Effects of silver nanoparticles on endothelial cells under physiologically-relevant dynamic flow conditions: Increased cytotoxicity and inflammation

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Introduction

With expanding applications of nanomaterials in innovative cardiovascular drugs and medical devices, there is a need for improved test methods to evaluate their safety prior to clinical use. The complex physiological environment makes it difficult to predict potential clinical outcomes using traditional *in vitro* biological evaluation approaches and non-human *in vivo* testing, which do not account for hemodynamic conditions. Advanced *in vitro* test methods, such as organ-on-a-chip microphysiological systems (MPS) that incorporate controlled fluid flow regimes, have shown potential in toxicological research and may provide better prediction of clinical outcomes in the safety evaluation and risk assessment of nanomaterials.

Objectives

To evaluate the toxicity of citrate-coated 10 nm silver nanoparticles (AgNPs) on human cerebral microvascular endothelial cells under physiologically-relevant dynamic flow conditions using an endothelium-on-a-chip fluidic platform.

Materials and Methods

Nanoparticle Characterization

- Citrate-coated 10 nm AgNPs (nanoComposix).
- AgNP size and morphology characterization by transmission electron microscopy (TEM).
- AgNP hydrodynamic size and zeta potential analysis by dynamic light scattering (DLS).

Endothelium-on-a-chip Model (Figure 1A)

- Human cerebral microvascular endothelial cells (hCMEC/D3) (Millipore Sigma).
- Endothelium-on-a-chip model was established using hCMEC/D3 cells and an ibidi µ-Slide I Luer and Pump System (ibidi).

Experimental Procedure (Figure 1B)

- Day 0: Cells seeded in collagen-coated µ-Slide I Luer chamber and cultured 72 h in static conditions.
- Day 3: Endothelial-seeded µ-Slide connected to pump system and unidirectional dynamic flow slowly increased to 10 dyn/cm^{2}. hCMEC/D3 cells treated with AgNPs (0-40 µg/mL) under 10 dyn/cm^2 unidirectional dynamic flow conditions. Static experiments were performed concurrently.
- Day 4: After 24 h exposure, cells and supernatant collected for biological assays:
  - PrestoBlue™ HS Cell Viability Reagent and Nitric Oxide (NO) Assay Kit (Invitrogen)
  - Caspase-Glo® 1 Inflammasome Assay and CytoTox96® Non-Radioactive Cytotoxicity Assay (lactate dehydrogenase, LDH) (Promega)
  - Human Endothelial cell-specific molecule 1 (ESM1 or Endocan) ELISA kit (Abcam).
  - V-PLEX Plus Human Proinflammatory Panel (Meso Scale Diagnostics)

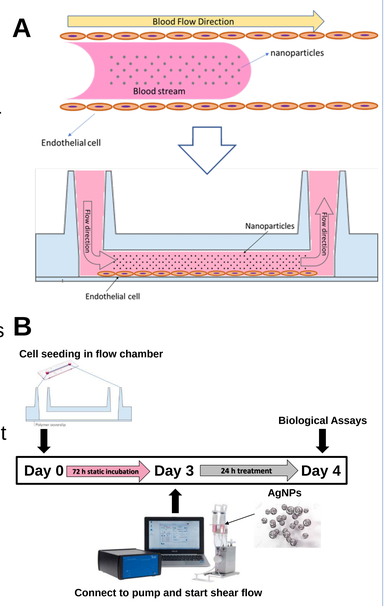

Figure 1. Schematic of endothelium-on-a-chip model (A) and experiment design (B).

Results

Nanoparticle Characterization

- TEM images showed uniform, spherical morphology of AgNPs (~9.83 nm) (Figure 2).
- Hydrodynamic size of AgNPs increased in cell culture medium compared to PBS. The hydrodynamic size increased after 24 h incubation in cell culture medium under static conditions due to possible aggregation, while incubation in cell culture medium under dynamic conditions showed reduced effect of aggregation on hydrodynamic size.

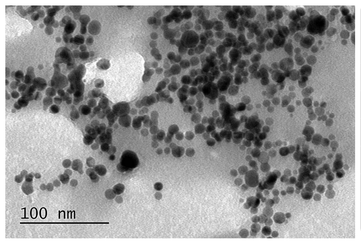

Figure 2. TEM image of 10 nm AgNPs.

AgNP Characterization by TEM and DLS

Condition	Size (nm)
TEM	9.83 ± 1.83
PBS	25.71 ± 3.35
Medium	61.08 ± 2.53
24h in static medium	127.50 ± 3.30
24h in dynamic medium	87.18 ± 0.58

Table 1. AgNP size measurements from TEM images, and hydrodynamic size characterization in PBS and complete cell culture medium.

Concentration and Fluid Dynamic-Dependent Toxicity of AgNPs

- Increased accumulation of AgNPs in hCMEC/D3 cells under dynamic flow conditions compared to static conditions was observed (Figure 3A).
- Cell viability was markedly reduced in hCMEC/D3 cells exposed to AgNPs under dynamic flow compared to static conditions at higher concentrations (Figure 3B).

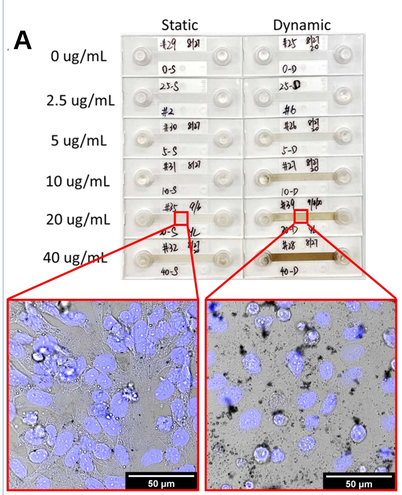

Figure 3. Accumulation of AgNPs in the endothelium-on-a-chip system. Cell nuclei are shown in blue and the dark spots are aggregaties of AgNPs in the microscopy images (A). Relative viability change of hCMEC/D3 cells after exposure to AgNPs under static and dynamic conditions (B), * denotes significant difference compared to static condition and # denotes significant difference compared to its own untreated control group, n=3, p<0.05.

Concentration and Fluid Dynamic-Dependent Toxicity of AgNPs

- Stress markers LDH and NO, inflammation-mediated Caspse-1, endothelial cell-specific pathological indicator ESM-1, and proinflammatory cytokines (IL-1β, IL-6, IL-8, and TNF-α) were considerably elevated under dynamic flow condition at higher concentrations (Figure 4).

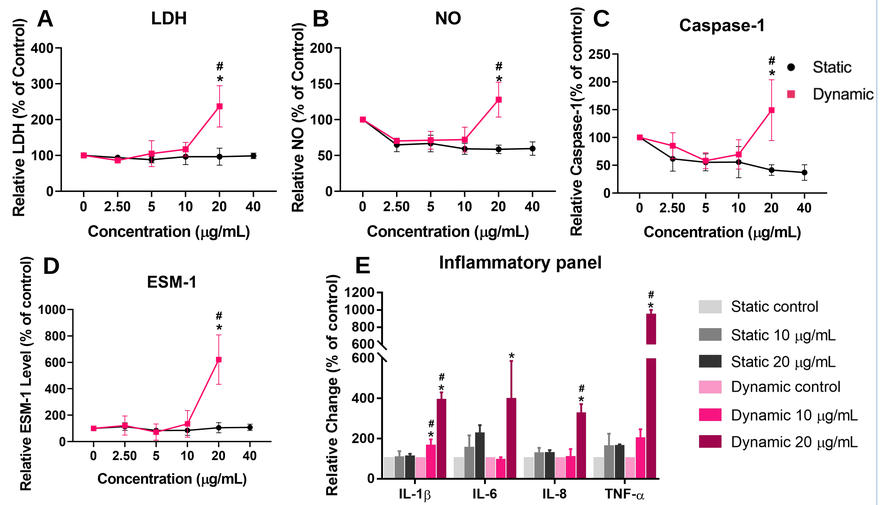

Figure 4. Relative changes of LDH (A), NO (B), Caspase-1(C), and ESM-1 (D) in hCMEC/D3 cells after exposure to AgNPs under static and dynamic conditions, n=3. Relative changes of proinflammatory cytokines, IL-1β, IL-6, IL-8, and TNF-α after AgNPs exposure at 0, 10, and 20 µg/mL under static and dynamic conditions. * denotes significant difference compared to static condition and # denotes significant difference compared to its own untreated control group, n=3, p<0.05.

Conclusions

- **Dynamic flow conditions reduce aggregation of 10 nm AgNPs in cell culture medium compared to AgNPs in static conditions.**
- **Toxicity of 10 nm AgNPs on hCMEC/D3 cells is concentration and fluid flow-dependent.**
- **Increased LDH, NO, Caspase-1, and ESM-1 in hCMEC/D3 cells exposed to AgNPs under dynamic flow conditions suggest possible inflammatory response-related toxicity.**
- **Elevated proinflammatory cytokines further confirmed the inflammatory-related toxicity of AgNPs under dynamic flow conditions.**
- **Further investigation is needed to better understand the underlying mechanisms.**

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