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Original article

Inhibition of neutrophil oxidative burst and NETosis by fullerene-like inorganic tungsten disulfide nanoparticles with preservation of cell viability

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Summary

Introduction. Tungsten disulfide (WS_2) nanoparticles possess unique physicochemical properties, making them promising candidates for biomedical applications. While previous studies have demonstrated the in vitro biocompatibility of WS_2 in various cell lines, their effects on neutrophil function remain unexplored.

Methods. Human neutrophils were isolated via dextran sedimentation and exposed to increasing concentrations of inorganic fullerene-like WS_2 (IF-WS_2) (12.5–200 µg/mL). After incubation, cells were stimulated with phorbol 12-myristate 13-acetate (PMA), calcium ionophore (CaI), or N-formylmethionyl-leucyl-phenylalanine (fMLP). Cell viability was assessed by flow cytometry, reactive oxygen species (ROS) production was measured using luminol-based chemiluminescence, and neutrophil extracellular trap (NET) formation was quantified using Sytox Green fluorescence.

Results. IF-WS_2 had no significant effect on neutrophil viability at any tested concentration. However, ROS production was inhibited in a concentration-dependent manner, especially under PMA stimulation. IF-WS_2 also significantly reduced both spontaneous and stimulus-induced NETosis.

Conclusion. IF-WS_2 nanoparticles attenuate key neutrophil functions, including oxidative burst and NETosis, even at low concentrations. These findings suggest their potential utility as immunomodulatory agents in inflammatory and autoimmune diseases.

Key words: tungsten disulfide, WS_2 nanoparticles, neutrophils, ROS, NETosis, immunomodulation, nanomedicine

Introduction

Tungsten disulfide (WS_2) nanoparticles represent a class of inorganic nanomaterials with a distinctive polyhedral and multilayered morphology, which endows them with considerable potential across a wide spectrum of applications, including biomedical use [1]. Together with molybdenum disulfide (MoS_2), WS_2 belongs to the broader group of transition metal dichalcogenides (TMDs), recognized for their exceptional physicochemical characteristics [2]. Since the seminal

work by Professor Reshef Tenne in 1992, which first introduced cylindrical and polyhedral WS_2 nanoparticles, scientific interest in these materials has steadily intensified due to their superior mechanical resilience, chemical inertness, and excellent tribological properties [3]. These features have enabled the integration of WS_2 nanoparticles into various technological and industrial domains such as environmental remediation, the development of nanocomposites, energy storage systems, sensor technologies, and solar energy applications. Furthermore, the discovery of their unique optical behavior has opened new avenues for their utilization in photonic devices and photocatalysis [4].

Neutrophils, constituting approximately 50–70% of leukocytes in peripheral blood, are the most prevalent immune cells and play a pivotal role in the innate immune system. They are swiftly recruited to inflammatory sites via interactions with endothelial adhesion molecules, particularly P- and E-selectins [5]. Beyond their established function in early microbial defense, neutrophils also modulate adaptive immune responses and are critically involved in the pathogenesis of numerous inflammatory and autoimmune diseases, including rheumatoid arthritis, chronic pulmonary disorders, sepsis, atherosclerosis, and malignancies [5–8].

One of the hallmark mechanisms of neutrophil activation is the oxidative burst. The process is characterized by a rapid release of reactive oxygen species (ROS) generated by organelles such as mitochondria, lysosomes, and the endoplasmic reticulum. Nanoparticles that are internalized by neutrophils may modulate ROS production either directly, by stimulating NADPH oxidase and pattern recognition receptors, or indirectly, through pathways involving calcium mobilization and redox-sensitive transcription factors. Although ROS are essential for effective pathogen elimination, excessive or prolonged ROS generation can trigger inflammatory cascades and contribute to tissue damage [9].

Apart from producing ROS, activated neutrophils can also undergo a unique form of cell death known as NETosis, which is characterized by the release of neutrophil extracellular traps (NETs). These web-like structures, composed of chromatin and granule proteins, are designed to trap and neutralize pathogens [10]. While beneficial for host defense, uncontrolled or excessive NETosis has been associated with the development of various chronic inflammatory and autoimmune diseases [6, 11].

Although earlier studies have indicated that WS_2 nanoparticles are largely biocompatible in various in vitro cell cultures, such as epithelial cells [12], hepatocytes, macrophages [13], and peripheral blood mononuclear cells (PBMCs) [14], their interactions with neutrophils, particularly regarding innate immune effector mechanisms, remain insufficiently investigated. In our previous paper, we provided evidence that inorganic fullerene-like WS_2 nanoparticles (IF-WS_2) exerted a significant immunomodulatory role in the culture of human PBMCs [14]. The present study aims to evaluate the effects of IF-WS_2 nanoparticles on human neutrophil viability, uptake dynamics, and functional activity, with a special emphasis on their capacity to influence ROS production and NETosis in response to appropriate stimuli.

Methods

Isolation of neutrophils

Peripheral venous blood was collected from healthy donors into vacuum-sealed tubes containing K_2EDTA as an anticoagulant. Neutrophils were isolated using dextran sedimentation with a 3% (w/v) dextran solution. The leukocyte-rich plasma layer was carefully aspirated and layered over a Lymphoprep gradient (density 1.077 g/mL; PAA Laboratories, Vienna, Austria). Following centrifugation at 2200 rpm for 20 minutes at room temperature, the PBMC layer was discarded. The granulocyte-enriched pellet was treated with an ammonium chloride-based lysis buffer (NH_4Cl, $KHCO_3$,

Na_2EDTA) to remove residual erythrocytes. The resulting granulocyte population, consisting predominantly of neutrophils, was washed and resuspended in calcium- and magnesium-free Hanks' Balanced Salt Solution (HBSS; Sigma-Aldrich, Steinheim, Germany). Cell number was determined manually using a hemocytometer (Neubauer chamber) under a light microscope. Cell viability, which consistently exceeded 95%, was assessed using a 1% trypan blue solution. The same procedure was used to determine cell viability in the culture assay.

Reactive Oxygen Species (ROS) production

ROS production was quantified using luminol-enhanced chemiluminescence. Human neutrophils were resuspended in HBSS medium supplemented with Ca^{2+}, Mg^{2+}, 0.5% heat-inactivated human serum, and 10 mM HEPES buffer (1 M stock). Cells were seeded into 96-well plates designed for luminescence (Sarstedt, Nümbrecht, Germany) in the presence of increasing concentrations of IF-WS_2 (12.5, 25, 50, 100, and 200 μg/mL), or in WS_2-free control medium. IF-WS_2 nanoparticles were kindly provided by Dr Dušica Stojanović (University of Belgrade, Faculty of Technology and Metallurgy, 11000 Belgrade, Serbia).

After the 1-hour incubation at 37°C, luminol was added, followed by stimulation with either PMA (50 nM; Sigma-Aldrich, St. Louis, MO, USA) or fMLP (1 μM; Sigma-Aldrich, St. Louis, MO, USA). Chemiluminescence intensity, reflecting ROS generation, was measured using a plate-reading luminometer (Synergy HT, BIOTEK Instruments, Winooski, VT, USA).

Apoptosis and necrosis assay

Apoptotic and necrotic cells were identified using an Annexin V–fluorescein isothiocyanate (Annexin V-FITC)/propidium iodide (PI) staining kit (R&D Systems, Minneapolis, MN, USA). Neutrophils (2.5×10^5 cells/150 μL per well) were cultured in 96-well flat-bottom plates (Sarstedt, Nümbrecht, Germany) containing HBSS medium supplemented with Ca^{2+}, Mg^{2+}, 1% heat-inactivated human serum, and 10 mM HEPES.

Following the 6-hour incubation at 37°C in a humidified atmosphere with 5% CO_2, cells were harvested and stained according to the manufacturer's instructions. Briefly, cells were washed, resuspended in the binding buffer, and stained with Annexin V-FITC and PI. After a 20-minute incubation in the dark at room temperature, cells were analyzed by flow cytometry (Attune, Thermo Fisher Scientific, Waltham, MA, USA). The final sample volume was adjusted to 300 μL using the binding buffer.

The proportions of viable (Annexin V^-/PI^-), early apoptotic (Annexin V^+/PI^-), late apoptotic (Annexin V^+/PI^+), and necrotic cells (Annexin V^-/PI^+) were quantified and expressed as percentages.

NET formation assay

NETosis was quantified by fluorescence detection using Sytox Green, a membrane-impermeable DNA-binding dye (Invitrogen, Thermo Fisher Scientific, Waltham, MA, USA). Human neutrophils were cultured in HBSS+ medium in the presence or absence of IF-WS_2 nanoparticles at concentrations of 12.5, 25, 50, 100, and 200 μg/mL. Triton X-100 (Sigma-Aldrich, St. Louis, MO, USA) was used as a positive control to induce complete membrane permeabilization, representing 100% NETosis.

After a 1-hour preincubation with IF-WS_2, neutrophils were stimulated with either PMA (50 nM), calcium ionophore A23187 (CaI, 1 μM; Sigma-Aldrich), or were left unstimulated (negative control). Four hours later, Sytox Green was added to each well at a final concentration of 500 nM, followed by the 15-minute incubation at 37°C in the dark.

Fluorescence intensity was measured using a microplate reader (Synergy HT, BIO-TEK Instruments, Winooski, VT, USA) and used as an indirect measure (proxy) of extracellular DNA release and the extent of NET formation.

Statistical analysis

All data are presented as mean ± standard deviation (SD) or as representative values. Statistical analyses were performed using GraphPad Prism 10 software (GraphPad Software Inc., La Jolla, CA, USA). Differences between treatment groups were assessed using repeated measures analysis of variance (RM-ANOVA) followed by Tukey's multiple comparisons test, or paired Student's *t*-test, where appropriate. A *p-value* < 0.05 was considered statistically significant.

Results

Cytotoxicity of IF-WS_2 in human neutrophils

Human neutrophils were exposed to increasing concentrations of IF-WS_2 nanoparticles (12.5, 25, 50, 100, and 200 μg/mL) for six hours. Cell viability, as well as rates of apoptosis and necrosis, were assessed using Annexin V-FITC/PI staining followed by flow cytometry. At all tested concentrations, no statistically significant differences were observed in the proportions of viable (Annexin V^-/PI^-), early apoptotic (Annexin V^+/PI^-), late apoptotic (Annexin V^+/PI^+), or necrotic (Annexin V^-/PI^+) cells compared to the untreated control group.

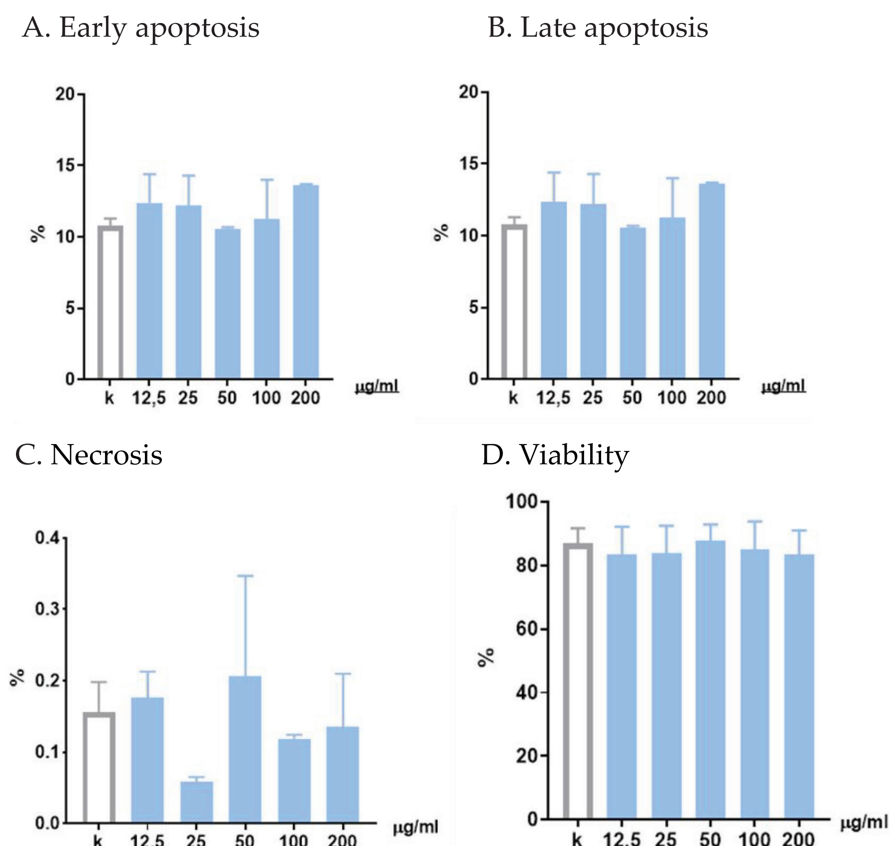

Figure 1. Cytotoxicity of IF-WS_2 in neutrophil cultures. Flow cytometry analysis of cell viability and apoptosis/necrosis after a 6-hour exposure to IF-WS_2 nanoparticles (12.5–200 μg/mL). Data are presented as mean ± SD from three independent experiments. No significant differences were observed compared to untreated controls ($p > 0.05$, one-way ANOVA).

Effect of IF-WS_2 on ROS production

Neutrophils were incubated with IF-WS_2 nanoparticles at five concentrations (12.5–200 µg/mL) and subsequently stimulated with PMA (50 nM) or fMLP (1 µM). ROS production was measured using luminol-based chemiluminescence.

In cultures stimulated with PMA, a reduction in chemiluminescence intensity was observed at concentrations of 50 µg/mL and above. In cultures stimulated with fMLP, ROS levels did not differ significantly between IF-WS_2-treated and untreated cells.

Effect of IF-WS_2 on NETosis

Neutrophils were pre-incubated with IF-WS_2 (12.5–200 µg/mL) for one hour, followed by stimulation with PMA (50 nM), CaI (1 µM), or no stimulation. NET formation was quantified using Sytox Green fluorescence after four hours of incubation. Fluorescence values were normalized to the Triton X-treated positive control (100%).

In PMA-stimulated cultures, a concentration-dependent reduction in NET-associated fluorescence was observed, reaching 38% of the control value at 200 µg/mL. In CaI-stimulated cultures, fluorescence intensity was significantly

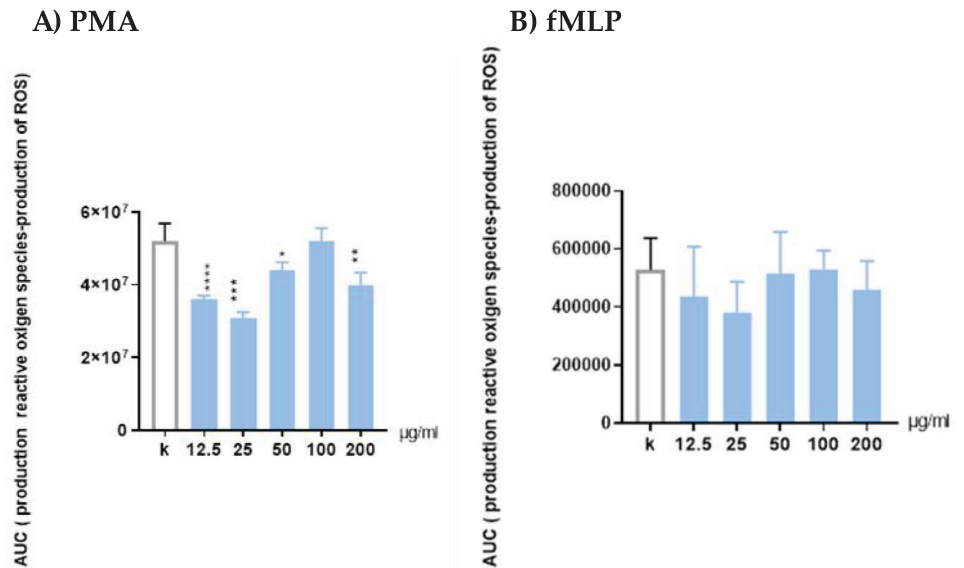

Figure 2. Chemiluminescence-based quantification of ROS production in IF-WS_2-treated neutrophils following stimulation with PMA or fMLP. Results are expressed as mean percentage values relative to the control (± SD) from three independent experiments. *$p < 0.05$ (ANOVA with Dunnett's post hoc test)

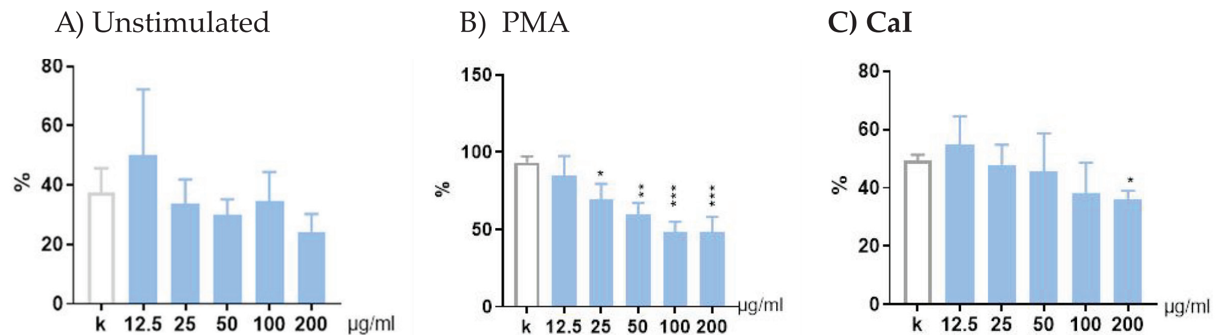

Figure 3. Sytox Green fluorescence intensity reflecting NET formation after IF-WS_2 exposure and stimulation with PMA, CaI, or no stimulus. Results are presented as mean ± SD from three replicates. *$p < 0.05$; ***$p < 0.005$ vs. control (ANOVA with Dunnett's post hoc test)

reduced only at 200 µg/mL. In unstimulated cultures, no significant differences in NETosis were observed between IF-WS_2-treated and untreated groups.

Discussion

The findings of this study provide new insights into the effect of IF-WS_2 nanoparticles in the context of innate immunity and neutrophil function. Previous studies on the biocompatibility of IF-WS_2 nanoparticles have been demonstrated in various epithelial, hepatic, and immune cell lines [14–17]. However, data on their interactions with granulocytes remain limited. In this study, we evaluated the cytotoxic potential, impact on ROS production, and modulation of NETosis in human neutrophils, revealing multiple concentration-dependent effects of IF-WS_2 that may be relevant for biomedical applications.

A key requirement for the biomedical application of nanomaterials is a favorable safety profile. In our study, IF-WS_2 nanoparticles did not compromise neutrophil viability across a broad concentration range (12.5–200 µg/mL), as demonstrated by Annexin V/PI staining after six hours of incubation. The absence of significant apoptosis or necrosis aligns with previous reports describing the low cytotoxicity of IF-WS_2 in other cell types, including keratinocytes [17], bronchial epithelial cells [16], hepatocytes, and macrophages [16]. This distinguishes IF-WS_2 from several other nanomaterials, such as carbon nanotubes and silver nanoparticles, that often exert dose-dependent cytotoxic effects on leukocytes. The preservation of neutrophil viability also suggests that IF-WS_2 does not interfere with early apoptotic signaling. Since neutrophils undergo spontaneous apoptosis as part of immune homeostasis and inflammation resolution, our findings imply that IF-WS_2 does not prolong the neutrophil lifespan, which might otherwise risk sustained inflammatory activation [18].

The oxidative burst represents a primary effector function of neutrophils and a hallmark of their activation [19]. We observed that IF-WS_2 nanoparticles significantly suppressed ROS production in PMA-stimulated neutrophils in a dose-dependent manner. This inhibitory effect was not evident in fMLP-stimulated cells, suggesting that IF-WS_2 may selectively interfere with NADPH oxidase-dependent signaling rather than G-protein-coupled receptor (GPCR) pathways. These findings are consistent with previous reports indicating that IF-WS_2 possesses intrinsic antioxidant activity, capable of scavenging free radicals and modulating intracellular redox balance [20]. Interestingly, contrasting data from tumor cell models indicate that IF-WS_2 can enhance ROS generation under conditions of mitochondrial targeting [21]. This discrepancy may reflect context-specific effects of IF-WS_2 nanomaterials, dependent on cell type, nanoparticle localization, or the activation status of redox systems. The specificity of ROS inhibition in PMA-stimulated neutrophils suggests that IF-WS_2 could modulate oxidative responses relevant to inflammation without suppressing essential physiological ROS functions involved in microbial killing [22].

Beyond ROS production, NETosis is a well-established neutrophil function involved in host defense as well as in various pathological processes. NETosis is triggered by diverse stimuli, including PMA and calcium ionophores, and involves chromatin decondensation, plasma membrane rupture, and extrusion of nuclear content [23, 24]. In our study, IF-WS_2 significantly inhibited NETosis in both PMA- and CaI-stimulated neutrophils, with a more pronounced reduction observed at concentrations ≥ 50 µg/mL. Notably, suppression was stronger in PMA-stimulated cells, consistent with the inhibitory effect on ROS production and suggesting the involvement of ROS-dependent NETosis pathways. While NETs are essential for pathogen clearance, excessive or persistent NET formation

has been implicated in the pathogenesis of autoimmune diseases (e.g., systemic lupus erythematosus, rheumatoid arthritis), thrombosis, and cancer metastasis [25]. Thus, the ability of IF-WS_2 to inhibit both spontaneous and stimulus-induced NET release highlights its potential as a nanotherapeutic with anti-inflammatory and anti-thrombotic applications. Similar anti-NET effects have been observed with other nanomaterials, such as cerium oxide nanoparticles and gold nanoclusters [26], although the underlying molecular mechanisms remain poorly defined.

A study by Domenica et al. reported that IF-WS_2 could induce NET formation under basal conditions, in contrast to our findings [27]. This inconsistency may be due to methodological differences, such as variations in nanoparticle size, surface modifications, or endotoxin contamination, as well as differences in donor variability and in vitro culture conditions. Our IF-WS_2 nanoparticles were confirmed to be endotoxin-free and were suspended in a controlled environment, reducing the likelihood of such confounding factors.

The dual inhibition of ROS production and NETosis observed in our study suggests that IF-WS_2 may act by targeting upstream signaling pathways, such as protein kinase C (PKC), ERK1/2, or calcium-dependent mechanisms. The finding that CaI-induced NETosis was only partially inhibited at the highest concentration of IF-WS_2 supports the hypothesis that IF-WS_2 affects both NOX-dependent and NOX-independent forms of NET formation, albeit to varying degrees.

The immunomodulatory effects of IF-WS_2 nanoparticles, as demonstrated in this and our previous study [14], may offer translational value across a range of clinical scenarios. In autoimmune and inflammatory diseases where aberrant NET formation contributes to tissue damage, IF-WS_2 may serve as a suppressor of pathological neutrophil activation. Furthermore, in oncology, accumulating evidence suggests that NETs facilitate tumor cell extravasation and metastatic dissemination. Inhibiting NETosis via IF-WS_2 could thus represent an adjuvant strategy in cancer therapy.

Nonetheless, further research is required to clarify the molecular mechanisms underlying IF-WS_2-mediated suppression of neutrophil activation. Future directions should include transcriptomic and proteomic profiling, mapping of intracellular signaling pathways, and validation in animal models. It will also be essential to investigate the pharmacokinetics, tissue distribution, and long-term safety of IF-WS_2 formulations intended for clinical use.

Conclusion

This study provides the first evidence that IF-WS_2 nanoparticles, at concentrations ranging from 12.5 to 200 μg/mL, do not compromise neutrophil viability, while exerting a significant inhibitory effect on ROS production and NET formation under both unstimulated and stimulated conditions. The dose-dependent suppression of oxidative burst and NETosis suggests a potential role for IF-WS_2 nanoparticles in the modulation of innate immune responses. These findings warrant further investigation into the underlying mechanisms and highlight IF-WS_2 as a promising candidate for future biomedical applications targeting neutrophil-driven inflammation.

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Ethical approval. The Ethics Committee of the University of East Sarajevo, Faculty of Medicine Foča, Foča, Republic of Srpska, Bosnia and Herzegovina, approved the study and informed consent was obtained from all individual respondents. The research was conducted according to the Declaration of Helsinki.

Conflicts of interest. The authors declare no conflict of interest.

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Inhibicija oksidativnog praska i NEToze neutrofila neorganskim nanočesticama volfram-disulfida sličnim fulerenu uz očuvanje ćelijske vitalnosti

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Uvod. Nanostrukture volfram-disulfida (WS_2) posjeduju jedinstvena fizičko-hemijska svojstva, što ih čini obećavajućim kandidatima za primjenu u biomedicini. Iako su prethodne studije pokazale *in vitro* biokompatibilnost WS_2 sa različitim ćelijskim linijama, njihov uticaj na funkciju neutrofila do sada nije ispitan.

Metode. Humani neutrofili su izolovani metodom sedimentacije dekstranom i izloženi rastućim koncentracijama WS_2 (12,5, 25, 50, 100 i 200 µg/mL). Nakon inkubacije, ćelije su stimulisane uz pomoć PMA, CaI ili fMLP. Vijabilnost ćelija je ispitivana protočnom citometrijom, proizvodnja ROS-a mjerenjem hemiluminiscencijom uz upotrebu luminola, a formiranje NET-ova kvantifikovano je pomoću Sytox Green boje.

Rezultati. WS_2 nije imao značajan efekat na vijabilnost neutrofila ni na jednoj ispitivanoj koncentraciji. Međutim, proizvodnja ROS je bila inhibirana na način zavisan od koncentracije, naročito pri stimulaciji uz pomoć PMA. IF-WS_2 je takođe značajno smanjio i spontanu i stimulisanu NETozu.

Zaključak. WS_2 nanostrukture inhibiraju ključne funkcije neutrofila, uključujući oksidativni odgovor i NETozu, čak i pri niskim koncentracijama. Ovi rezultati ukazuju na njihov potencijal kao imunomodulatornih agenasa u inflamatornim i autoimunim bolestima.

Ključne riječi: volfram-disulfid, WS_2 nanočestice, neutrofili, reaktivne vrste kiseonika, NEToza, imunomodulacija, nanomedicina