Silver nanoparticles augment releasing of pyrogenic factors by blood cells stimulated with LPS

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Running title: Pyrogenicity of silver nanoparticles
Abstract

Silver nanoparticles (AgNPs) have cytotoxic properties via generation of reactive oxygen species which are involved in the generalized sickness behavior of the host including fever and lethargy among others. The aim of the present study was to investigate the impact of AgNPs on the ability of rat peripheral blood mononuclear cells (PBMCs) to release fever mediating factors after stimulation with lipopolysaccharide (LPS). Body temperature and motor activity of the Wistar rats were measured by biotelemetry system. Rat PBMCs were stimulated with LPS and after that, the cells were washed and incubated alone or with AgNPs. The final supernatants were injected intraperitoneally. The levels of endogenous pyrogens such as interleukin-1β (IL-1β), IL-6 and tumor necrosis factor-α (TNF-α) released from the PBMCs into the final supernatants were also estimated. The results indicated that injection of the supernatants from the cells stimulated with LPS induced fever and inhibited motor activity. These effects were potentiated by the presence of AgNPs during the final incubation. The presence of the AgNPs also resulted in significant increases in levels of endogenous pyrogens. The augmentation of fever in the rats by the AgNPs treatment of the cultures seemed to be primarily associated with the changes in interleukin-1β levels.

keywords: silver nanoparticles, fever, motor activity, lipopolysaccharide, peripheral blood mononuclear cells, pyrogenic factors, interleukin-1β, interleukin-6, tumor necrosis factor-α
Introduction

Nanotechnology is the general term for the creation, manipulation and application of structures in the nanometer size range. Nanoparticles are defined as structures having dimensions of roughly 1-100 nm, whose unique properties enable their novel application [1]. The range of applications of nanoparticulate as drug carriers includes the treatment of all diseases of major public health concern, such as cancer, diabetes and severe infections [2]. Generally, nanoscale drug delivery systems have the ability to improve the pharmacokinetics and increase biodistribution of therapeutic agents to target organs [3]. Moreover, this technology allows for release of the drug in a controlled manner and increases bioavailability by facilitating drug transport across critical and specific barriers, including blood-brain barrier [4]. Nowadays, nanotechnology has developed numerous types of nanoparticulate drug carriers, such as liposomes, micelles, dendrimers, nanotubes, nanoshells, nanoemulsions and metallic nanoparticles such as silver nanoparticles [5, 6, 7].

Presently, silver nanoparticles (AgNPs) are emerging as a new generation of antibacterial agent, which has been used in medical applications (e.g. wound dressings, surgical instruments and bone prostheses) [8, 9]. Among metallic nanomaterials including copper, zinc, titanium, magnesium, gold and silver, AgNPs have proved to be most effective in killing bacteria, viruses and other eukaryotic microorganisms [10]. These nanostructures can act on the living cells at the nano-level resulting not only in the biologically beneficial effects, but also may provoke some undesirable perturbations. AgNPs have a large surface area per unit mass and this property makes nanosystems very reactive in the cellular environment [11]. In consequence, AgNPs have cytotoxic properties via a generation of the reactive oxygen species (ROS) and induction of the intracellular oxidative stress [12]. It is well known that ROS are involved in the generalized sickness behaviour of the host including fever and lethargy, and these activities are related to the induction of the signal transduction leading to
the upregulation of the transcription factors such as nuclear factor kappa B (NF-κB) [13]. Intracellular upregulation of NF-κB is necessary for the induction of the synthesis of the pyrogenic and pro-inflammatory cytokines [14]. However, to the best of our knowledge, there is no research focusing on the pyrogenic and sickness behaviour properties of the silver nanoparticles.

Fever is a common response to infection, inflammation, injury and trauma. It is a regulated rise in body temperature (Tb) that results from an upward resetting of variables controlling the set-point of thermoregulation [15]. An increase of Tb during fever has several advantages over infections: inhibition of bacterial growth, increase in bactericidal activities of neutrophils and macrophages, T cells proliferation and differentiation, B cells proliferation and the production of antibodies or stimulation of acute-phase protein synthesis [16, 17]. The initial step in the cascade of events leading to fever is believed to be a stimulation of a large number of various immune types of cells, i.e., monocytes, macrophages and neutrophils by exogenous stimuli, called exogenous pyrogens [18]. These stimuli are represented by bacteria wall components such as lipopolysaccharide (LPS) or lipoteichoic acid, viral components such as double-stranded RNA and bacterial DNA (CpG-DNA) [19, 20]. Stimulation of immune cells by any of these various pyrogens leads to synthesis of pro-inflammatory mediators, called endogenous pyrogens. The most important mediators of fever and other symptoms of sickness are cytokines such as interleukin-1β (IL-1β), IL-6, interferon-γ (IFN-γ) and tumor necrosis factor-α (TNF-α) [15, 18, 21-24]. Since it has been known that macrophages stimulated with LPS release the pyrogenic factors [25, 26], and there are data showing that AgNPs may influence some immunologic activities, we have studied the effect of AgNPs on the releasing of these fever mediating factors by the PBMCs stimulated in vitro with LPS. From our findings, we conclude that AgNPs can potentiate
production of pyrogenic factors by PBMCs that have been already stimulated with LPS but have only a small effect on the unstimulated cells.

Experimental Procedures

Animals
Male Wistar rats [Strain: Wistar Crl: WI(Han)] (8-12-wk-old, 200-250 g) were purchased from the Experimental Medicine Centre at the Medical University of Bialystok and allowed to acclimatize for 10 days before the start of any experiments. The rats were housed in individual polycarbonate cages in a room maintained at constant relative humidity (60 ±5%), temperature (24 ±1°C), and with a 12-hr light-dark period. All rats had ad libitum access to standard rodent laboratory food (Labofeed B, Morawski, Poland) and filtered drinking water. The Local Bioethical Committee for Animal Care approved all procedures (Permission # 4/2011).

Body temperature (Tb) and motor activity measurement
The Tb of rats was monitored (with accuracy of ±0.1°C) using battery-operated miniature biotelemeters (PhysioTel® model TA10TA-F40, Data Sciences International, USA) implanted intra-abdominally as described previously [27]. The animals were anaesthetized with a ketamine/xylazine mixture (87 and 13 mg kg−1, respectively) injected intramuscularly. An incision was made in the abdomen and a telemetry transmitter was placed into the abdominal cavity for the continuous monitoring of the physiologic variables. All surgical procedures were done at least one week before the start of experiments. Motor activity of the rats was measured by changes in position of the implanted transmitter over the receiver board. These result in a change of the signal strength detected by the receiver and recorded as a "pulse" or "count" of the activity.
Rat PBMCs fraction preparation

Blood was collected from anesthetized rats by cardiac puncture into the solution of ethylenediamine tetraacetic acid (EDTA, MP Biomedicals; cat. no. 160054). The whole blood was diluted 1:1 (vol/vol) with phosphate buffered saline (PBS, pH 7.4) at room temperature. A 5 mL portion of this blood was then carefully layered onto 3.5 mL of the separation medium (Ficoll-Paque Plus, Amersham Biosciences; cat. no. 17-1440-02) in a 10 mL centrifuge tube and centrifuged (35 min, 400 x g, room temperature). The PBMCs fractions were isolated, pooled, and then washed twice in 2 vols PBS before being suspended at $10^6$ cells mL$^-1$ in complete RPMI 1640 containing 2 mM L-glutamine (Sigma-Aldrich; cat. no. R5886) and heat-inactivated 10% fetal bovine serum (FBS), 100 g streptomycin mL$^-1$, and 100 U penicillin mL$^-1$ (PAA Laboratories GmbH, Cölbe, Germany).

Culture conditions and stimulation

Freshly isolated PBMCs were seeded into 60-mm petri dishes (at $10^6$ cells mL$^-1$ - in 10 mL volume) and then pre-incubated for 1 hr at 37°C in a 5% CO$_2$ incubator maintained with a 95% humidity. All culture conditions were identical in each protocol. The $10^6$ cells were stimulated with LPS (1 µg mL$^-1$) for 4 h. Then, the cells were washed twice in PBS (1000 x g, 5 min), suspended in the fresh pathogen-free RPMI medium 1640 and incubated for the next 2 h with AgNPs (25 µg mL$^-1$; assigned as experimental incubation; cells+LPS+AgNPs). In separate experiments, the $10^6$ cells were stimulated only with AgNPs (25 µg mL$^-1$) for 2 h and not treated with LPS (assigned as reference incubation I; cells+AgNPs). In the separate cultures the cells were stimulated for 4 h with LPS (1 µg mL$^-1$; assigned as reference incubation II; cells+LPS). Control cells were incubated with pyrogen-free saline for 4 h as a control vehicle (control incubation; cells + saline). After completion of all variants of the stimulation, cells were collected from the plates by using a scraper and washed twice in PBS (1000 x g, 5 min). Then, the cells were suspended in fresh pathogen-free RPMI medium.
1640 and incubated for 2 h. The final supernatants were aspirated, centrifuged for 5 min at 1500 x g and stored at -50°C. All procedures resulted in a greater than 95% viable PBMCs in the suspension by trypan blue dye (Sigma-Aldrich; cat. no. T6146) exclusion assay. LPS derived from *Escherichia coli* (0111:B4, Sigma Chemicals; cat. no. L-2630) and AgNPs (Sigma Aldrich; cat. no. 576832) were used for the stimulation of the cells. LPS was diluted in a pyrogen-free saline. Serum-free RPMI 1640 medium were used in the case of cell cultures incubated with AgNPs to avoid aggregation. Before exposure, a stock solution of AgNPs (1 mg mL⁻¹) was prepared aseptically in the serum-free RPMI 1640 medium followed by bath sonication (Sonic 1, POLSONIC, Poland) for 10 min at room temperature to avoid nanoparticles agglomeration. Prior to administration to the cells, the final nanoparticles suspension was vortexed for 15 seconds.

**Cell viability - MTT assay**

MTT assay was performed to estimate toxic effects of AgNPs on the PBMCs. The cells were seeded into 96-well plates at a density of 5x10⁴ cells/well and stimulated with AgNPs (5, 12.5, 25, 35 and 50 µg mL⁻¹) for 2 hr in the same culture condition as described above. After exposure to AgNPs, the culture media was removed and replaced with fresh media (RPMI medium 1640 without phenol red; 100 µL/well). The cells were thereafter treated with 25 µL of MTT (3-[4,5-dimethylthiazol-3-yl]-2,5-diphenyl tetrazolium bromie; Sigma Aldrich; cat. no. M5655, 5 mg mL⁻¹ in PBS) and incubated at 37 °C for 4 hr. Non-treated cells were used as control. After that, the formazan crystals were dissolving with 100 µL of dimethyl sulfoxide (Sigma Aldrich; cat. no. D2438). Then absorbance at two wavelengths (570 nm and 630 nm) was recorded using ELISA reader (Multiskan FC; Thermo Scientific, USA). All absorbance values were corrected against blank wells which contained growth media alone. All measurements were done in duplicate in three independent experiments.
Silver nanoparticles analysis by scanning electron microscopy (SEM) and Dynamic Light Scattering technique (DLS)

The size and shape of the dry AgNPs powder were analyzed by the scanning electron microscopy (SEM - Quanta 3D FEG; Carl Zeiss, Göttingen, Germany) in the secondary electron (SE). The working parameters used were: high voltage - HV = 30.0 kV and working distance - WD = 10.1 mm. It is a high-resolution microscope, which allows studying of the structure of nanomaterials. On the basis of the resulting image the diameters of AgNPs were measured using the program ImageJ (Java-based image processing program developed at the National Institutes of Health; USA).

Dynamic Light Scattering method (DLS - Zetasizer Nanoseries S4700, Malvern Instruments, Worcestershire, UK) was used to determine the size distribution profile of nanoparticles in the solution (serum-free RPMI 1640 medium). For this, the 1 mg mL-1 stock AgNPs suspension in the deionized water was sonicated for 15 minutes in a water bath sonicator (Sonic 1, POLSONIC, Poland) before further dilution in serum-free culture medium to a final concentration of 25 μg mL-1. Then, the final nanoparticles suspension was vortexed for 15 seconds, sonicated for 15 minutes in a water bath sonicator and finally, it was vortexed again for 15 seconds immediately before DLS analysis. The samples were loaded into quartz microcuvette and three measurements were performed, for which the mean result was recorded.

Elemental analysis of the AgNPs was performed using the energy-dispersive X ray spectroscopy (EDX - Quantax 200; Carl Zeiss, Göttingen, Germany).

Cytokine assays

The final culture supernatants were analyzed for the release of IL-1β, IL-6 and TNF-α from the PBMCs. The levels of these cytokines were determined by a standard sandwich ELISA
kit from R&D Systems (cat. no. R6000B, RLB00, RTA00, respectively) according to the manufacturer’s instructions. Colorimetric changes in the assays were detected using Multiskan FC ELISA plate reader (Thermo Scientific, USA). The sensitivity of the IL-1β, IL-6 and TNF-α kits were, respectively, less than 5 pg mL⁻¹, 21 pg mL⁻¹ and less than 5 pg mL⁻¹.

In vivo treatment protocols

Separate groups of animals were injected intraperitoneally (ip) at 8:00 am with 2 mL of the supernatants from the control, reference I, reference II, or experimental incubation systems. An additional set of control rats was injected IP with RPMI 1640 vehicle while another set was injected IP with AgNPs suspended in RPMI 1640 at 25 µg mL⁻¹.

Statistical analysis

All values are reported as means ± standard error mean (S.E.M.) and were analyzed by analysis of variance (ANOVA) followed by the Student’s t-test with the level of significance set at P<0.05. For the Tb and motor activity measures, recordings were taken at 5-min intervals. For data presentation, these 5-min temperature and motor activity recordings were then pooled into 20-min or 5-hr averages, respectively.

Results

Characterization of silver nanoparticles

Dry powder of AgNPs from Sigma Aldrich were analyzed by the scanning electron microscopy. According to the SEM micrographs, shapes of the particles were approximately spherical, with a tendency to agglomerate (Figure 1A). The diameters of AgNPs measured using the program ImageJ on the basis of the resulting image indicated that nanoparticles had
a diameters in the range of 32-98 nm. Moreover, analysis of 100 nanoparticles showed an average particle size of 67.6 [± 17] nm (data not shown). In agreement with data obtained from the Sigma Aldrich, SEM images of the anhydrous AgNPs samples showed primary particle sizes of < 100 nm diameter.

DLS technique was used to determine the size distribution profile of nanoparticles in the solution (serum-free RPMI 1640 medium). The obtained results indicated that the tested colloid contained nanoparticles with the sizes of 298 [± 160] nm (92%), 46 [±9] nm (4,2%) and 4660 [± 869] nm (3,8%) (Figure 1B).

EDX studies showed the presence of >99.9% silver in the samples used here (data not shown).

Figure 1

Cell viability

Viability percentage measured by MTT assay on PBMCs exposed to 5, 12.5, 25, 35 and 50 µg AgNPs mL−1 for 2 h was dose-dependent (Figures 2). The results demonstrated that AgNPs at a dose of 5, 12.5 and 25 µg mL−1 during 2 h incubation did not have a toxic effect on PBMCs in comparison to non-treated cells. On the other hand, the higher dose (35 and 50 µg mL−1) of AgNPs were 24 [± 2] % and 44 [± 3] % more cytotoxic (P < 0.05 and P < 0.01 respectively) after 2 hr incubation. Therefore, a dose of 25 µg mL−1 was selected for experimentation.

Figure 2

Changes in the body temperature after injections of the tested supernatants

Rats are nocturnal animals revealing low daytime and high nighttime Tb and motor activity. Animals injected with AgNPs in RPMI showed normal Tb circadian rhythms (12-hr average
daytime Tb of non-treated rats = 37.25 [± 0.09]°C and those treated with AgNPs in RPMI = 37.24 [± 0.09]°C; data not shown). Injection of supernatants from the control or reference incubation I cultures gave rise to 12-hr average Tb values of, respectively, 37.22 [± 0.10]°C and 37.18 [± 0.09]°C (Figure 3). This indicated that neither regimen led to a culture medium that could induce alterations in rat Tb. The occasional transient increase in rat Tb at 08:00 am (injection time) was most likely due to the process of handling the rats itself. During night hours, there were no differences in Tb among the rats in all tested groups (data not shown).

Figure 3

As can be seen in Figure 4, injection of the reference incubation II (cells+LPS) supernatants induced increases in rat Tb that started to increase 2.5 h post-injection and lasted 3 hr (10:30-13:30). On the other hand, administration of the supernatants from the experimental incubation (cells+LPS+AgNPs) augmented this elevation in Tb. These rats displayed a fever that started to increase 2 hr after the injection but now lasted for 5 hr (10:00-15:00). Among rats that received the reference incubation II material, the 3-hr average Tb was 37.29 [± 0.10]°C; whereas in rats treated with supernatants from the experimental incubation system, the 5-hour average Tb was 37.81 [± 0.14]°C, the levels significantly different from one another (P < 0.001).

Figure 4

A presence of AgNPs in the cultures did not affect the lethargy subsequently associated with the treatment of PMBC with LPS alone

As shown in Figure 5, the motor activity of rats (from 10:00-15:00) that had been injected with culture supernatant of the reference incubation II or experimental incubation was
significantly lower than that in rats that received medium from the control incubation (1.18 ± 0.17 counts). The values associated with the reference incubation II or experimental incubation, i.e., 0.81 [± 0.08] and 0.68 [± 0.13] counts, respectively, did not significantly differ (P = 0.36).

Figure 5

AgNPs augment secretion of IL-1β into culture of PBMCs stimulated with LPS but have no effect on release of IL-6 and TNF-α

Stimulation of PBMCs with LPS only (reference incubation II) provoked release of cytokines into the culture medium (Figure 6). Levels of IL-1β in control (cells + saline) and reference incubation I (cells + AgNPs) samples were similar (64 [± 2] and 67 [± 4] pg mL-1, respectively) (Figure 6A). With reference incubation II cultures (cells + LPS), the IL-1β level reached 201 [± 13] pg mL-1; P < 0.001 between respective groups). The addition of AgNPs in the experimental incubation system (cells + LPS + AgNPs) further augmented this release of IL-1β to 378 [± 10] pg mL-1 (Figure 6A; P < 0.001 between the two groups). In contrast, a presence of AgNPs did not influence LPS-induced elevations of IL-6 and TNF-α (Figure 6B and 6C).

Incubation of the PMBCs with AgNPs (reference incubation I; cells+AgNPs) itself increased the level of IL-6 to 161±14 pg mL-1 compared to the control value of 41±7 pg mL-1 (cells+saline). In contrast, LPS stimulated IL-6 release to a level of 580 [± 18] pg mL-1 (P < 0.001). The addition of AgNPs did not significantly alter this LPS effect (level now of 603 [± 13] pg mL-1; P=0,36; Figure 6B).

TNF-α release by PBMCs in the control (cells+saline) and reference I incubations (cells+AgNPs) was at levels of 10 [± 7] and 12 [± 1] pg mL-1, respectively (Figure 6C). LPS alone significantly stimulated TNF-α secretion (reference II incubations; cells+LPS) to 454
[± 17] pg mL-1 (P < 0.001). The addition of AgNPs in the incubation did not significantly affect the LPS-induced effect (level now of 361 [± 68] pg mL-1; P=0.09 between the two incubation variants).

Figure 6

Discussion

Silver nanoparticles were characterized by SEM and DLS technique. It can be seen that the results obtained from SEM analysis and the DLS method are not comparable. The size of the dry AgNPs powder analyzed by the SEM (67.6 [±3]nm; Figure 1A) was significantly smaller in comparison to the diameters of nanoparticles measured by DLS technique (298 [±160]nm and 46 [±9]nm (4.2%); Figure 1B). Moreover, the DLS analysis demonstrated the diameter sizes, which were considerably larger than of that indicated in product’s information (< 100 nm; Figure 1B). Because of the fact, that the SEM technique allowed to determine the primary size and morphology of AgNPs only in dry samples, the size characterization of AgNPs in RPMI 1640 medium had to be carried out using DLS method. It is known that nanoparticles size and agglomeration are dependent on the type of suspension media. For example, Murdock et al. [28] showed that the diameters of AgNPs size of 80 nm increased from 250 nm in deionized water to 743 nm in serum-free RPMI 1640 medium. Similarly, other also demonstrated that in the DLS technique larger aggregates were observed in the deionized water or in the RPMI 1640 medium in comparison to the results from the SEM and transmission electron microscopy (TEM) methods. The discrepancy may be attributed to particle aggregation in the DLS measurements while SEM or TEM allow to latitude for eliminating clumps of particles [29, 30].

Presented data demonstrate that silver nanoparticles (~300 nm) added to the culture of LPS-stimulated blood mononuclear cells enhanced the LPS-induced production of the pro-
inflammatory cytokine IL-1β, and the supernatants from that culture, when administered to rats, elicited significantly greater febrile responses than that of supernatants derived from the cell culture stimulated with LPS alone. Moreover we have shown that neither the sterile suspensions of AgNPs nor supernatants from PBMCs co-cultured with AgNPs are pyrogenic. It also appeared that AgNPs possessed an \textit{in vitro} potential to stimulate release of IL-6 from PBMCs, however it did not potentiate an LPS-induced releasing effect on IL-6. Since the supernatants derived from cells+AgNPs culture did not evoke a febrile response in the rats (Figure 4), we conclude, therefore, that the enhanced fever in the rats injected with cells+LPS+AgNPs culture supernatants was due to the greater concentration of IL-1β rather than IL-6, although the two cytokines, as was mentioned earlier, are considered equally the most potent endogenous pyrogens.

Our studies correspond to the data presented by Zampronio \textit{et al.} [25], who showed that rat peritoneal macrophages stimulated with LPS (10 µg mL-1) for 30 min released the pyrogenic factors into the supernatants. Injections of supernatants, which showed pyrogenic properties, also provoked lethargy measured as a decrease in motor activity of rats. Depression of motor activity is correlated with the increase in Tb (fever) of rats. Both reactions belong to the non-specific symptoms, which are known as the sickness behavior that accompany responses of the host to infection and inflammation [31].

There are data demonstrating that AgNPs have the inflammatory activities. Carlson \textit{et al.} [32] showed in \textit{in vitro} studies that rat alveolar macrophages exposed to 5, 10 and 25 µg AgNPs mL-1 (sizes of 15, 30 or 55 nm) for 24 h produced significant levels of pro-inflammatory cytokines, such as IL-1β and TNF-α. On the other hand, some authors suggest that AgNPs can also have the anti-inflammatory properties. Tian \textit{et al.} [33] showed that treatment of murine infected burns with AgNPs increased the rate of healing and decreased scarring. It has been accompanied by the increased expression of anti-inflammatory
cytokines such IL-10 and reduced expression of IL-6. Santoro et al. [34] demonstrated that murine RAW264.7 monocyte/macrophage cell line stimulated with AgNPs (20, 40 and 60 µg mL⁻¹) for 4, 8 or 12 h did not induce pro-inflammatory cytokines expression, such as IL-1β or IL-6. The explanation of these different results needs further examination. One could consider the characteristics of the AgNPs themselves, such as surface properties (charge, and specific area) or morphology (shape and crystal structure) [35]. The size of nanoparticles, type of the cells and the time of in vitro stimulation could also have had effect on the production of pro-inflammatory cytokines.

Recently, there is an increasing number of reports indicating potential toxic characteristics of AgNPs, which is associated with inflammatory potential of necrotic or apoptotic cell death by the nanoparticles. Orlowski et al. [36] using an in vitro model of mouse peritoneal monocytes (RAW 264.7 cell line) and mouse keratinocyte cell line 291.03C showed that AgNPs were toxic in concentration-dependent manner. However, necrosis were the dominating cell death mechanism in monocytes while in keratinocytes Orlowski et al. observed rather apoptosis than necrosis. Moreover, murine keratinocytes exposed to AgNPs underwent apoptosis characterized by a high activity of caspase-9 – an indicator of mitochondrial apoptotic pathway. In contrast, murine monocyte RAW 264.7 cell line showed more necrosis than apoptosis and high activity of caspase-1 – an enzyme involved in inflammatory reactions. These results indicate that effects of AgNPs depend on the type of exposed cells. [36]. Park et al. [37] using an in vitro model of mouse peritoneal monocytes (RAW 264.7 cell line) proposed a ‘Trojan – horse mechanism’ of AgNPs cytotoxicity. After exposure to commercially produced AgNPs powder, cell viability decreased, which is correlating with the increasing sub G1 fraction and it indicates for cellular apoptosis. Moreover, AgNPs decreased intracellular glutathione level, increased nitric oxide (NO) secretion and increased TNF-α in protein and gene levels [37]. It is well known, that
nanoparticles induce toxicity through oxidative stress and inflammation by generating reactive oxygen species (ROS) in cells. Glutathione protects cells against exogenous and endogenous toxins, including ROS and it is the primary antioxidant responsible for maintaining the reducing intracellular microenvironment that is essential for normal cellular function and viability [38]. Moreover, NO is a signaling molecule that plays a key role in the pathogenesis of inflammation. It gives an anti-inflammatory effect under normal physiological conditions. On the other hand, NO is considered as a pro-inflammatory mediator that induces inflammation due to over production in abnormal situations [39]. Finally, TNF-α is also known to stimulate several signaling pathways leading to inflammation, apoptosis and tissue degradation [40]. Summarizing, phagocytosis of AgNPs stimulate inflammatory signaling through the generation of ROS, followed by the activated cells induced secretion of TNF-α. Finally, the increase of TNF-α cause damage of cell membrane and apoptosis [37].

As described above, it is known, that AgNPs may have significant toxic effects on cells that primarily occur in a dose-dependent manner. However, there have been also reports on the size dependence of the cellular toxicity of AgNPs. The size of nanoparticles plays a critical role in the cellular uptake processes from the intracellular membrane to the nuclear membrane [41]. Kim et al. [42] using an in vitro model of mouse preosteoblast cell line (MC3T3-E1) and rat adrenal medulla derived cell line (PC12) demonstrated that AgNPs with the sizes of approximately 10, 50, and 100 nm have the potential to exert cytotoxic effects. Moreover, the smallest sized AgNPs (10 nm size) had a greater apoptotic effect against the MC3T3-E1 cells than other-sized AgNPs (50 and 100 nm). Other researchers have also suggested the higher cytotoxicity of smaller particles compared to larger ones, which was related to the amount of ROS generated at the relatively larger surface area of small nanoparticles [43, 44]. The MTT assay has shown that tested AgNPs at a dose of 25 μg mL⁻¹
during 2 h incubation did not have a toxic effect on PBMCs (Figure 2). Moreover, the results of DLS technique showed that our nanoparticles were the size of about 300 nm. The findings of scientists suggested that particle features such as size and agglomeration status as well as the type of cells may contribute to nanoparticles biological impact [45]. Auffan et al. [46] demonstrated that single or smaller particles could pose a greater hazard compared to larger particles or agglomerates/aggregates. Furthermore, Lankoff et al. [47] showed that the aggregates of AgNPs size about 300 nm at a dose 10 and 50 μg mL-1 did not significantly effect on the viability of the following cell lines: A549 (human lung epithelial cell line), HepG2 (human liver cell line) and THP1 (human monocyte cell line) during 24 h incubation. Moreover, the THP1 cells showed significantly higher binding/uptake for less agglomerated particles. Because of the fact, that in our studies we incubated PBMCs with the agglomerated AgNPs size about 300 nm only for 2 h, we concluded that the tested aggregates of AgNPs had to large sizes in RPMI 1640 medium to be internalized by monocytes, induce necrosis and thus, inflammatory reaction. For these reason, we summarize that our AgNPs augment releasing of pyrogenic factors by PBMCs stimulated with LPS and moreover, these phenomenon does not affect cell viability.

It is well known, that both polymorphonuclear leucocytes (basophils, eosinophils and in particular neutrophils) as well as PBMCs (lymphocytes and monocytes/macrophages) are capable of synthesizing and releasing a variety of pro-inflammatory mediators, including cytokines [48]. However, in our experiments from all leukocytes, we have decided to stimulated only PBMCs with LPS and/or AgNPs. Firstly because, Dinarello et al. [49] showed that although neutrophils can synthesize and release endogenous pyrogen, it appeared that macrophages show in this respect 100 fold higher capacity. Moreover, the presence of neutrophils may have a suppressive effect on monocytes to release pro-inflammatory cytokines [49]. Secondly, recent studies have described cytokine production by granulocytes
with results that are controversial. Several groups have detected production of pro-inflammatory cytokines such as IL-1β, IL-6 and TNF-α [50, 51] and others reported no IL-6 production [52]. Similarly, the measured TNF-α secretion could not be confirmed by other authors [53]. Summarizing, PBMCs population seems more suitable for research on the production of pro-inflammatory cytokines than granulocytes.

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Figure legends

Figure 1. Scanning electron microscopy (SEM) image of silver nanoparticles (a scale illustrating 500-nm size is presented in the Figure 1A) in relation to the size distribution profile of nanoparticles in the solution (serum-free RPMI 1640 medium) measured by the Dynamic Light Scattering technique (DLS) (B).

Figure 2. Cell viability of AgNPs treated cultures relative to the controls. PBMCs were exposed to 5, 12.5, 25, 35 and 50 μg AgNPs mL⁻¹ for 2 hr. Values are expressed as means ±S.E.M. of three experiments. Asterisk indicates significant difference compared to the controls (*P<0.05,**P<0.01).

Figure 3. Changes of body temperature over time (h) of rats treated ip at 8:00 am (arrowhead) with the supernatants from control incubation (cells+saline; open circles) and the supernatants from reference incubation I (cells+AgNPs; close triangles) in comparison to non-treated animals (close circles). Values are means ± S.E.M. at 20-min averages (n indicates sample size in a respective group).

Figure 4. Changes of body temperature over time (h) of rats treated ip at 8:00 am (arrowhead) with the supernatants from reference incubation II (cells+LPS; open diamonds) and the supernatants from experimental incubation (cells+LPS+AgNPs; close diamonds) in comparison to Tb of animals administrated ip with the supernatants from control incubation (cells+saline; open circles). Values are means ± S.E.M. at 20-min averages (n indicates sample size in a respective group).

Figure 5. Changes in motor activity of rats shown in Figure 2. Values are means ± S.E.M. at 5-h averages from 10 am to 3 pm (n indicates sample size in a respective group of rats). Asterisk indicates significant difference (**P<0.01).

Figure 6. Concentration of IL-1β (pg mL⁻¹ A), IL-6 (pg mL⁻¹; B) and TNF-α (pg mL⁻¹; C) in the final supernatants aspirated after: control incubation of PBMCs with pyrogen-
free 0.9% saline (vertical bars), reference incubation I of cells with AgNPs (black column), reference incubation II of cells with LPS (horizontal bars) and experimental incubation (cells+LPS+AgNPs; white column). Values are expressed as means ±S.E.M. of three experiments. Asterisk indicates significant difference (**P<0.01, ***P<0.001).
Figure 2

Cell viability relative to the control [%]

- control
- 5 µg/ml
- 12.5 µg/ml
- 25 µg/ml
- 35 µg/ml
- 50 µg/ml

* * *
Figure 3

- **control (n=8)**
- **reference incubation I (cells + AgNPs) (n=5)**
- **control incubation (cells + saline) (n=4)**

Body temperature [°C]

07:00 08:00 09:00 10:00 11:00 12:00 13:00 14:00 15:00 16:00 17:00 18:00 19:00

Time [h]
Figure 4

- Reference incubation II (cells + LPS) (n=6)
- Control incubation (cells + saline) (n=4)
- Experimental incubation (cells + LPS + AgNPs) (n=5)
Figure 5

Motor activity [counts]

- Control incubation (cell + saline) n=6
- Reference incubation II (cells + LPS) n=6
- Experimental incubation (cells + LPS + AgNPs) n=5
Figure 6

A

IL-1β [pg/ml]

control incubation (cells + saline)

reference incubation I (cells + AgNPs)

reference incubation II (cells + LPS)

experimental incubation (cells + LPS + AgNPs)

B

IL-6 [pg/ml]

control incubation (cells + saline)

reference incubation I (cells + AgNPs)

reference incubation II (cells + LPS)

experimental incubation (cells + LPS + AgNPs)

C

TNF-α [pg/ml]

control incubation (cells + saline)

reference incubation I (cells + AgNPs)

reference incubation II (cells + LPS)

experimental incubation (cells + LPS + AgNPs)