Differences in Immunological Impact of Chemically and Biologically Synthesized Silver Nanoparticles

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***Abstract*— Silver nanoparticles (AgNPs) are widely used for their antimicrobial properties but pose risks like environmental contamination and potential harm to human health. Nanoparticles' small size facilitates translocation within the body, often bringing them into contact with blood. Most toxicological research focuses on chemically synthesized AgNPs (CAgNPs) and their effects on microbes and animal cells. Fewer studies explore biologically synthesized AgNPs (BAgNPs) on animal cells, and their impact on blood components is uncertain with varied findings due to differences in size and stability. This study examined BAgNPs' effects on blood components in healthy and diseased states, using algae *Parachlorella kessleri* for synthesis. Nanoparticle size and morphology were assessed via TEM and UV-Vis spectrophotometry. Exposure to BAgNPs resulted in an increased number of echinocytes, reduced neutrophils, and decreased leukocyte viability. Unlike CAgNPs, BAgNPs did not increase macrophage proliferation. Differences in biological properties between BAgNPs and CAgNPs stem from their colloidal stability in varying environments. CAgNPs, stabilized electrostatically, exhibited greater aggregation in environments with higher salinity and lower pH, diminishing their biological effects in human blood. Hence, electrostatically stabilized chemically produced AgNPs may not be suitable for biomedical applications.**

***Index Terms*— Immune system, blood cells, nanoparticle toxicity.**

I. Introduction

IN RECENT years, silver nanoparticles (AgNPs) have attracted considerable attention for their strong antimicrobial properties, resulting in their extensive use in medical, industrial, and consumer products [1]. AgNPs' efficacy in combating a broad spectrum of pathogens has been well-documented, making them valuable in areas such as wound healing, coatings for medical devices, and as additives in personal care products [2]. However, the growing utilization of AgNPs also raises concerns about their potential adverse effects, including environmental contamination and health risks to humans. During the manufacturing, use, or disposal of products containing AgNPs, particularly sprays or humidifiers, silver nanoparticles may be released into the air and enter the human body via the respiratory system [3], [4], [5]. Silver nanoparticles also have the ability to penetrate both damaged and intact human skin when using cosmetics containing AgNPs or through dermal contact with bandages and textiles containing nanosilver [6]. However, the most common route of silver nanoparticle exposure is oral ingestion. AgNPs are used in the food industry for packaging and storage to increase shelf life. Several studies have demonstrated that nanoparticles in packaging can migrate into the food. Silver nanoparticles are also used in water treatment, which can be another potential source of oral exposure [6]. It is reported that the daily intake of silver through diet can range from 27-90 μg, and sometimes even higher [7], [8].Via each route, AgNPs can translocate into the bloodstream, thereby reaching every organ in the body. This carries several potential risks, including cytotoxic effects, accumulation of silver in organs, and induction of inflammatory responses, all of which must be carefully considered before the application of AgNPs [9]. One of the key characteristics of nanoparticles is their size, which allows for easy translocation within biological systems. This unique property facilitates their interaction with various cellular components, including blood cells, upon entering the human body [10], [11], [12]. The majority of toxicological studies to date have focused on chemically synthesized silver nanoparticles (CAgNPs), investigating their impact on microbial organisms and mammalian cell lines [13], [14], [15]. Despite this extensive research, there are many gaps in knowledge regarding the effects of biologically synthesized silver nanoparticles (BAgNPs), particularly in the context of animal cells and blood components. BAgNPs offer a promising alternative to their chemically synthesized counterparts,

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as they are typically produced using environmental-friendly processes involving biological organisms such as bacteria, fungi, and algae. These biogenic methods not only reduce the reliance on hazardous chemicals but also often result in nanoparticles with unique properties that may influence their biological interactions [1], [16], [17]. However, the variability in nanoparticle size, shape, and stability arising from different synthesis methods can lead to inconsistent findings in their biological effects, necessitating a deeper exploration of their impact on blood components. This study aims to bridge this knowledge gap by investigating the effects of BAgNPs on blood components in both healthy and diseased states.

II. Material and Methods

A. Preparation of Chemically Produced AgNPs

A 10^{-3}M $AgNO_3$ solution was brought to a boil in a boiling flask using a water bath. At the first sign of boiling, a 1% sodium citrate solution was added. A reflux condenser was attached to the flask, and the contents were boiled for 1 hour [18].

B. Preparation of Biologically Produced AgNPs

Silver nanoparticles were prepared using the algae *Parachlorella kessleri*. Following a modified procedure, an extract was prepared from the algae and subsequently mixed with an AgNO3 solution to achieve a final silver concentration of 100 mg/L.

UV-vis spectrometry was used to characterize both chemically and biologically produced nanoparticles after each production process to ensure consistency. However, a comprehensive characterization using TEM, UV-vis, FT-IR, and EDS was performed only during the standardization of the biological synthesis method and has been published in Kadukova [19]. Therefore, these data are not repeated in this study.

C. Preparation and Incubation of Blood Samples

Blood samples (20 mL per patient) were collected into tubes containing both EDTA and heparin as anticoagulants. The collected blood was then divided into experimental groups based on the treatment conditions. For nanoparticle preparation, colloidal solutions were centrifuged (5000 rpm, 40 min), the supernatant was largely removed, and nanoparticles were resuspended by vortexing. The appropriate types of AgNPs were added to designated tubes to achieve a final silver concentration of 50 mg/L in the blood. The concentration of AgNPs was determined as the total silver concentration using atomic absorption spectroscopy. The proportion of silver ions converted into nanoparticles was calculated based on measurements using an ion-selective electrode. For the sample with silver ions, 10 μL of silver ion solution was added to the microtube to achieve a total silver concentration of 5 mg/L. In the sample with the biological extract, 10 μL of the *Parachlorella kessleri* algae extract was pipetted into the microtube. A control sample without any additives was also included in the study. All blood samples were incubated at 37°C for 3 hours under controlled conditions. Additionally, an untreated control sample was analysed immediately (0-hour control) to serve as a baseline for comparison.

D. Blood Smear Preparation and Staining

A drop of fresh whole blood was placed on a clean microscope slide and spread into a thin film, air-dried, and fixed in methanol. The slide was stained with May-Grünwald (3 min) and Giemsa-Romanowsky (20 min) stains, gently rinsed, and air-dried. Cellular morphology was assessed microscopically (DM1000, Leica Microsystems, Germany).

E. Analysis of Viability and Apoptosis of Blood Leukocytes

Dead cells were analyzed using the Muse™Count and Viability kit (Muse™Cell Analyzer; Millipore, USA) according to the manufacturer's instructions. The percentage of apoptotic cells in the blood was determined using the Muse Annexin-V & Dead Cell Assay kit™(Muse™Cell Analyzer; Millipore, USA). Briefly, phosphatidylserine on the surface of apoptotic cells was detected by a fluorescent dye (FITC) conjugated to Annexin-V and as a dead cell marker 7-AAD was used. As a result, late apoptotic (Annexin-V+/7-AAD+), early apoptotic (Annexin-V+/7-AAD−), live cells (Annexin-V−/7-AAD−) and dead cells (Annexin-V−/7-AAD+) were detected using Muse Cell Analyzer (Millipore, USA) and analyzed with MuseSoft 1.4.0.0 (Millipore).

F. Preparation of M1 and M2 Macrophages

Monocytes (THP-1) were sourced from the European Collection of Authenticated Cell Cultures. THP-1 cells were cultured in R10 medium (RPMI-1640 with L-glutamine and 10% (v/v) fetal bovine serum (FBS)) and incubated at 37°C in a humidified atmosphere containing 5% (v/v) CO_2. To differentiate and polarize the THP-1 monocytes into M1 (classically activated) and M2 (alternatively activated) macrophages, we followed the protocol established by Foey et al. [20].

G. Real-Time Analysis of Cell Proliferation

To continuously monitor the effect of nanoparticles on the proliferation of differentiated macrophages, we employed the xCELLigence system following the methodology described in the study by Amrichová et al. [21].

H. Statistical Analysis

Statistical analyses of the results were conducted using Minitab version 16 (Minitab Inc., 2013, State College, PA, USA). Differences between groups were assessed using the t-test and one-way ANOVA, followed by Tukey's method for post-hoc pairwise comparisons.

III. Results and Discussion

A. Basic Characterization of Biologically and Chemically Produced AgNPs

The size, shape, and surface coating of the nanoparticles were determined and evaluated, as these factors can significantly influence the final properties of the nanoparticles

TABLE I
THE CHARACTERISTICS OF BIOLOGICALLY AND CHEMICALLY PRODUCED AGNPS

	BAgNP	CAgNP
Size	9 ± 2 nm	20 - 90 nm
Shape	Spherical	Spherical
Surface	Biomolecular corona from components of the *Parachlorella kessleri* algae extract	Citrate anions

CAgNPs = chemically synthesized silver nanoparticles, BAgNPs = biologically synthesized silver nanoparticles;

TABLE II
AGGREGATION OF RED BLOOD CELLS (RBC)

Sample	Aggregation
BAgNP	+
CAgNP	-
Ag^+ ions	-
Extract	+
Control	-

+ positive reaction; - negative reaction

(Table I). The physicochemical properties of BAgNPs, including their size and stability, have been previously characterized and published in Kaduková [19] and Sedlakova-Kadukova, Demcakova [22].

B. The Effects of AgNP, Silver Ions, and Algal Extract on Human Blood

It was essential to determine the extent to which silver ions and the nanoparticles themselves contribute to the biological properties of AgNPs. AgNPs are known to influence immune responses through multiple mechanisms, including cytokine modulation, oxidative stress, and direct interactions with immune cells. Some studies indicate that the release of Ag^+ ions from the surface of AgNPs is the primary cause of the nanoparticles' toxicity and biological effects. These results showed that the presence of both AgNPs and Ag^+ ions led to increased ROS production, pro-inflammatory cytokines (IL-1β, IL-6, TNF-α) and apoptosis induction in immune cells [23], [24], [25]. These findings are consistent with reports that AgNPs can activate toll-like receptors on immune cells, leading to NF-κB signaling pathway activation and subsequent cytokine production [26], [27]. However, other studies suggest that the toxic effects of AgNPs and Ag^+ ions are different, and that the biological effects of AgNPs are due to the nanoparticles themselves, as the toxic effect pattern differed from that of silver ions [24], [28], [29]. Smaller, more stable nanoparticles tend to induce stronger pro-inflammatory responses due to increased cellular uptake and surface area, whereas larger, less stable nanoparticles may aggregate, reducing their immunotoxicity [30]. This size-dependent behavior influences their impact on immune cell viability, cytokine production, and oxidative stress levels, which aligns with our observations that BAgNPs, being smaller and more stable, elicited a stronger pro-inflammatory response compared to CAgNPs. As stated by Pratsinis et al. [31], the extent to which silver ions contribute to the effects of AgNPs differed between smaller nanoparticles (<10 nm) and larger AgNPs. Smaller AgNPs have a much higher surface-to-volume ratio, allowing a greater percentage of Ag^+ ions to be released, making the cytotoxic effect of AgNPs predominantly due to Ag^+ ions. For larger AgNPs, cytotoxicity is mainly due to the AgNP-cell interaction. However, based on the later results, it is clear that nanoparticles of the same size and shape have different toxicological characteristics related to their corona that is the highly probable factor responsible for this toxicity [32]. The biomolecular corona influences nanoparticle-cell interactions and immune modulation by altering protein adsorption and immune recognition [33], [34]. We aimed to determine the extent to which the biological effects of biologically produced AgNPs are influenced by the extract components. Some studies have shown that the extract alone did not elicit a significant response [35], [36]. However, there is evidence that nanoparticles prepared from plant extracts may exhibit biological activities similar to those of the extract [37]. Upon examining the microscopic slide, an increased level of red blood cell aggregation was observed in the sample containing BAgNP and extract (Tab. II). Several studies have also noted that silver nanoparticles can induce procoagulant activity [38], [39], [40], [41]. Conversely, red blood cells or their aggregation did not significantly differ compared to the control sample. Based on this, we can infer to some extent that the effect with BAgNP was likely more influenced by the algae extract corona than by the release of Ag^+ cations. The difference was also observable between different types of silver nanoparticles. BAgNP induced significant aggregation of red blood cells, whereas this effect was absent when using CAgNP. This distinction could be explained by the different sizes of AgNPs or by variations in their stability. As mentioned earlier, smaller AgNPs with higher stability tend to be more toxic.

BAgNP are smaller compared to CAgNP and are coated with a corona that provides higher stability even under varying conditions in blood [42]. Based on these factors, it can be hypothesized that CAgNP, after incubation in blood, may aggregate to sizes exceeding 100 nm, forming clusters that are considered micro-particles. Studies have also compared the effects of AgNPs on blood components and concluded that silver micro-particles had a much weaker effect compared to silver nanoparticles [40], [41]. These results would therefore explain why red blood cell coagulation was significant when using biologically produced AgNPs but not chemically produced AgNPs. The previous hypotheses were further supported by the results of differential leukocyte counts in blood smears. Based on changes in the percentage representation of leukocytes, we can infer which subgroups of white blood cells were more affected and killed by the presence of BAgNP, CAgNP, Ag^+ ions, or extract (Tab. III).

Differential leukocyte counts revealed that BAgNPs negatively affected phagocytic leukocytes (neutrophils and monocytes), leading to reduced viability and increased apoptosis. This observation aligns with findings that AgNPs trigger oxidative stress and ROS production, particularly in phagocytic cells due to their engulfment and intracellular

TABLE III
THE EFFECT OF DIFFERENT TREATMENTS ON THE DIFFERENTIAL LEUKOCYTE COUNTS

WBC (%)	BAgNPs *n=5*	CAgNPs *n=5*	Ag^{+}ions *n=5*	Extract *n=5*	Control *n=5*
Neutrophils	59,0 ± 2,50*	63,83 ± 1,26	66,00 ± 4,09	60,50 ± 1,80*	63,17 ± 1,26
Eosinophils	1,33 ± 0,58	1,50 ± 0,50	2,00 ± 1,00	1,67 ± 0,58	0,67 ± 0,29
Basophils	0,83 ± 0,29	1,33 ± 0,58	2,33 ± 0,29	2,50 ± 0,50	1,33 ± 0,58
Lymphocytes	35.5 ± 2.78*	27,83 ± 2,75	22,17 ± 4,07	32,67 ± 1,89*	28,67 ± 1,53
Monocytes	3,33 ± 0,29	5,50 ± 0,87	7,50 ± 0,87	2,67 ± 0,29	6,17 ± 0,76

degradation [30], [43], [44], [45]. In contrast, Ag+ ions primarily impacted lymphocytes, suggesting a distinct mechanism of cytotoxicity through direct genotoxic effects, as Ag+ ions are known to bind to DNA and protein molecules, disrupting cellular functions [13], [25], [28], [31]. While it might be expected that Ag^{+} ions would be cytotoxic to all leukocytes, lymphocytes appeared to be somewhat more sensitive to their presence for certain reasons. Based on these observations, we can see that the trend observed with biologically produced nanoparticles was more similar to that seen with the extract, rather than Ag^{+} ions, where an opposite trend in the differential leukocyte count was indicated. These results support our initial observation that the biological effects of BAgNPs are not primarily due to Ag+ ion release but are influenced by the nanoparticles' colloidal stability, surface properties, and biomolecular corona. Our findings provide further evidence that synthesis methods significantly influence the immunological behavior of AgNPs, contributing to their distinct effects on immune cells. The lower colloidal stability of chemically produced nanoparticles resulted in the formation of aggregates at the micro-particle level, which do not have the same biological effect as nano-sized particles [40], [41]. Another explanation could be that CAgNPs might have had some impact on immune cells, but this effect could not be observed through changes in the leukocyte differential count. In such a case, the effect of these CAgNPs would differ from that of BAgNPs, which could be explained by differences in their size, stability, or surface coating. These differences are also documented in the literature, and further experiments will be necessary to better understand the behavior of AgNPs in human blood. However, upon analyzing viability, we can see that the presence of CAgNPs also impacted the overall viability of leukocytes. Although this difference did not manifest in the differential leukocyte count, a statistically significant difference was observed between the sample containing CAgNPs and the control when measuring viability (Fig.1). We observed a similar level of viability after using BAgNPs, which also showed a statistically significant difference compared to the control sample. Based on these results, it can be inferred that both types of AgNPs caused a statistically significant reduction in the viability of white blood cells, which, however, manifested differently in the determination of the percentage representation of lymphocytes in the blood smear. The differences between these nanoparticles likely caused their varying effects on leukocytes or the mode of cell death. However, these results cannot be supported by the literature, as no study addressing this specific issue has been found so far.

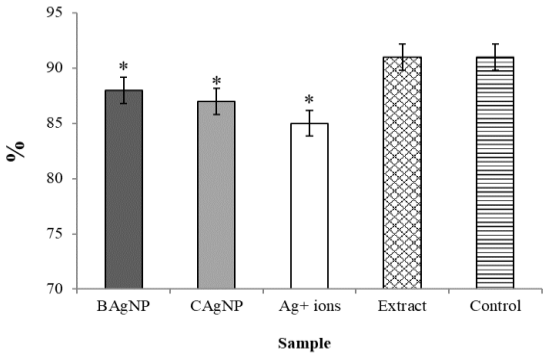

Fig. 1. Leukocyte viability rates in individual samples (∗ $P < 0.05$ vs. control; values are expressed as mean ± SD). BAgNPs - biologically synthesized silver nanoparticles; CAgNPs - chemically synthesized silver nanoparticles.

The most significant decline, which was statistically distinct from all other samples, was observed with the use of silver ions. As confirmed by the study by Greulich et al. [24], silver ions are highly toxic and can easily penetrate cells. Unlike AgNPs, all silver ions in the solution are readily available to cells, whereas, with AgNPs, only the silver atoms on their surface are accessible to cells. From these experiments, we found that the behavior of AgNPs and their impact on blood components differed significantly from the use of Ag+ ions, indicating that the primary effect of nanoparticles is due to the nanoparticles themselves, not merely as a source of Ag+ ions. The behaviour of biologically produced nanoparticles, based on the blood smear observations and differential count, more closely resembled the trend observed with the algae extract used in the production of BAgNPs. Differences were also observed between the nanoparticles themselves, likely due to variations in size and stability.

C. Comparison of the Effects of BAgNP and CAgNP on Blood Components in Health and Disease

In the next phase of our experiment, we focused on comparing the effects of biologically and chemically produced silver nanoparticles on the blood of healthy individuals and patients suffering from rheumatoid arthritis. Rheumatoid arthritis is one of the most common complex multifactorial autoimmune inflammatory disorders associated with joints. Due to chronic synovial inflammation, there is increased production of pro-inflammatory cytokines, production of autoantibodies, repeated activation of leukocytes, and gradual destruction of joint cartilage and bones. The treatment of this disease

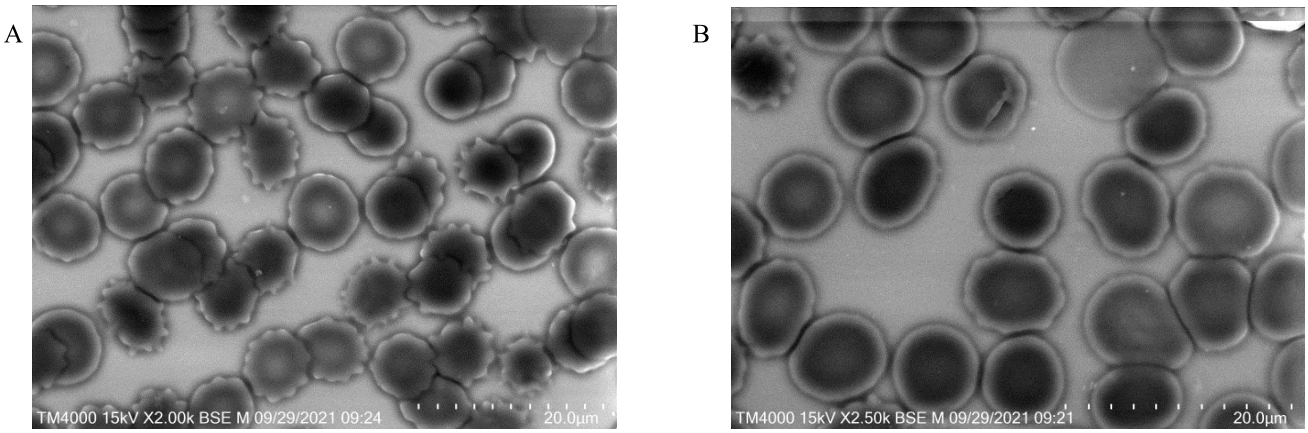

Fig. 2. Echinocytes visualized using a scanning electron microscope at 2500x magnification. A – erythrocytes after incubation with BAgNP; B – erythrocytes after incubation with CAgNP. BAgNPs - biologically synthesized silver nanoparticles; CAgNPs - chemically synthesized silver nanoparticles.

remains challenging, as it is often ineffective and accompanied by numerous side effects [46], [47]. Nanomedicine is one area that could provide targeted therapy for this and other inflammatory diseases [48]. Several studies have confirmed that the application of biologically produced AgNPs, particularly those prepared from extracts of various plants with anti-inflammatory effects, alleviated symptoms of rheumatoid arthritis in rats [46], [48]. These anti-inflammatory and anti-arthritic effects of BAgNPs could help reduce chronic inflammation and aid in the treatment of patients with rheumatoid arthritis. Despite these claims, most studies investigating the impact of AgNPs on immune cells and their response report that the nanoparticles predominantly had cytotoxic effects and induced an inflammatory reaction. This significantly contradicts the studies on the effect of AgNPs on arthritis. One possible explanation is that most studies examining the impact on immunity and blood components used chemically produced nanoparticles, whereas nearly all studies on the anti-arthritic effect of nanoparticles utilized biologically produced nanoparticles. Another possibility is the fact that the blood components and immune system of a healthy patient may behave differently compared to someone suffering from chronic condition such as arthritis. Continuous immune system activation, higher production of pro-inflammatory cytokines, and antibody production significantly affect not only immune function but also the entire organism. Therefore, it is important to consider the different conditions of a diseased patient. Certain studies have also indicated that, for example, oncology patients may be more susceptible to clot formation, and treatment involving AgNPs would need to be carefully considered in such cases [41].

In line with the first part of the experiment, we assessed blood smears from both patient groups after incubating their blood with BAgNP and CAgNP. Notably, a higher degree of erythrocyte coagulation was observed exclusively after exposure to BAgNP in all patients, consistent with findings in the study by Bian et al. [41]. This effect was not observed in samples incubated with CAgNP or in the control group. Additionally, we observed that BAgNP induced morphological changes in erythrocytes across all patients. Blood smears incubated with BAgNP showed a marked increase in echinocyte formation compared to those treated with CAgNP or the control, where the occurrence of echinocytes was rare or absent (Figure 2). This phenomenon is not isolated, as previous studies have also confirmed that AgNP can induce morphological alterations in erythrocytes [49], [50]. The question arises as to why enhanced procoagulant activity and morphological changes in red blood cells were observed with BAgNP but not with CAgNP. It is possible that the lower colloidal stability of CAgNP led to aggregation, resulting in the formation of larger silver particles. This could explain the significantly lower occurrence of echinocytes observed after the use of CAgNP compared to BAgNP.

More noticeable differences were observed in the differential leukocyte count. We hypothesized that AgNP would predominantly affect phagocytic cells, particularly neutrophils and monocytes, as these cells are likely to recognize the foreign material, engulf it, and subsequently come into direct contact with AgNP. Once inside the phagolysosome, AgNP can dissolve, releasing a substantial amount of silver at once, which may lead to a strong cytotoxic effect and potentially result in cell death [51]. A similar trend was observed in both patient groups: the presence of AgNP led to a reduction in the percentage of neutrophils (the most abundant phagocytic cells). The most pronounced effect was observed following exposure to BAgNP in blood samples. The percentage of neutrophils in the blood of healthy patients decreased significantly by 12% compared to the control sample, dropping from 59% to 47% (Figure 3A). A similar trend was observed in the blood of patients with arthritis, where the percentage of neutrophils decreased significantly by 8.5% compared to the control (Figure 3B). However, this marked trend was not observed after exposure to CAgNP, where the leukocyte percentages remained comparable to the control. The most likely explanation is the larger size and lower stability of CAgNP, which may have resulted in lower cytotoxic activity compared to BAgNP, thus supporting our previous assumptions. By comparing the differential leukocyte count between both patient groups, we observe that neutrophil levels in patients with rheumatoid arthritis are significantly lower than in healthy

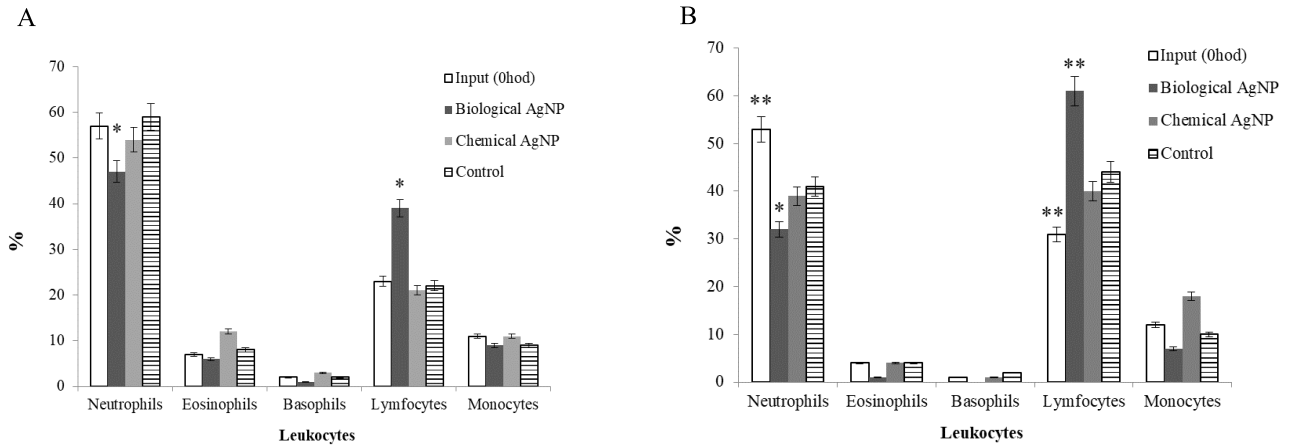

Fig. 3. Differential leukocyte counts in the blood of a healthy patient cohort (A) and in the blood of a patient with rheumatoid arthritis (B) following AgNP exposure; (*P < 0,05; **P < 0,01 vs. control; data presented as mean ± SD).

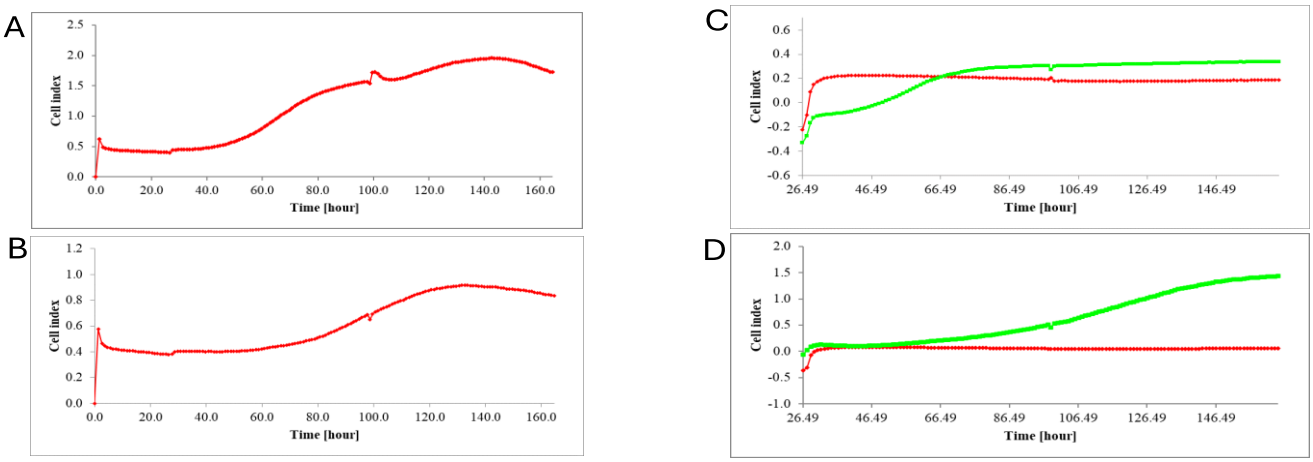

Fig. 4. Changes in the cellular index of M1 (A) and M2 (B) macrophages in the control samples. C: Changes in the cellular index of M1 macrophages (C) and M2 macrophages (D) in the sample with BAgNP (red) and CAgNP (green). BAgNPs - biologically synthesized silver nanoparticles; CAgNPs - chemically synthesized silver nanoparticles.

patients, despite similar baseline values. However, in the blood of patients with rheumatoid arthritis, a reduction in neutrophil count occurred even due to incubation alone, which was evident not only in samples treated with AgNP but also in the control. This is likely due to the heightened sensitivity of neutrophils resulting from increased activation associated with chronic inflammation. Neutrophils, when activated in response to chronic inflammation, can exhibit increased responsiveness and a tendency to undergo apoptosis, particularly due to the ongoing exposure to inflammatory cytokines. This heightened state of activation makes them more susceptible to various stimuli, leading to significant functional changes, including altered immune responses and a higher propensity for cell death [46]. This suggests that certain blood components in patients with chronic inflammatory diseases may be more susceptible to environmental influences, which should be considered when determining appropriate therapy.

D. Comparison of the Effects of BAgNP and CAgNP on the Proliferation of M1 and M2 Macrophages

Macrophages are crucial components of the immune system, with M1 macrophages playing a role in pro-inflammatory responses and M2 macrophages being involved in anti-inflammatory processes and tissue repair. Understanding how these nanoparticles affect macrophage proliferation can provide insights into their potential therapeutic uses or toxicological impacts. Since macrophages are among the first immune cells to encounter silver nanoparticles upon exposure in the human body [30], it is crucial to study their interactions. Both control samples of M1 and M2 macrophages exhibited similar trends in their cell index curves. In the initial hours, cell adhesion was observed, followed by a plateau phase lasting until approximately the 40th to 50th hour, after which significant cell proliferation occurred, reflected in the gradual increase in the cell index (Figures 4A and 4B). M1 macrophages, being adherent cells, had a higher cell index compared to M2 macrophages. The xCELLigence technology is also employed to determine the toxicity of silver nanoparticles. However, the methodology has a drawback: silver is a conductive metal. Since the instrument records electrical impedance, interference from the presence of AgNPs can affect cell index values, which must be taken into account [52]. For evaluating the effects of AgNPs, THP-1 cell line was used. These cells have the potential for continuous proliferation and serve as a

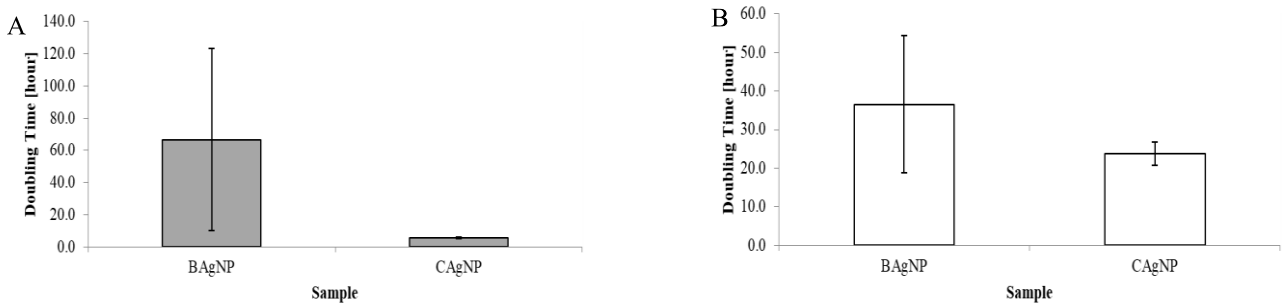

Fig. 5. Doubling time of M1 macrophages (A) and M2 macrophages (B) in samples with BAgNP and CAgNP. BAgNPs - biologically synthesized silver nanoparticles; CAgNPs - chemically synthesized silver nanoparticles.

suitable model for assessing the toxic effects of AgNPs. After stabilizing conditions for 24 hours and adding AgNPs to the cells, an increase in the cell index was observed for both types of nanoparticles. However, their progression subsequently differed, despite both samples receiving silver at the same concentration of 50 mg/L (Figure 4C). After using biological AgNPs, there was no significant change in the cell index from 30 hours onwards, which may be related to a decrease in cell proliferation of M1 macrophages. In contrast, in the sample with chemical AgNPs, the cell index gradually increased and maintained an upward trend. This difference was also evident in the doubling time. Despite both samples receiving the same amount of silver, the doubling time of M1 macrophages in the presence of BAgNP was nearly 12 times longer compared to CAgNP application (Figure 5A). A similar effect was observed after adding biological and chemical nanoparticles to M2 macrophages. Although the total amount of silver in the solution was the same, changes in the cell index and the course of the curve differed after using BAgNP and CAgNP. Similar to M1 macrophages, with M2 macrophages, the application of BAgNP led to an initial increase in the cell index values that subsequently did not change significantly. However, after applying CAgNP, the cell index values gradually increased, and the curve maintained an upward trend throughout the measurement period (Figure 4D).

Although the change in doubling time for M2 macrophages followed a similar pattern as observed for M1 macrophages, cell division was nearly twice as slow after exposure to biologically synthesized AgNPs compared to CAgNPs (Figure 5B). While silver can interfere with impedance-based measurements, the distinct differences in curve progression and doubling times between BAgNP and CAgNP treatments suggest that these effects are primarily due to differences in cell proliferation, rather than artifacts caused by silver conductivity on the E-plate. Notably, both BAgNP and CAgNP contained silver at the same concentration. In both M1 and M2 macrophages treated with BAgNPs, we observed an initial increase in the cell index followed by a plateau, indicating stagnation in proliferation. This effect occurred even in tumor-derived cells with high proliferative capacity. These findings support our hypothesis that BAgNPs exert a stronger antiproliferative effect than CAgNPs, likely due to their smaller size and higher colloidal stability. Moreover, the ability of BAgNPs to inhibit the proliferation of tumor cells may suggest potential anticancer applications. Recent advancements in nanomedicine further reinforce the translational potential of our findings. For example, functionalized AgNPs have been utilized in cancer diagnostics via surface-enhanced Raman spectroscopy [53], while innovative macrophage-derived nanovaccine platforms have shown promise in enhancing antiviral immunity [54]. Immune-adjuvanted metal–organic frameworks represent another promising strategy for harnessing nanomaterials to modulate tumor immunity [55]. Additionally, nanomaterials have shown therapeutic promise in tissue regeneration, as exemplified by immunoregulatory hydrogels that promote bone healing through localized inflammation control [56]. Our results contribute to this growing body of research, highlighting the influence of nanoparticle origin and stabilization methods on immune cell behavior and supporting the potential role of BAgNPs in precision immunotherapy.

IV. Conclusion

The colloidal stability of AgNPs within biological environments is a critical factor, as aggregation can profoundly influence their biological activity. Our results suggest that due to the diverse environmental conditions encountered within the human body, sterically stabilized, biologically produced nanoparticles offer a more advantageous option for biomedical applications. These nanoparticles better maintain their non-aggregated state and biological efficacy compared to their electrostatically stabilized counterparts. While size and stability are key determinants of nanoparticle activity, our study focused primarily on their biological effects. However, as different synthesis methods can yield nanoparticles with variable size, shape, and surface properties, standardization of biogenic synthesis protocols is crucial to ensure reproducibility and reliability of biological outcomes.

Furthermore, while this study utilized BAgNPs synthesized specifically from *Parachlorella kessleri*, we acknowledge that nanoparticles produced using other biological organisms (such as bacteria, fungi, or higher plants) may exhibit different physicochemical characteristics and immunological effects. Thus, future comparative studies involving diverse biogenic sources are needed to assess the generalizability of our findings. Although our study provides valuable comparative data on the immunomodulatory effects of biologically and chemically synthesized AgNPs, we recognize the potential of further mechanistic insights that could be gained through additional analyses, such as qPCR and Western blot. These

advanced techniques would allow for a deeper understanding of the molecular pathways involved in immune modulation and help establish a more direct causal link between nanoparticle characteristics (e.g., size, stability, and surface properties) and their biological activity.

Our findings underscore the critical importance of the synthesis and stabilization methods employed in nanoparticle production, as these factors significantly influence colloidal stability and, consequently, the overall biological activity of the nanoparticles within the organism. A comprehensive understanding of AgNP properties, colloidal stability, and their biological impacts is essential for their effective application in biomedical fields. Appropriately engineered AgNPs hold potential for personalized therapeutic applications, such as inducing inflammation in immunosuppressive disorders, inhibiting excess immune responses in inflammatory disorders, or reducing tumor cell proliferation.

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