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Bioprinting of a human neuron model for quantifying pollutant toxicity

Running title: Human neuron model for pollutant toxicity

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Abstract

Although pesticides are widely used in modern agriculture, growing evidence indicates that chronic exposure to these environmental contaminants contributes to the development of neurodegenerative disorders. This concern is amplified by the global rise in aging populations and the corresponding increase incidence in neurodegenerative diseases. Among the most widely used pesticides, organophosphates, particularly chlorpyrifos (CPF), are of significant concern due to their extensive agricultural use and well-established neurotoxic effects. Here, we present a 3-dimensional (3D) human neuron progenitor cell (hNPC) tissue model, fabricated using digital light processing (DLP)-based bioprinting. This platform enables precise spatial control over the cell density, geometry, and mechanical properties within a gelatin methacrylate (GelMA) scaffold, providing a tunable and biomimetic extracellular matrix. Using this system, we evaluated hNPC response to CPF exposure within a physiologically relevant 3D context. We further investigated CPF-induced neurotoxicity, specifically exploring acetylcholinesterase gene expression as a primary mechanistic etiology. Our findings demonstrate that our engineered 3D bioprinted constructs offer a more physiologically relevant and cost-effective platform for studying pollutant-induced neurotoxicity compared to conventional 2-D and *in vivo* rodent models, respectively. This work establishes a scalable 3D bioprinted system for mechanistic toxicology studies and advances predictive modeling of human neurological responses to environmental toxicants.

Keywords: Bioprinting; Human neuron tissue model; Pollutants; Toxicity; Chlorpyrifos

1. Introduction

Pesticides are important to contemporary agricultural systems, however, mounting evidence implicates chronic exposure in the pathogenesis of neurodegenerative and neurodevelopmental disorders.^{1,2} This concern is compounded by the increase of aging populations: while advances in healthcare have extended human lifespan, this has driven a parallel rise in the increase in neurodegenerative diseases.

Organophosphates are among the most used pesticides worldwide. Both acute and chronic exposure is toxic to the nervous system, and can lead to neurological diseases.³ Specifically, chlorpyrifos (CPF), an organophosphate pesticide, is of particular concern. It is one of the most recognized pesticides worldwide, used as an insecticide on corn, soybeans, wheat, and many fruit trees. It is highly effective against termites, fleas, earthworms, and more.⁴ CPF's primary mechanism of toxicity is through irreversible inhibition of acetylcholinesterase (AChE), the enzyme responsible for hydrolysis of neurotransmitter acetylcholine. In recent years, epidemiological evidence has suggested that occupational or environmental exposure to CPF is implicated with Alzheimer's Disease (AD).^{5,6,7}

Mechanistic investigation of CPF-induced neurotoxicity has historically relied upon reductive two-dimensional (2D) *in vitro* culture systems and *in vivo* rodent models.⁸ While these models have been informative, each carries inherent limitations that constrain their translation to humans. Conventional 2D cultures fail to recapitulate the complex organization of the brain, disallowing the investigation of cell-cell and cell-matrix interactions.^{9,10} *In vivo* models preserve systemic physiological complexity, but their species divergencies lower their relevancy.¹¹ There is an urgent need for human-relevant *in vitro* models capable of recapitulating the cellular architecture and biochemical environment of the neuronal brain tissue, within which the neurotoxicity trajectory of CPF exposure can be properly characterized.¹²

In this study, we utilized digital light processing (DLP)-based bioprinting to create cell-encapsulated hydrogel scaffolds. DLP allows for controlled shape, size, cell density, and mechanical properties.^{13,14} The matrix material used is gelatin methacrylate (GelMA), where the methacrylate functional groups allow for a controllable and reproducible scaffold. By leveraging the unique properties of GelMA, we can enhance the structural

and functional fidelity of the tissue models. Using these models, we evaluated the effects of CPF on human neuron progenitor cells (hNPCs) and investigated the primary degradation mechanism. The progenitor-like state was chosen over maturing the neurons because the hNPCs are particularly vulnerable to environmental toxins, and they can differentiate into multiple cell types which enable assessment of toxin-induced alterations. Furthermore, we modulated the mechanical properties of the matrix by varying the stiffness to include a soft or stiff tissue model. This biologically relevant feature of the neural microenvironment is known to influence neural progenitor cell behavior, including proliferation, differentiation, and cellular signaling. Changes in tissue mechanics have been implicated in neurodegenerative disorders,^{15,16} and our platform provides a method to study those changes. This study advances our ability to investigate mechanisms of neurotoxicity using both known and emerging neurotoxins, helping to bridge the translational gap between traditional in vitro models and the complex neurons within the human brain. By providing a more biomimetic neural microenvironment, these models may improve the prediction of human responses to toxic exposure and support the development of more effective therapeutic strategies.

2. Methods

1) Cell Culture

Human iPSC-Derived Neural Progenitor Cells (hNPCs) were obtained from StemCell Technologies (Cat # 200-0620) and were cultured using STEMdiff™ Neural Progenitor Medium kit (Cat # 05833, StemCell Technologies) with 100 U/mL penicillin-streptomycin (Cat# P4333, Sigma-Aldrich). This combination is referred to as media throughout this study. Before plating the cells, Matrigel (Cat # 354230, Corning) was used to coat the plates overnight. Media was changed every day, and the cells were passaged every 3 days by disassociating with Accutase (Cat # A6964, Sigma-Aldrich) and seeded 1:3 to new plates. Cells were incubated at 37 °C under 5% CO₂.

2) Bioink Composition

The bioink consisted of 10%(w/v) gelatin methacrylate (GelMA), 0.6%(w/v) lithium phenyl-2,4,6-trimethylbenzoylphosphinate (LAP, Cat # 900889, Sigma-Aldrich), Dulbecco's phosphate-buffered saline (DPBS, Cat # 14190144, Thermo Fisher), and

cells. GelMA was synthesized according to previously published protocols.¹⁷ Briefly, gelatin (Cat# 2500, Sigma Aldrich) was dissolved in a 0.25 carbonate-bicarbonate buffer solution. Methacrylic anhydride (Cat # 276685, Sigma Aldrich) at 0.095 mL/g ratio was added for 1 hour at 50 °C. GelMA purification was done using dialysis in MilliQ water with 12-14 kDa tubings (Cat # 132706, Spectrum Labs) for one week at 37 °C. It was further collected, lyophilized, and dissolved in DPBS into 20% (w/v) stock solution and filtered (Cat # SCNY00040, Millipore Sigma) to ensure sterility.

3) Bioprinting

Bioprinting was conducted using an in-house DLP bioprinter equipped with a 365 nm LED light source at 6.8 mW/cm² light power for soft scaffolds and 10.8 mW/cm² for stiff ones. Polydimethylsiloxane (PDMS) sheets were sectioned and mounted onto glass slides to form the bioink reservoir, while methacrylated glass coverslips served as the printing substrate, prepared using an established protocol.¹³ A brain-shaped mask 250 µm in height and 500 µm in diameter was used for all scaffolds unless otherwise specified. The bioprinting process is as follows: hNPCs were collected by lifting and centrifuging for 5 minutes at 200 g and counted with Trypan Blue (Cat # 15250061, Thermo Fisher). Bioink was added to the cells to create bioink, and 5 µL of the bioink was placed into the bioink reservoir. Soft samples were created using light intensity of 136 mJ/cm² (or 6.8 mW/cm²) and stiff ones at 324 mJ/cm² (or 10.8 mW/cm²) and immediately placed into media. Total cell density was 50 million/mL. Soft samples were exposed for 20 seconds, stiff ones were exposed for 30 seconds.

4) Young's Modulus

Young's modulus was quantified using CellScale's MicroTester. Samples were subjected to three compression cycles by a 0.3048 mm diameter stainless steel beam and platens. Custom MATLAB scripts processed force and displacement data to calculate the overall compressive modulus.

5) Live/Dead Staining

Cells were stained with ApexBio's Live/Dead kit (Cat # K2081). Calcein-AM (excitation: 490 nm, emission 515 nm) was prepared at a working concentration of 2 mM to stain viable cells and Propidium Iodide (excitation 535 nm, emission 617 nm) was prepared at

a working concentration of 1.5 mM to stain dead cells. Bioprinted scaffolds were stained for 30 minutes in the incubator, then immediately washed and imaged.

6) RT-qPCR

hNPCs were collected from bioprinted scaffolds by dissociating the hydrogel with Trizol (Cat # 15596026, Thermo Fisher) and mechanical pipetting. Total RNA was extracted using Direct-zol RNA MicroPrep Kit (Cat # R2062, Zymo Research) following manufacturer's protocol. To ensure RNA quality was pure enough to proceed, the 260/280 nm ratios were characterized using a plate reader (Tecan), and only ratios above 1.7 were used. Protoscript FirstStrand cDNA synthesis kit (Cat # E6300, New England BioLabs) was used to synthesize cDNA from the extracted RNA. RT-qPCR was performed with Luna Universal Master Mix (Cat # M3003, New England BioLabs) in a Quantstudio 3 machine (ThermoFisher). Raw data was analyzed using the Ct threshold method against housekeeping gene GAPDH, and fold change was compared between dosed and control groups. All primers used were purchased from Integrated Data Technologies and designed using NCBI's Primer-BLAST tool.

PAX6 Forward primer sequence: TGTCCAACGGATGTGTGAGT

PAX6 Reverse primer sequence: TTTCCCAAGCAAAGATGGAC

Nestin Forward primer sequence: AACAGCGACGGAGGTCTCTA

Nestin Reverse primer sequence: TTCTCTTGTCCCGCAGACTT

SOX2 Forward primer sequence: TACCAGCATGTCCTACTCGCAG

SOX2 Reverse primer sequence: GAGGAAGAGGTAACCACAGCG

β -amyloid Forward primer sequence: CCTTCTCGTTCCTGACAAGTGC

β -amyloid Reverse primer sequence: GGCAGCAACATGCCGTAGTCAT

Acetylcholinesterase Forward primer sequence: GTTCTCCTTCGTGCCTGTGGTA

Acetylcholinesterase Reverse primer sequence: ATACGAGCCCTCATCCTTCACC

β -site APP cleaving enzyme (BACE1) Forward primer sequence:
GTGAGGTTACCAACCAGTCCTTC

β -site APP cleaving enzyme (BACE1) Reverse primer sequence:
CGTGGATGACTGTGAGATGGCA

GAPDH Forward primer sequence: 5-GAG TCA ACG GAT TTG GTC GT-3

GAPDH Reverse primer sequence: 5- TTG ATT TTG GAG GGA TCT CG-3

7) Toxin Dosing

Stock solutions of toxin compounds were prepared in dimethyl sulfoxide (DMSO) or deionized water, selected based on compound solubility, and stored at -20°C until use. Chlorpyrifos (Cat # 32212, Restek) was dosed into the scaffolds at $500\text{ }\mu\text{g/L}$ concentration. Benzo[a]pyrene (BaP, Cat # 50-32-8, MedChemExpress) was dosed into the scaffolds at $100\text{ }\mu\text{g/L}$. hNPC tissue models were constructed in 96-well plates with 3-6 replicates each. All toxins were dosed into scaffolds directly after printing. Samples were incubated for 3 days or 5 days at 37°C with 5% CO_2 . Drugs were dissolved into media and introduced using $200\text{ }\mu\text{L}$ total volume in the wells. Side wells not containing sample were filled with PBS to combat evaporation. All drugs dosed contained less than 0.5% DMSO in each well, and the control conditions contained the matched DMSO amount present in dosed samples.

8) Cell Viability Assay

The viability of hNPC cells in the 3D bioprinted tissues were done using Cell Titer Glo (Cat # G7570, Promega). Cell Titer Glo was added in a 1:1 ratio with cell media and incubated away from light. For 3D samples, incubation time was increased to one hour to allow the buffer to penetrate the sample, while 2D cells were incubated for 10 minutes. Samples were then measured using Tecan plate reader in a quartz well plate using 1-second integration time.

9) Statistical Analysis

Graphpad Prism was used to perform statistical analysis. The data are presented as mean and error bars are standard deviation, unless otherwise specified. Comparison was performed with Student's t-test or One-Way ANOVA, and significance is determined if p values are less than 0.05.

3. Results

Throughout this study, we utilized human neuron progenitor cells (hNPCs). A 3D GelMA-based hNPC scaffold was bioprinted with an in-house DLP bioprinter. As shown in Fig. 1A schematic, a defined mask is reflected using UV light onto the bioink reservoir by digital micromirror devices (DMD) and dictates the scaffold pattern. After light irradiation, the hNPCs are encapsulated in GelMA, and they are cultured in the incubator to proliferate, migrate, and interact with their environment (Fig. 1B-E). To ensure the hNPCs can grow well in the scaffold, we measured their viability at day 0, 3, and 7. We found their growth profile reflects robust proliferation throughout the week (Fig. 1F).

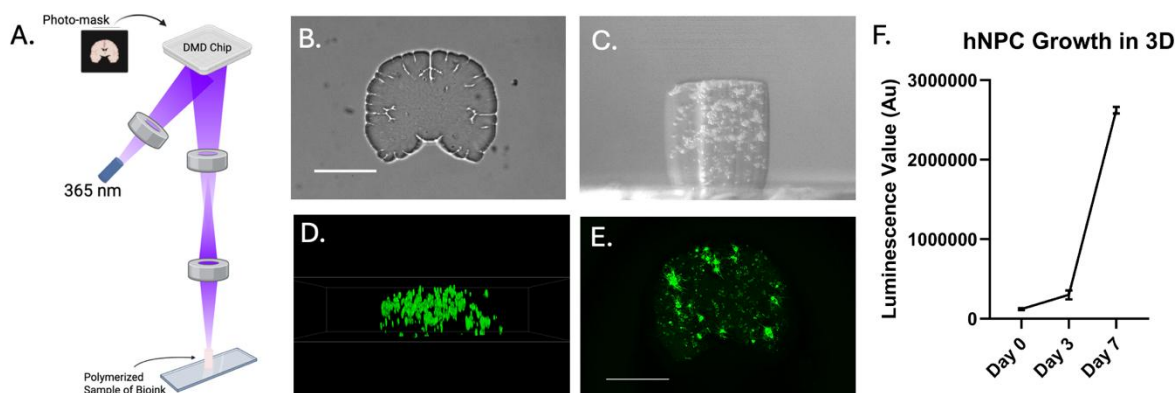


Fig. 1. hNPC constructs were created using DLP bioprinting. A. Schematic of DLP bioprinting setup. B. Acellular model of the scaffold (top view). C, D. Brightfield (C) and Confocal (D) side image of hNPC distribution throughout the bioprinted sample. E. hNPC-laden tissue after 7 days in culture with visible axons sprouting. F. hNPC growth profile in 3D at day 0, 3, and 7. Scale bar = 250 μ m, error bars represent standard deviation.

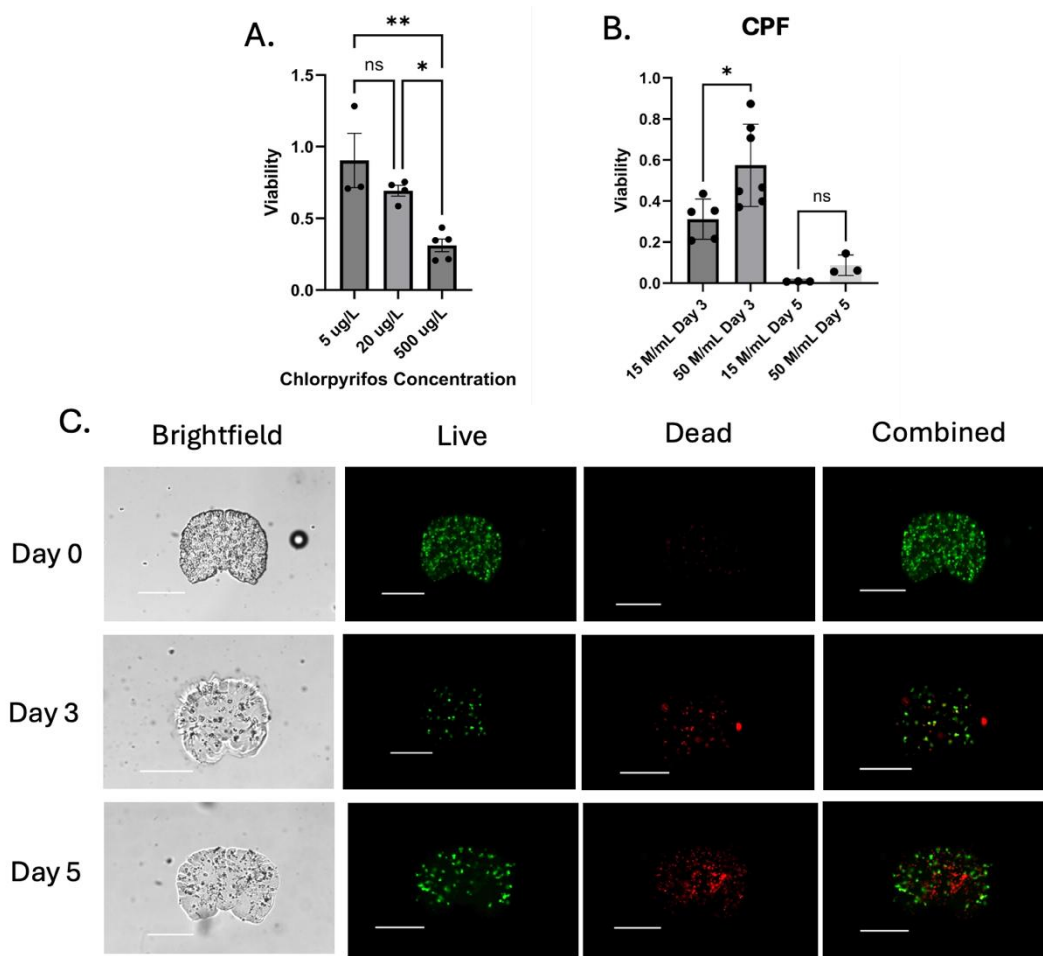


Fig. 2. Parameter dependence of the bioprinted hNPC tissue models. A. Concentrations of 5, 20, and 500 $\mu\text{g/L}$ CPF were dosed into the samples. B. Cell densities of 15 million/mL and high cell density 50 million/mL were printed and dosed with CPF for 3-days and 5-days. C. Live/dead staining of CPF-dosed bioprinted samples at day 0, 3, and 5. Scale bar = 250 μm , error bars represent standard deviation. Ns = not significant, * $p \leq 0.05$, ** $p \leq 0.01$.

After incubation under normal culture conditions for 48 hours, the scaffolds received CPF dosing for 3 days. This mimics physiological scenarios, where CPF is able to penetrate the epidermis to enter tissues or organs.¹⁸ CPF-treated scaffolds received a broad spectrum of potential neurotoxic responses, including: 5, 20, and 500 $\mu\text{g/L}$ per scaffold into fresh media. The lower concentrations (5, 20) were chosen to assess subtle or early cellular effects, while the 500 $\mu\text{g/L}$ concentration was included to

evaluate accumulation of long-term exposures (Fig. 2A). As expected, the lower concentrations of CPF had a higher cellular viability (** $p \leq 0.01$).

Two exposure durations, 3 and 5 days, were evaluated to investigate both early and prolonged effects of CPF exposure on the neural samples. The 3-day time point was selected to assess acute cellular responses and initial toxicity, while the 5-day time point allowed evaluation of transcriptional and cellular responses following continued exposure. Given the cytotoxic effects observed following CPF exposure, the gene expression profiles at Day 5 were interpreted in the context of the surviving cell population and may reflect a transcriptional stress response to continued CPF exposure rather than homeostatic physiological adaptation. Comparing multiple exposure durations enabled characterization of the temporal progression of CPF-induced neurotoxicity within the 3D microenvironment, and follows published precedent.¹⁹ Human CPF exposure occurs primarily through dietary intake, typically through low dose CPF exposures across the lifespan.²⁰ To better mimic the translational relevance, the experiments moving forward were designed with the 5-day exposure period.

Initial studies utilized a cellular density of 15 million cells per mL. However, native neural tissue is characterized by a higher cellular density and extensive cell-cell interactions, the final tissues were fabricated with 50 million cells per mL. The 50 M/mL density observes higher viability compared to the lower, with a significant (* $p \leq 0.05$) increase at Day 3 (Fig. 2B). We visually observe this using a live/dead staining in Figure 2C. This high cellular density better recapitulates the *in vivo* neural microenvironment.²¹

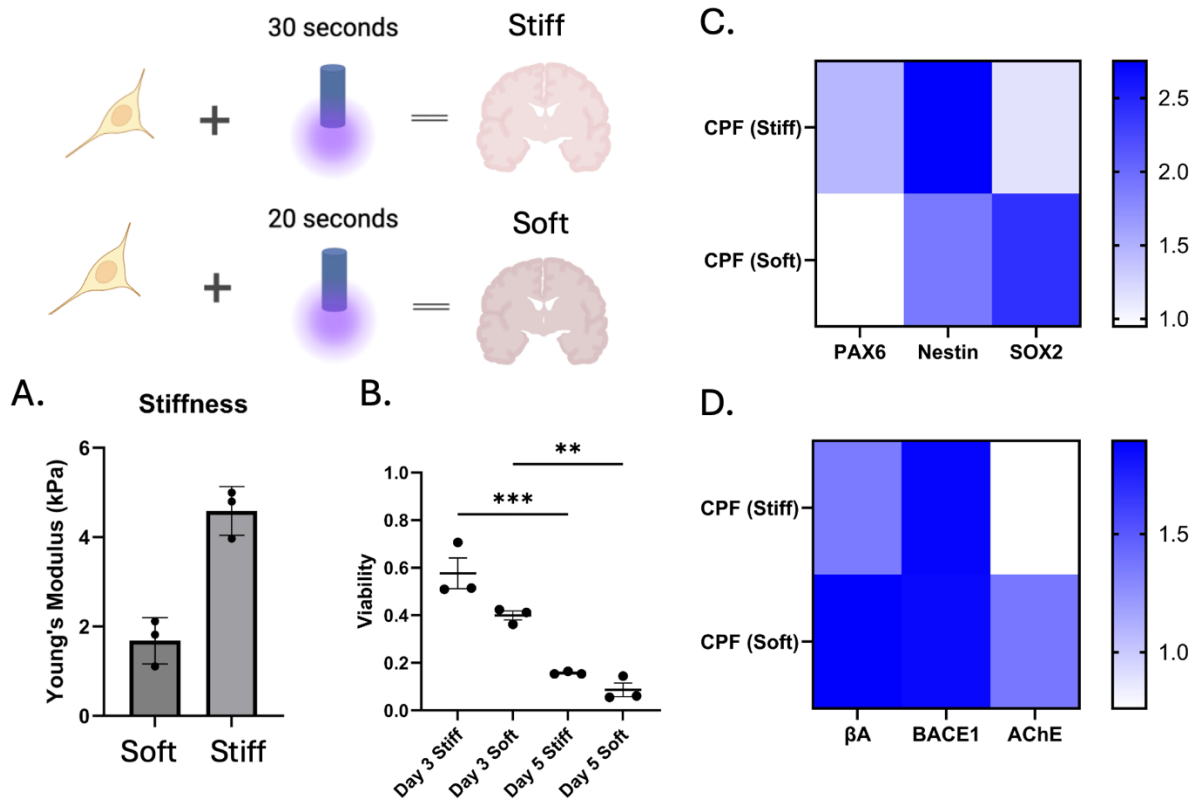


Fig. 3. Characterization of bioprinted stiff versus soft hNPC neuron tissue models. A. Mechanical stiffness of stiff and soft models, as shown by Young's Modulus. B. Stiff samples with CPF-dosing at day 3 and 5, compared with soft samples. C. RT-qPCR results of neuronal markers PAX6, Nestin, and SOX2. D. RT-qPCR results of Alzheimer's Disease-associated markers β eta Amyloid, BACE1, AChE. Error bars represent standard deviation, ** $p \leq 0.01$, *** $p \leq 0.001$.

By varying the parameters of the 3D printing process, we created two groups: one soft hNPC group with 2 kPa, and another stiff group with 4 kPa (Fig. 3A). Overall, samples dosed for 3 days survive significantly better than samples dosed for 5 days. This trend was observed in both the stiff and soft groups. The stiffer groups dosed had a greater cellular viability compared to the soft groups (Fig. 3B).

To elucidate cellular mechanisms underlying their responses to CPF, we evaluated established hNPC proliferation and prognosis markers. We investigated neuronal markers PAX6, Nestin, and SOX2. We found the stiffer model upregulates PAX6 and Nestin compared to the soft model, which upregulated SOX2 (Fig. 3C). Furthermore, we

investigated three AD-associated markers, β eta Amyloid, BACE1, and AChE (Fig. 3D). The soft group upregulated these markers more so in comparison to the stiff group, indicating two different responses to CPF injury.

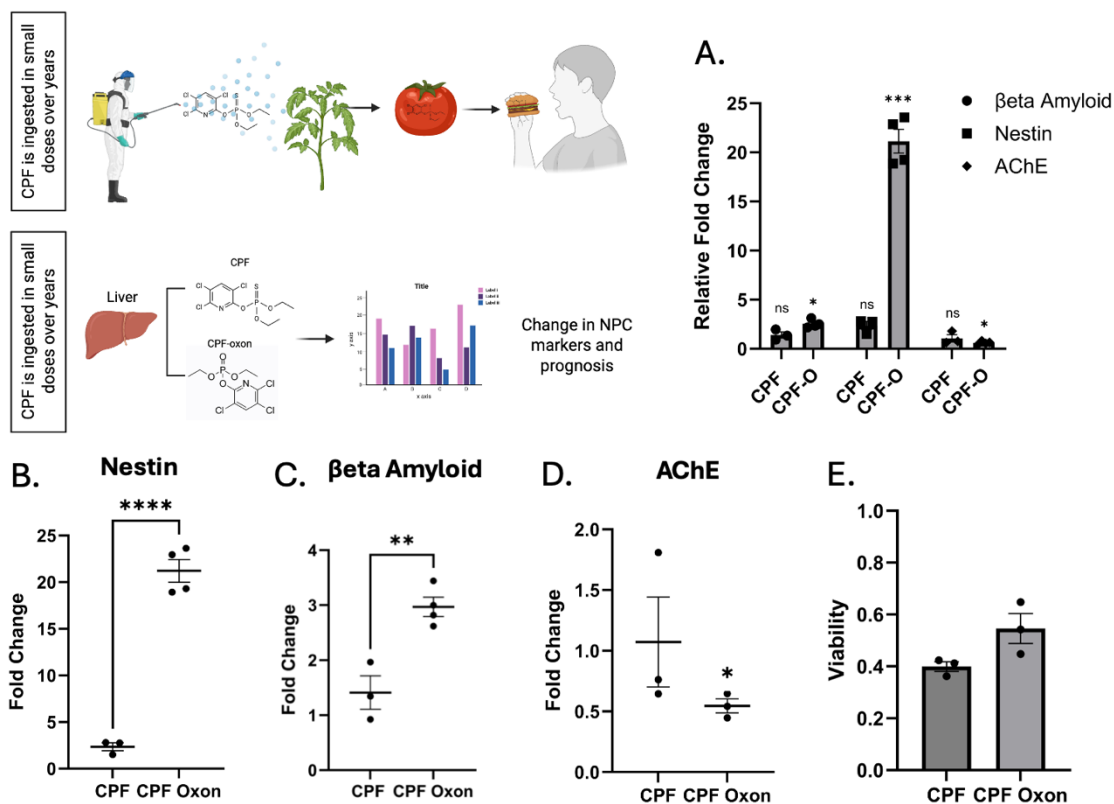


Fig. 4. Mechanistic studies of CPF and its metabolite CPF-Oxon. A. RT-qPCR of AD-associated markers comparing CPF and CPF-oxon normalized to non-CPF dosed samples. B, C, D. RT-qPCR comparing CPF and CPF-Oxon dosed samples with Nestin, β eta Amyloid, and AChE markers. E. Cellular viability of the bioprinted neuron tissue after CPF and CPF-Oxon dosing. Scale bar = 250 μ m, error bars represent standard deviation. ns = not significant, * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$.

To further investigate the CPF's toxicity on hNPCs, we must incorporate the physiological degradation mechanism into our model. CPF will enter the body and be metabolized by the liver into two components: CPF and CPF-oxon (CPF-O). The bioprinted tissue model does not include hepatic metabolism, therefore, we further included the metabolized form CPF-O into our experiments. CPF and CPF-O undergo

different mechanisms in injuring hNPCs: CPF relies on oxidative stress mechanisms whereas CPF-O relies on inhibition of AChE activity.²² We explored the qPCR markers associated with AD on both CPF dosed samples and CPF-O dosed samples (Fig. 4A-D). However, comparing the CPF-O dosed samples with their non-dosed control, all three AD-associated markers were significantly upregulated. Nestin, the cellular stress marker, had a 20-fold significant increase. Similarly, β Amyloid had a 4-fold significant increase, indicating more β Amyloid plaques were deposited. AChE was downregulated significantly, indicating the CPF-O-dosed group of hNPCs do not break down acetylcholine as effectively as CPF. It is important to note that the mRNA expression does not directly reflect the enzyme activity. Comparing the CPF dosed samples with their non-dosed control, all three AD-associated markers had no upregulation. Strictly from a viability perspective, CPF had a larger viability decrease compared to CPF-O (Fig. 4E). The larger viability loss may reflect generalized cellular stress or oxidative injury leading to broader toxicity rather than more selective neurodegenerative pathways. Although the parent compound CPF appeared to be more potent, the results point to CPF-O having a much stronger AD-associated transcriptional response despite its less overall toxicity. These results strongly suggest that the two compounds are engaging in fundamentally different cytotoxic and neurotoxic mechanisms, and that the CPF-O metabolite is driving the AD-relevant pathology.

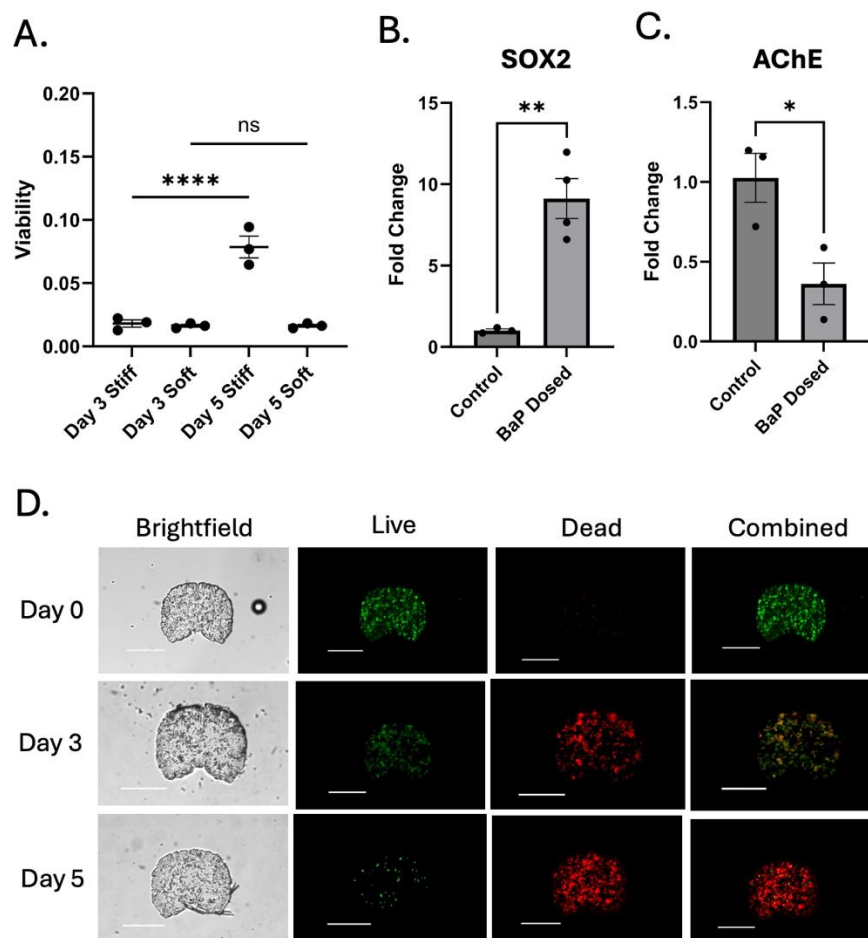


Figure 5. Application of the hNPC tissue model for environmental toxicology screening using Benzo[a]pyrene. A. BaP-dosed stiff and soft samples viability at day 3 and day 5. B. RT-qPCR of SOX2 marker on control samples compared to BaP-dosed samples. C. RT-qPCR of AD-associated marker on control samples compared to BaP-dosed samples. D. Live/dead staining of BaP-dosed samples at Day 0, 3, and 5. Scale bars are 250 μ m, error bars represent standard deviation. Ns = not significant, * $p \leq 0.05$, ** $p \leq 0.01$, **** $p \leq 0.0001$.

To further test our neuron toxicity model, we utilized a second environmental pollutant, Benzo(a)pyrene (BaP). BaP is a polycyclic aromatic hydrocarbon that is prevalent in cigarette smoke, charcoal-based foods, car exhausts, and waste.²³ Occupational exposure typically occurs through dermal contact or inhalation, and due to its high lipophilicity, BaP can cross the blood-brain-barrier. This exposure has become associated with cognitive impairments and neurodegenerative-like diseases.^{24,25} Using

our established model, we dosed BaP into both stiff and soft samples for 3 and 5 days. From a viability perspective, we examined that the stiffer samples survived better at day 3 compared to the soft samples, with similar viability rates at day 5 (Fig. 5A). Using RT-qPCR, we find BaP-dosed samples upregulate injury marker SOX2 significantly and downregulate AChE marker. We visualize degradation in viability using a live/dead staining (Fig. 5D). Although we did not further investigate BaP's degradation mechanism in our model, the same workflow as CPF can be used to elucidate it.

4. Discussion

1) Modulating bioprinting parameters creates a physiologically relevant hNPC tissue model

Conventional 2D cellular models do not reveal the complex interactions of cell-cell relations in a 3D architecture.^{26,27,28} Using our bioprinting platform, we created a physiologically relevant high cell density hNPC model that can represent cellular injury response to toxins. Mechanical stiffness is an important consideration, as it dictates cellular fate, drives migration, and differentiation.²⁹ To represent a soft or stiff condition, we engineered the 3D-bioprinted models to match physiological stiffnesses (softer samples around 2 kPa, and stiffer samples around 4 kPa). Using our platform, we can effectively engineer various parameters such as stiffness by simply modulating printing settings. Before characterizing the samples biologically, we performed live/dead staining and proliferation assays to ensure the hNPCs grow effectively inside the biomaterial.

2) CPF-induced injuries can be mechanistically probed using the hNPC model

To probe cellular expressions after being exposed to CPF-induced injury, we performed RT-qPCR. In the stiff hNPC CPF-dosed group, both paired box 6 (PAX6) and neuroepithelial stem cell protein (Nestin) were increased compared to the soft group. PAX6, a transcription factor involved in hNPC development, neuronal differentiation, and proliferation, was examined following CPF exposure. Consistent with previously reported findings,³⁰ PAX6 expression showed a trend toward upregulation following neuronal injury, suggesting a potential role in tissue repair. This difference in PAX6

upregulation may reflect the greater regenerative capacity of stiffer hNPC tissue, which retain a more plastic and proliferative state conducive to injury-driven repair.

Nestin, an intermediate filament protein expressed in hNPCs, was significantly upregulated (* $p \leq 0.05$) in CPF-dosed samples relative to controls. This significant increase is indicative of an injury-induced activation of neural progenitor populations, consistent with the established role of Nestin as a marker of progenitor cell mobilization in response to injury. The upregulation observed suggests that CPF exposure induced a cellular stress state that triggers repair mechanisms. Like PAX6, the stiffer samples upregulate Nestin significantly compared to the softer samples.

SRY-box 2 (SOX2) is a transcription factor crucial for the maintenance of neural progenitor cell identity. The stiff CPF-dosed and non CPF-dosed samples were similar in SOX2 expression, indicating CPF did not cause differentiation and the neural progenitor cells maintained their cellular identity through the process. In the soft samples, SOX2 was upregulated, suggesting an stiffness-related change in stemness programs, potentially reflecting a decrease in differentiation cues or an attempt to maintain their progenitor identity.

Nestin, SOX2, and PAX6 are markers associated with hNPCs, and represent various aspects of progenitor biology including proliferation, remodeling, stemness, and neural lineage commitment states. Previous studies have demonstrated that the expressions of these markers are regulated by matrix mechanics, highlighting the influence of biomechanical cues on hNPC fate.^{31,32} The differential expressions of these genes following CPF or neurotoxin exposure may reflect distinct transcriptional responses that arise due to its soft or stiff environment. When interpreted alongside cell viability, these changes provide insight into how matrix stiffness may influence hNPC response to toxin stress and highlight the importance of personalized models to study disease. We went on to investigate three AD-associated markers within both stiff and soft samples. In our soft samples, we found a non-significant upregulation of AChE, indicating that acetylcholine is being broken down in comparison to the stiffer samples. Furthermore, looking into β Amyloid accumulation, we found a non-significant upregulation,

indicating the beginning stages of AD development. β Amyloid accumulation has been implicated in the etiology of AD.^{33, 34} BACE1, a β -secretase³⁵ which is involved in plaque formation in the brain, was upregulated in both groups.

Paired with the injury markers, the AD-associated markers allow us to visualize a bigger picture of both the stiff and soft models. From our data, we can conclude that CPF is harmful to hNPCs, and their injury mechanisms vary depending on their stiffness. The soft group of hNPCs attempt to maintain progenitor identity and regulate self-renewal through upregulation of SOX2, whereas the stiffer group activate cytoskeletal remodeling and have a structural-based injury response through Nestin. The further upregulation of AD-associated markers suggests the softer group is more susceptible to AD-associated injury compared to the stiffer group.

3) The high-throughput bioprinting platform can be used to screen potential neurotoxins

We explored using a second class of pollutants, polycyclic aromatic hydrocarbons, specifically BaP. BaP was dosed into both stiff and soft samples and was incubated for 3 and 5 days. The stiffer samples survived better compared to the soft, particularly at day 5. Looking into the soft hNPC sample response to injury, SOX2 was significantly upregulated (** $p \leq 0.01$), indicating injury triggered its cellular repair state. ACHE expression was significantly downregulated (* $p \leq 0.05$) in the BaP-dosed sample.³⁶

The next step to create a more physiological and relevant model will be adding more cellular types, including astrocytes. The hNPC-astrocyte interplay is an important consideration specifically about injury and injury-related pathways. Our model and findings should be interpreted as a base model, intended to investigate the effects of compounds known to access the brain, and to be improved upon by adding more cell types, a blood-brain-barrier, and dynamic culture. Eventually, we aim to use this platform to screen a variety of pollutants for their neurotoxicity.

5. Conclusion

This study presents a precise and robust 3D bioprinting platform incorporating human neuron progenitor cells within biomimetic hydrogels, successfully replicating mechanical features of physiological tissue and elucidating CPF-induced neurotoxicity. The model is capable of longitudinal studies regarding cellular viability, toxicity, and transcriptomic information that traditional 2D models cannot. It serves as a toxicological *in vitro* neurotoxicity platform that can elucidate the cellular response to injury, specifically regarding CPF, but also other pollutants. While further integration of more cellular types is needed, this model represents a valuable leap towards understanding the relationship between environmental pollutants and neurodegeneration.

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Conflict of interest

The authors declare no competing interests.

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Formal analysis: Emmie Yao, Steven Chen

Investigation: Emmie Yao, Steven Chen, Grace Lu, Changyu Sun

Methodology: Emmie Yao, Steven Chen, Grace Lu, Changyu Sun

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Resources: Emmie Yao, Quyen T. Nguyen

Supervision: Quyen T. Nguyen

Visualization: Emmie Yao, Steven Chen, Grace Lu, Changyu Sun

Writing—original draft: Emmie Yao, Steven Chen

Writing—review & editing: Quyen T. Nguyen

Ethics approval and consent to participate

None.

Consent for publication

None.

Availability of data

The data that support the findings of this study are available upon reasonable request.

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Appendix

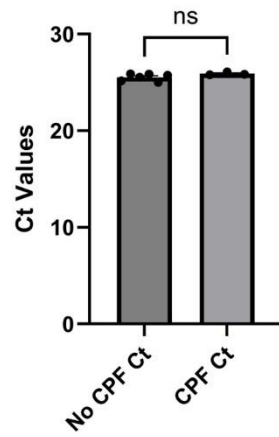


Figure A1. Ct values extracted from real-time qPCR of GAPDH housekeeping gene shows no significant difference between no-CPF and CPF-dosed samples.