NHUM ($p=0.0003$) and between the NHUM and C ($p=0.0005$) groups.

According to Magalhaes et al. [21], IL-10 opposes the actions of proinflammatory cytokines and apparently suppresses both proliferative and inflammatory responses in the brain by reducing the synthesis of proinflammatory cytokines. In monocytic cells, IL-10 influences antigen presentation, the release of immune mediators, and phagocytosis.

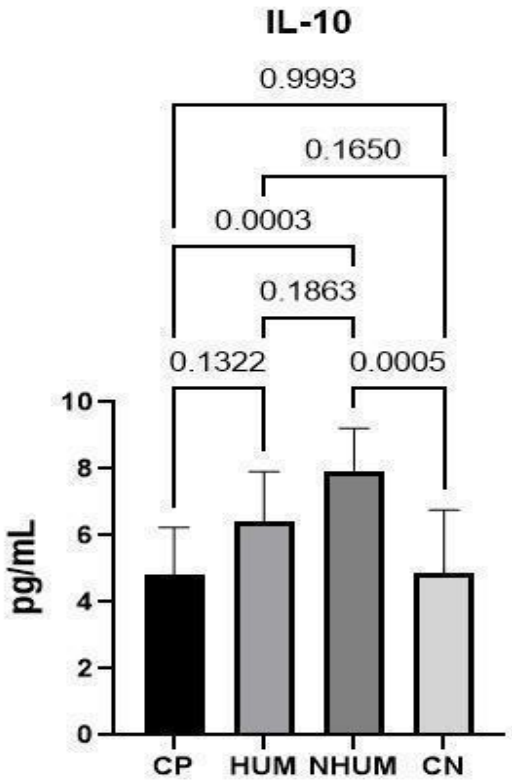


**Fig. 10. Quantitative analysis using IL-10 flow cytometry**

In this study, there was no significant difference between the C and PC groups (p=0.9993). However, there was an increase in the IL-10 concentration in the NHUM group, in contrast to the changes in the other inflammatory cytokines, demonstrating that the changes in the experimental model of AD were resolved. These data were also confirmed by immunohistochemistry (IHC) when anti-GAPF was used; the results were positive for C, strongly positive for PC, positive for HUM, and weakly negative for the NHUM group, as indicated by the brown color.

According to Magalhães et al. [21], an imbalance between proinflammatory and anti-inflammatory cytokines may be an important phenomenon in AD. In their study, the authors described that there was a seven- to tenfold increase in IL-1β production compared with that in control subjects.

This was also the case in the present study, in which inflammatory cytokines were expressed at higher levels in the PC group than in the C group. IL-10, which is an antagonist of inflammatory cytokines, did not significantly differ among the groups, but it was expressed at a greater concentration after treatment in the NHUM group.

In this study, we observed that the concentrations of most of the inflammatory cytokines were significantly greater in the HUM group than in the NHUM group (p=0.0495), thus demonstrating the anti-inflammatory effects of nanoparticle-loaded HUM.
Consistent with the findings of Pauluk et al. [22], who reported in their study, nanoparticles have gained attention due to their ability to control drug/compound release, improving the pharmaceutical and pharmacokinetic parameters of the loaded drug.

## 4. CONCLUSION

The experimental model of AD used in this study was effective due to the higher concentrations of most inflammatory cytokines in the PC group. In addition, there was a difference between the PC and NHUM groups regarding the inflammatory cytokines IL-6 and IFN, which were greater in the PC group than in the control group, confirming that there was an inflammatory process. IL-10,

which is an anti-inflammatory cytokine, was differentially expressed between the PC and NHUM groups but was more abundant in the NHUM group. This finding indicates that the inflammatory process was in the resolution phase. Consistent with the immunohistochemistry findings, both anti-TAU and anti-GAPF antibodies were strongly positive in the PC group, weakly positive in the NHUM group, and positive in the HUM group. Thus, treatment of AD patients with HUM and NHUM was effective.

## CONSENT

It is not applicable.

## ETHICS APPROVAL

This study was approved by the ethics committee on the use of animals of the Midwestern State University protocol nº 009/2021 – CEUA/UNICENTRO and was conducted in strict compliance with the guidelines set forth by the National Centre for the Replacement, Refinement & Reduction of Animals in Research (NC3Rs) and the ARRIVE guidelines.

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## COMPETING INTERESTS

Authors have declared that no competing interests exist.

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