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# Evaluation of CuO nanoparticle toxicity on 3D bioprinted human iPSC-derived cardiac tissues

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## Abstract

Particulate matter has been identified as a significant environmental threat to human health. As one of its components, copper oxide nanoparticles (CuO NP) have been found highly potent in cytotoxicity. However, the elucidation of its mechanism is still limited. This study investigated the toxicity of CuO NP toward a cardiac tissue. To better recapitulate the species-specific tissue phenotype and toxin response, we developed a human induced pluripotent stem cells (iPSC)-derived cardiac micro-tissue. With the precise deposition of the cell and scaffold material enabled by rapid 3D bioprinting, the cardiac micro-tissue showed a mature phenotype and was incorporated with a force gauge to enable contraction measurement. We discovered an $LD_{50}$ of 7.176 g/mL from the CuO NP treatment outcome of the micro-tissue with a downward trend in tissue force as toxicity increased. We also identified mitochondrial damage and activation of extrinsic apoptosis as a significant pathway to mediate the tissue toxicity.

## Introduction

According to the World Health Organization (WHO), air pollution is the single largest environmental threat to human health [1,2]. In 2015, ambient air pollution was responsible for 4.2 million deaths worldwide, and is projected to cause 6 to 9 million deaths by 2060 [3]. Mounting epidemiological evidence on the negative health effects of air pollution suggests that the morbidity and mortality is increased in non-communicable diseases, such as cardiovascular and respiratory diseases [[1], [2], [3]]. When air pollution is evaluated, small particulate matter (PM), a mixture of solid and liquid particles of organic and inorganic substances suspended in air, is considered to be a key player in contributing to disease [1,2,4]. Of these particles, nanoparticles (NP, PM less than 100 nm in diameter), are being increasingly recognized as a significant health hazard. In the reports published between 2005 and 2021, WHO determined that enough epidemiological evidence had been reached to connect NP exposure with a variety of short and long term health effects, and recommended general guidelines for the particulates [1]. However, the mechanisms leading from NP exposure to long term health effects, particularly cardiovascular diseases, are not well studied or understood.

Current hypotheses suggest that through a variety of pathways, nanoparticles can increase the amount of reactive oxygen species (ROS) to a level above what cells can handle, leading to oxidative stress [4]. In one proposed mechanism, inhaled NPs are translocated across the alveolar membrane into the bloodstream and interact with the vascular endothelium before settling in secondary organs [[4], [5], [6]]. Once in the secondary organ, oxidative stress may be induced by 1) free radical generation on the surface of the NP; 2) cytokine and oxidative mediator release from activated inflammatory cells; and 3) interaction of the NP and cells [4].

Most current studies evaluating NP toxicity in heart tissue are either in animal models or 2D culture models, which both have their limitations. Although animal models can showcase complex organ response, intrinsic species-species differences often make results and dosing studies non-translatable. For example, mouse models have a shorter QT rate (found using an electrocardiogram, it is the length between the beginning of the Q wave, signifying the depolarization of the ventricular myocardium, to the end of the T wave, signifying the repolarization of the ventricular myocardium) [7]; a higher heart beating rate that is 10 times faster than the average human; and differences in both ion handling and pharmacokinetic properties [8,9]. Although 2D human cell culture models are an appealing *in vitro* model, they lack important extracellular-cell, cell-cell, and cell-tissue interactions, and thus do a poor job replicating an in vivo heart response [8,10,11]. As adult human cardiomyocytes are difficult to source in large quantities, many researchers use stem cell-derived cardiomyocytes [11,12]. The replication of the microenvironment is especially important in this context, as stem-cell cardiomyocytes are well known for their immature phenotype, a core issue addressed by many researchers by both physical [[12], [13], [14], [15], [16], [17]] and chemical [18] cues. Most consistently, stem cell cardiomyocytes that receive both physical alignment and force loading from tissue contraction display mature phenotypes [14,17]. In a previous study by our group, we showcased that by using a light-based 3D bioprinter, we were able to print a 3D cardiac micro-tissue to better deliver alignment and force loading, leading to a micro-tissue exhibiting a mature phenotype at 7 days [17]. Briefly, we used an in-house developed micro-continuous optical printing (μCOP) system, a bioprinting method advantageous for its superior printing resolution, speed, and biocompatibility in comparison to ink-jet and extrusion-based bioprinters [[19], [20], [21], [22]]. A multi-layer hydrogel was formed from UV-activated prepolymer solution, and the optical patterns were projected by the μCOP system to form a high resolution cardiac micro-tissue the size of a well in a 96-well plate. A pillar model was incorporated with the micro-tissue to output functional data such as contraction force and rate that are significant parameters impacted by environmental factors.

For this study, we aimed to test the impact of copper oxide (CuO) NPs on 3D cardiac micro-tissues. CuO NPs are believed to be one of the more toxic metal oxide nanoparticles [[23], [24], [25], [26]], yet are widely used in many commercial fields including batteries, surfactants, paints and textiles, antimicrobial agents [27,28]. Thus, the need for greater understanding of the toxicological effect of CuO NPs is paramount, so that we can develop better protective measures against these NPs. *In vitro* studies into CuO NPs have been performed on an array of human cell lines, including lung and liver cancer cells, bronchial and airway epithelial cells, and skin keratinocyte cells, where cytotoxicity occurred primarily from oxidative stress and led to DNA damage [25,29,30]. However, there are limited *in vitro* studies investigating the impact of the NPs in human cardiac cells [31], and even fewer in 3D cardiac tissue [32]. In their study, Lu et al. investigated the interplay between the endothelial and cardiomyocytes in the heart, concluding that upon endothelial damage, CuO NPs contributed to an increase in oxidative stress and ultimately impacted the contractile function of the cardiac tissue [32]. However, the nanoparticles in Lu et al.'s study were not stabilized by any dispersant or surfactant. Before the NPs reach cardiac tissue in an in vivo setting, interactions with proteinaceous bodily fluid will occur, such as blood or lung epithelial, and proteins can adsorb onto the NP [[33], [34], [35]]. Protein adsorption has been shown to alter NP physicochemical properties and decrease toxicity [36,37]. For this reason, CuO were coated with bovine serum albumin (BSA) to mimic the adsorption of a model protein. BSA is often used as a substitute for human serum albumin due to its properties and structural similarities [38]. To this end, we decided to add to this growing research by dosing the bioprinted cardiac micro-tissues with CuO NPs at concentrations of 0 $\mu$g/mL, 0.1 $\mu$g/mL, 1 $\mu$g/mL, 10 $\mu$g/mL, and 100 $\mu$g/mL, respectively. We evaluated the impact of increasing dose on the

viability of the micro-tissues utilizing the CCK-8 assay. We then utilized our pillar model to quantify the impact of toxicity on beating frequency and force output. Lastly, we investigated the pathways of cardiac toxicity.

## Section snippets

### Methacrylated coverslip preparation

Glass coverslips were functionalized with methacrylate groups to facilitate covalent bonding to hydrogels. Briefly, clean 5 mm round coverslips were incubated overnight at room temperature on a rocker in a solution of 1.75%(v/v) 3-(Trimethoxysilyl)-Propyl Methacrylate (TMSPMA) (Cat. # M6514-50ML, Sigma-Aldrich), 10.50%(v/v) 1:10 acetic acid: ethanol, and 87.75%(v/v) 100% ethanol. The next day, coverslips were washed twice with 100% ethanol, twice with MilliQ water, and then left to dry at room …

### The cardiac micro-tissue and dosing

Cardiac micro-tissues were developed and characterized for maturity in a previous study [17]. Using the μCOP printer, the micro-tissues were formed quickly into three layers (Fig. 1A, Supplementary Figure S1). The bottom layer consists of a thin (125 $\mu$ m) hyaluronic acid glycidyl methacrylate (HAGM) and polyethylene glycol diacrylate (PEGDA) composite to prevent cell spreading by human cardiac fibroblasts. The second layer consist of high concentration of gelatin methacrylate (GelMA) pillars of …

## Discussion

This study successfully showcased the utility of the bioprinted iPSC cardiac micro-tissue platform for evaluating the toxic impact of the environmental pollutant, CuO NPs. Because 3D tissues recapitulate the cell-cell, cell-tissue, and cell-extracellular matrix interactions that influence cell behavior, it is vital to evaluate the phenotypic and genotypic responses of cells to NPs in these formats. However, to date, only one study has been previously published investigating the behavior of CuO…

## Conclusion

In this study, we developed a human iPSC-derived cardiac microtissue using a μCOP system to better recapitulate species-specific tissue phenotype and toxin response to particle matter pollutants. The mature cardiac microtissue incorporated a force gauge for contraction measurement and was used to investigate the toxicity of CuO NPs. CuO NPs were synthesized with a diameter of 60±23 nm, and BSA was successfully coated on the NPs to replicate protein adsorption in the bloodstream. Our findings…

## CRediT authorship contribution statement

**Kathleen L. Miller:** Conceptualization, Methodology, Validation, Formal analysis, Investigation, Writing – original draft, Writing – review & editing, Visualization. **Izaac Sit:** Conceptualization, Methodology, Validation, Formal analysis, Investigation, Writing – review & editing, Visualization. **Yi Xiang:** Methodology, Validation, Formal analysis, Investigation, Writing – review & editing. **Jerry Wu:** Methodology, Validation, Investigation. **Jacob Pustelnik:** Methodology, Validation, Investigation. …

## Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.…

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