Brief Report

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Midbrain-like spheroid generation and α-synuclein quantification from 3D midbrain neurospheres for Parkinson's disease research

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Abstract

Objectives: Traditional 2D *in vitro* models often fail to recapitulate the complex architecture and microenvironment of the human midbrain, limiting their translational relevance in drug discovery for neurological disorders such as Parkinson's disease (PD). To address this, 3D culture systems are gaining traction due to their improved physiological relevance. However, advanced 3D models such as organoids and assembloids often suffer from reduced reproducibility and increased methodological complexity, making them less suitable for early-stage drug screening. Our objective was to develop a streamlined and reproducible 3D Neurosphere-based method for generating midbrain-like spheroids.

Methods: We developed 3D Neural Progenitor Cells (NPCs) neurospheres using U-bottom ultra-low attachment plates. NPCs (12,800 cells/well) were seeded in (ULA) 96-well plates, cultured in NPC expansion media for 3 days, and then transitioned to a dopaminergic differentiation medium for 21 days. Immunostaining was performed to check for midbrain differentiation and presence of dopaminergic markers and ELISA was used to quantify α-synuclein levels in both 2D and 3D NPC cultures.

Results: Resulting spheroids averaged 210 μm in diameter and were maintained in culture for up to 70 days. Immunostaining confirmed expression of midbrain and dopaminergic markers (FOXA2, DAT), while ELISA quantification revealed elevated α-synuclein levels compared to 2D cultures.

Conclusions: This Neurosphere-based model provides a straightforward and scalable platform for the generation and differentiation of NPC-derived spheroids, enabling consistent spheroid measurement and characterization of α-synuclein levels, thereby supporting its utility in preclinical drug or neuroprotective compound screening applications for Parkinson disease.

Keywords: 3D cell culture; spheroids; neurospheres; Parkinson's disease; midbrain model and α-synuclein

Introduction

Parkinson's disease (PD) is complex neurodegenerative condition affecting nearly 11.77 million people globally as per 2021 estimates. Due to multifactorial nature, generating a single standardized *in vitro* model for studying disease progression and evaluating prospective molecular leads remains a challenge [1]. Most pre-clinical models used are based on monolayer cultures of cell lines like SH-SY5Y (neuroblastoma cells), PC-12 (rat medullary pheochromocytoma), LUHMES (Lund human mesencephalic cells), iPSCs (induced pluripotent stem cells) and iPSC-s derived mature neurons and glial cells [2]. These models have the advantage of being simple, easy to maintain, cost-effective and generally pliable to be standardized for most drug-screening assays.

However, these models don't recapitulate the complex cytoarchitecture of the brain. Growth on flat surface affects cell shape, division polarity (division characteristics which dictate spatial arrangement of organelles during division into daughter cells in an established asymmetry) for the cell type, altered gene & protein expression. It is also reported to have a generally less diverse phenotypes of cells [1, 3].

To overcome these limitations, several 3D cell culture models have been put forward, ranging from the simplest

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ones like spheroids to more complex ones like organoids, assembloids, organ-on-chip (OOC) cultures, and organotypic cultures. Higher 3D models enable complex characterization and cell interaction studies, like neuron-glial interactions and vasculature changes. However, they often yield inconsistent results, even among cultures from the same cell batch [4].

In comparison to this, neurospheres are freely floating aggregates which expand upon exposure to growth factors and differentiate uniformly. These have better reproducibility and are easier to standardize for drug-screening assays compared to more agitation-based or ECM-based 3D culture models. Neural spheroids also have uniform shape and are easier to immunostain [5].

In the present study, neurospheres were created from neural progenitor stem cells (NPCs), which serve as an intermediate stage in the differentiation process from pluripotent stem cells to fully differentiated neurons. NPCs enable quicker differentiation compared to induced pluripotent stem cells (iPSCs), which can also be utilized but necessitate longer time for differentiation and maturation as well as higher degree of patterning.

NPC spheroids were generated using two cell lines validated of neurodegenerative disease research [6]: ACS 5006, a reporter NPC cell line from healthy umbilical cord tissue, and ACS 5001, a cell line derived from the dermal tissue of a 63-year-old Caucasian PD patient. Spheroid generation was done using Ultra-low attachment (ULA) microplates (widely used for spheroid generation). This approach is also used for midbrain organoid generation. Spheroids were maintained on but generally as a starting step after which the they're transferred into bigger vessels or encapsulation in ECMs [7]. The spheroids were maintained on NPC expansion media for 1 week followed by culturing on differentiation media for 3 weeks at 70 % media change rate with progressively more media being left behind every week of differentiation (without crossing over 150 µl working volume per well of 96-well plate).

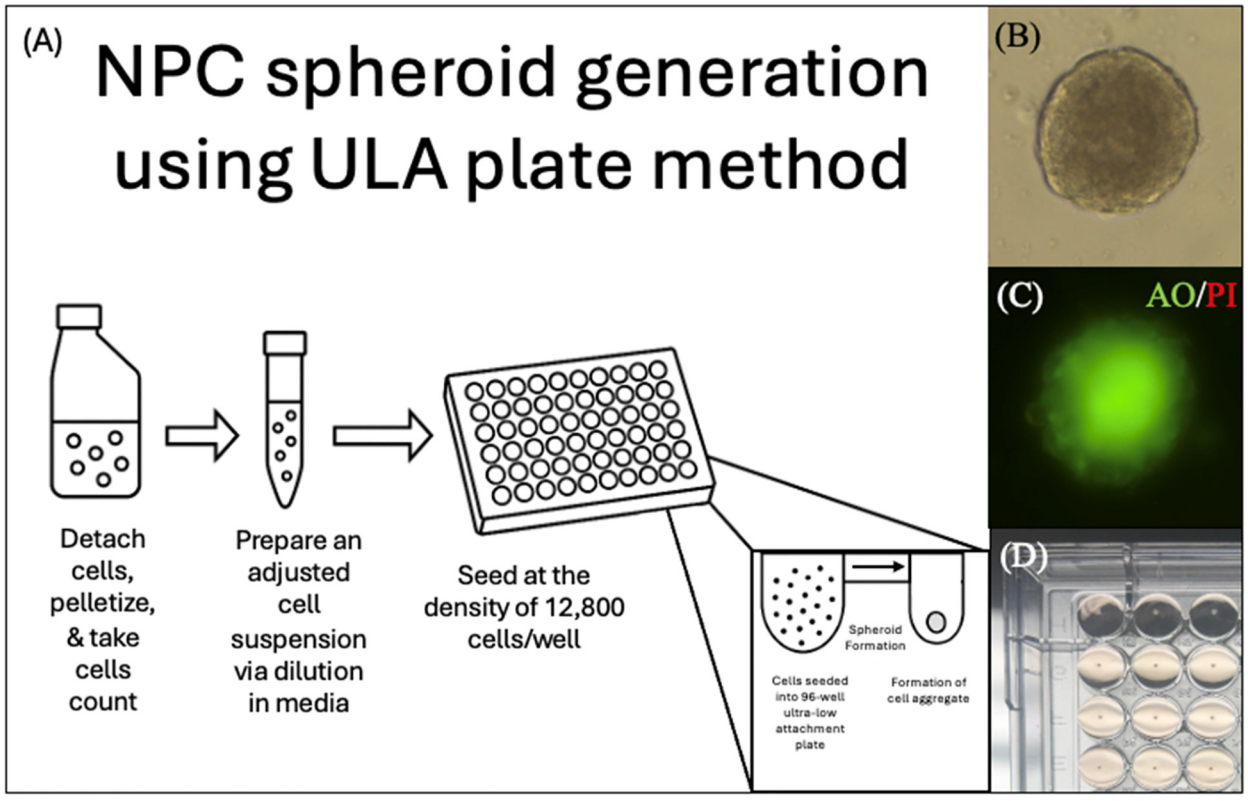
After this mark, spheroids could be immunolabeled for midbrain and dopaminergic differentiation markers and can be cultured for an extended periods of up to 50 days. Spheroid diameters were recorded by routinely brightfield imaging spheroids at 5-day intervals and measurement using Fiji (ImageJ) software. It was observed that differentiation led to a gradual reduction in spheroid diameter over the 21 day-period.

α-synuclein quantification from spheroids was carried out using a quantitative ELISA method. α-synuclein levels from both monolayer and spheroid cultures of both cell lines were measured post differentiation. The patient cell line recorded higher α-synuclein levels, and the spheroidal cultures overall had higher α-synuclein expression compared to 2D cultures.

The spheroid generation method has been validated for various NPC cell lines and can be effective to characterize spheroid health markers (like spheroid diameter) or PD disease markers (like α-synuclein). This method accelerates early-drug screening workflows for PD research.

Results

1. **Spheroid generation using Neural Progenitor Cells**: Both the cell lines yielded uniform NPC spheroids after 24 h of incubation. Figure 1A shows flow charts of spheroids generation. At the 12,800 cells/well seeding density, the spheroids yield an average diameter of 210 µm after 1 day which initially increases with time and then gradually plateaus upon the establishment of a necrotic core (Figure 1E and F).
2. **Spheroid diameter changes over time:** As neural stem cells (NSCs) differentiate into mature neurons, their volume typically decreases. Some neural spheroid cultures may shrink or enlarge during differentiation. Several factors influence these diameter changes, including differentiation type/cell fate, seeding density, nutrient, electrolyte or growth factor gradients, presence of vascularization, and the culture agitation based/extracellular matrix (ECM) embedded approach [8, 9]. The result of spheroid diameter changes over time are shown in (Figure 1E and F).
3. **Immunolabeling for Mid brain differentiation of spheroids**: After 21 days of differentiation, the spheroids were fixed in 10 % formaldehyde solution (in PBS) for 30 min, followed by permeabilization using 0.2 % Triton X-100 solution (in PBS) and blocking under 5 % BSA (in PBS). They were probed with primary antibody (1:1,000 dilution) overnight at 4 °C followed by incubation of secondary antibodies (1:2,000 dilution). The spheroids stained positive for FOXA2, an early midbrain floorplate marker during midbrain fate specification (Figure 2). They also stained positive for DAT, a specific dopaminergic neuron marker and GIRK2, a specific A9 dopaminergic marker (Figure 3). This indicates presence of subtrantia nigral dopaminergic neurons further validating our model for PD research.
4. **α-synuclein quantification using Sandwich ELISA:** α-synuclein levels (in pg/mL) were quantified by assaying 300 µg/mL protein lysates (made in 1X Cell Extraction buffer PTR) of 2D & 3D NPC cultures.



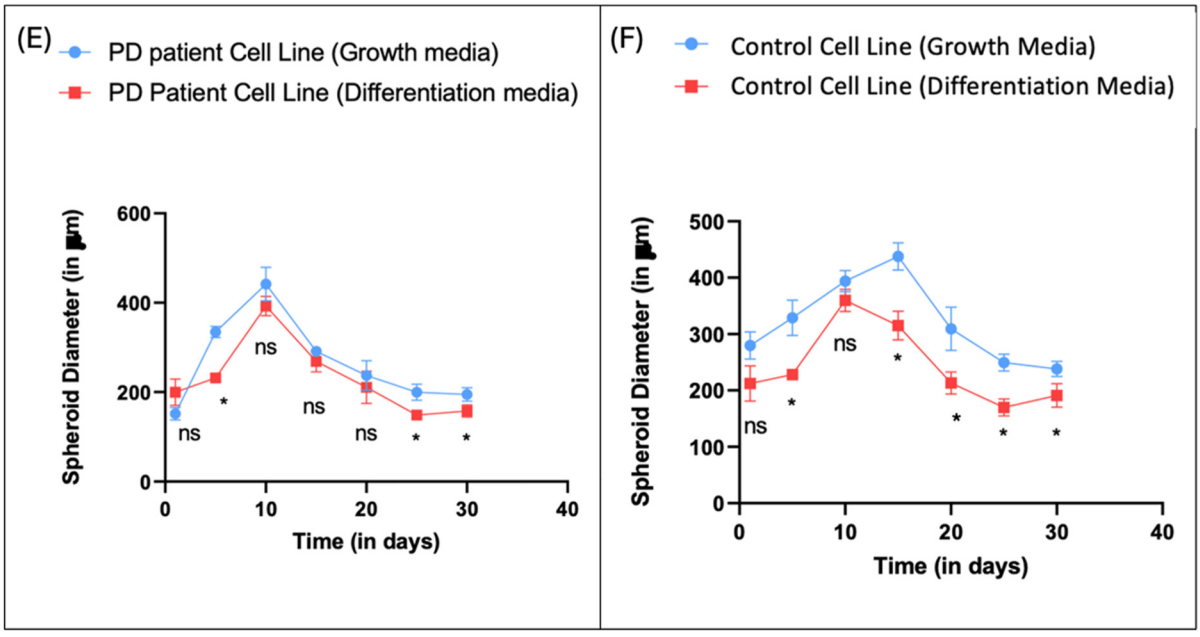


Figure 1: Spheroid generation method: (A) neural progenitor cells (NPCs) derived from healthy (ACS 5006) and PD patient (ACS 5001) lines were cultured in Matrigel-coated flasks. Cells were enzymatically detached using accutase, centrifuged at 1,000 rpm for 5 min at room temperature, counted using trypan blue exclusion method, and made into an adjusted cell suspension such that 12,800 viable cells/well could be seeded into ultra-low attachment 96-wellplate and incubated overnight in standard cell culture conditions (37 °C, 5 % CO_2, 95 % humidity). Spheroids generate approximately within 24 h of incubation and can be imaged easily using (B) Brightfield microscopy, or (C) fluorescence microscopy [in present example, they were stained with acridine orange-propidium iodide (AO-PI)]. (D) Image of spheroids in wells of ULA microplate. Spheroid diameter changes over time in (E) PD patient cell line (ACS 5001), and (F) control cell line (ACS 5006): NPC spheroids were imaged periodically under Brightfield microscopy. Spheroid diameters were quantified using Fiji (ImageJ), with pixel-distance calibrated to microscope scale bars. Multiple angle measurements per spheroid were averaged per time point. (A complete stepwise procedure is given in Supplementary Material protocol 3).

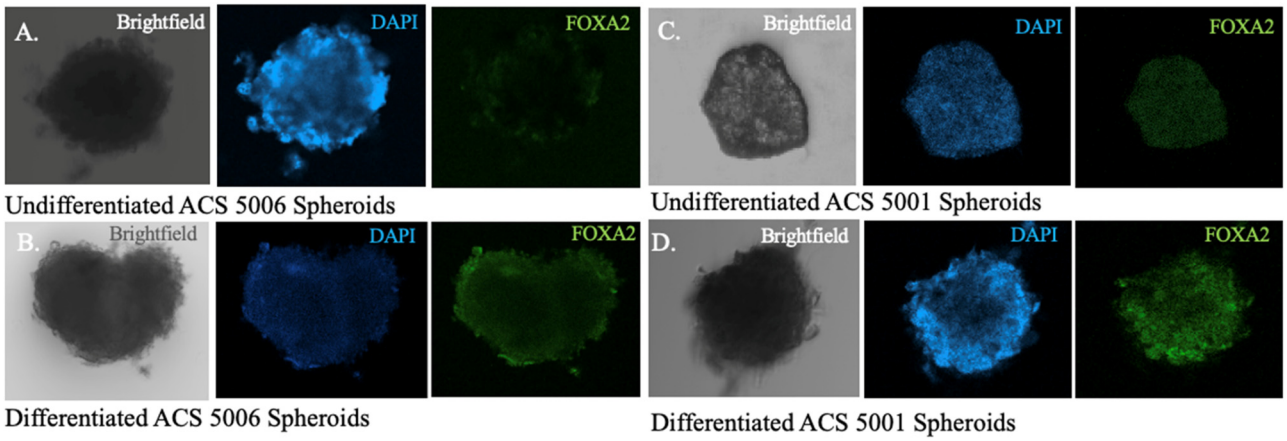


Figure 2: Confirmation of differentiation with immunocytochemistry (ICC): (A) & (C): images of un-differentiated control cell line spheroid & PD patient cell line spheroid respectively in Brightfield, DAPI and green filters. (B) & (D): Images of differentiated control cell line spheroid & PD patient cell line spheroid respectively in Brightfield, DAPI and green filters. NPC-derived spheroids (PD and control) were maintained in dopaminergic differentiation media for ≥21 days. Spheroids were fixed in 10 % formaldehyde (in PBS), permeabilized with Triton X-100, blocked with BSA, and immunostained with anti-FOXA2 (1:1,000, Abcam ab108422). Hoechst 33,342 and fluorescent secondary antibody (1:2,000 dilution, Abcam ab150077) were used for visualization under DAPI and green channels (please refer Supplementary Materials for complete list of chemicals used).

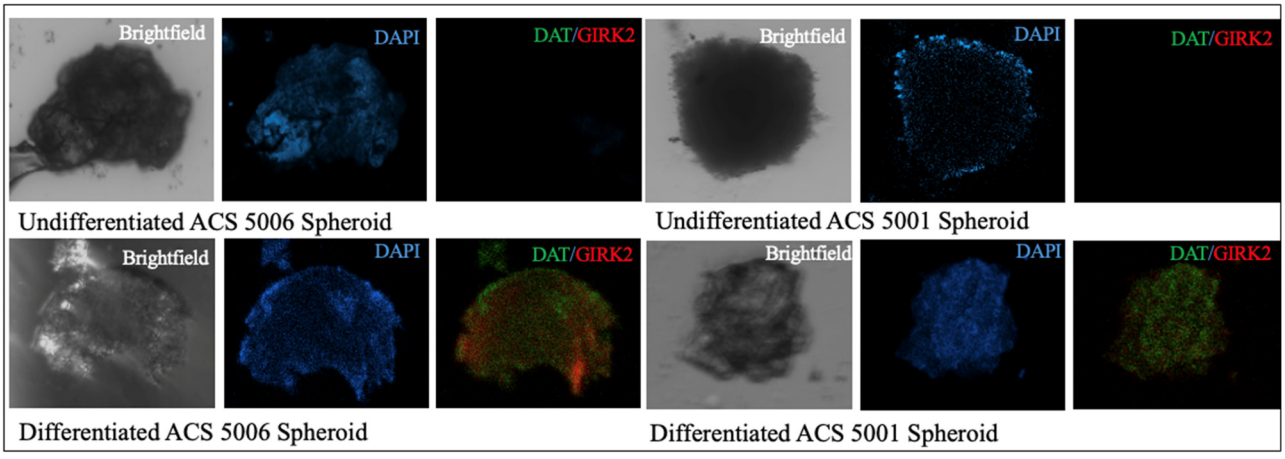


Figure 3: Confirmation of differentiation with immunocytochemistry (ICC): (A) & (B): images of un-differentiated control cell line spheroid & PD patient cell line spheroid respectively in Brightfield, DAPI and green-red filters. (C) & (D): Images of differentiated control cell line spheroid & PD patient cell line spheroid respectively in Brightfield, DAPI and green-red filters fixed spheroids were immunostained with anti-DAT and anti-GIRK2 (both 1:1,000; Abcam ab184451 & Abcam ab65096), followed by fluorophore-tagged secondary antibodies (1:2,000 dilution, Abcam ab150131 and Abcam ab150077). Fluorescent imaging was performed using DAPI (nuclei), green (DAT), and red (GIRK2) channels to assess dopaminergic marker expression in differentiated vs. undifferentiated spheroids (please refer to Supplementary Materials for complete list of chemicals used).

Both the cell lines showed higher α-synuclein levels in spheroidal cultures (Figure 4). This can be explained due to higher maturation of NPCs in spheroids compared to monolayer cultures. This typically results in higher cell-cell interaction and greater degree of differentiation, which may have been resulted in higher α-synuclein production [10]. The patient cell line (ACS 5001) reporting higher α-synuclein levels is expected as it is derived from a PD patient.

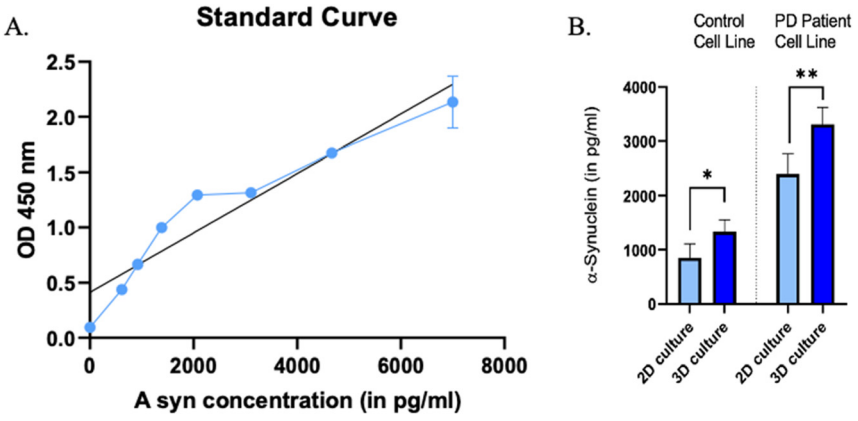


Figure 4: Sandwich ELISA for α-synuclein quantification: α-Synuclein levels were quantified from lysates of 2D monolayers and 3D spheroids using a commercial ELISA kit (Abcam ab260052). Lysates were prepared per Supplementary Material protocols 4 and 5, quantified using Bradford assay and loaded as 5 µg sample per well. Absorbance was measured at 450 nm to generate standard curves and sample comparisons. (A) Standard curve of α-synuclein concentration vs. absorbance at 450 nm. (B) Comparison of α-synuclein levels in differentiated 2D & 3D cultures.

Discussion & conclusions

Due to translational inconsistencies found between 2D cell cultures & animal models, development of robust 3D models for PD has been increasingly incentivized. Various methods put forth to generate midbrain-like organoids (MLOs) such as ECM-based systems, agitation methods, and hybrid approaches have been able to more accurately represent PD-characteristics like neuromelanin-positive dopaminergic neuron occurrence [11, 12]. The promotion of 3D cultures aims to reduce animal testing and enhance translation of findings across studies, even going as far as being included as valued components of 'New Approach Methodologies' (NAM) for IND & NDA submissions [13]. However, these complex models often exhibit significant variability attributable to factors like ECM composition, differentiation scheme or starting cell type/source.

Spheroids present a simplified model with more predictable differentiation timelines and provide an intermediate level of complexity between 2D cultures and full-fledged organoid systems [5, 14]. They offer a middle ground showcasing greater reproducibility, drug resistance & biomarker heterogeneity comparable to *in vivo* conditions, making them well-suited for early-stage drug discovery.

In this study, we developed neurospheres by seeding neural progenitor cells (NPCs) into ultra-low attachment (ULA) microplates. We demonstrated that these spheroids differentiate into substantia nigra-like dopaminergic neurons, as evidenced by positive immunostaining for GIRK2 [see Figure 3C and D]. Additionally, we present a straightforward protocol for quantifying α-synuclein levels in the spheroids using a standard ELISA assay. This method enables the assessment of treatment effects on α-synuclein expression within a 3D context and allows for direct comparisons with traditional monolayer cultures (see Figure 4B).

Despite these advantages, the model does have limitations. The neurospheres do not capture intercellular interactions – such as those between neurons and glial cells – or the dynamic influence of ECM components, owing to their free-floating nature. Moreover, spheroid growth plateaus after a few days due to the formation of a necrotic core, limiting long-term culture and expansion. In addition, a relatively large number of spheroids must be pooled to obtain sufficient protein concentrations for reliable α-synuclein detection and quantification.

Overall, while not a substitute for more complex organoid systems, the described method is a rapid and scalable approach for generating dopaminergic neurospheres and assessing PD-related molecular changes. It requires significantly less time for dopaminergic differentiation and requires fewer exogenous cues compared to traditional differentiation outflows. Additionally, the ECM-free culture system eases spheroid harvesting for subsequent analyses like lysate preparation/molecular profiling. This Neurosphere model offers a valuable tool for accelerating the screening of early phase therapeutic interventions for PD. Disease induction by alpha synuclein can be done in the spheroids and the neurosphere model developed can be used to test neuroprotective molecules or compounds on Parkinson's Disease markers such as dopaminergic neurons differentiation, α-synuclein level, accumulation of phosphorylated α-synuclein (pSer129) and aggresomal levels in the 3D spheroids derived from healthy and PD patient cell lines.

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Author contributions: Ameya Joshi: Conceptualization, Data curation, Formal analysis, writing-original draft, writing-review & editing. **Erik Moore**: Data curation, writing-review & editing. **Surabhi Shukla**: Conceptualization, Funding acquisition, Supervision, Data curation, Formal analysis, writing-original draft, writing-review & editing.

Use of Large Language Models, AI and Machine Learning Tools: Not applicable.

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