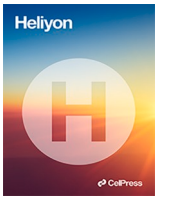
Heliyon 11 (2025) e42735

Contents lists available at ScienceDirect

Heliyon

journal homepage: www.cell.com/heliyon



Research article

Enhancing NADPH to restore redox homeostasis and lysosomal function in G6PD-deficient microglia

Abir Mondal [a], Soumyadeep Mukherjee [a], Prince Upadhyay [a], Isha Saxena [a], Soumya Pati [b,**], Shailja Singh [c,*]

[a] Department of Life Sciences, School of Natural Sciences, Shiv Nadar Institution of Eminence, Delhi NCR, India
[b] Amity Institute of Neuropsychology & Neurosciences, Amity University, Noida, UP, 201313, India
[c] Special Centre for Molecular Medicine, Jawaharlal Nehru University, Delhi, India

ARTICLE INFO

Keywords:
G6PD deficiency
Metabolites
Phytochemicals
Oxidative stress
Therapeutics

ABSTRACT

Microglia, the immune cells of the central nervous system (CNS), play key roles in neurogenesis, myelination, synaptic transmission, immune surveillance, and neuroinflammation. Inflammatory responses in microglia can lead to oxidative stress and neurodegeneration, contributing to diseases like Parkinson's and Alzheimer's. The enzyme glucose-6-phosphate dehydrogenase (G6PD) is essential for producing nicotinamide adenine dinucleotide phosphate hydrogen (NADPH), which neutralizes oxidative stress. G6PD deficiency has been linked to several disorders, including neurological conditions. Our study shows that G6PD deficiency in microglia reduces NADPH levels, disrupting redox balance and lysosomal function. To address this, we explored alternative metabolic pathways by targeting enzymes like isocitrate dehydrogenase 1 (IDH1) and malic enzyme 1 (ME1), both crucial for NADPH production. Supplementing metabolites such as citric and malic acid improved NADPH levels, while small molecules like dieckol and resveratrol enhanced IDH1 and ME1 expression. The combination of these approaches restored redox homeostasis and lysosomal function, offering potential therapeutic strategies for G6PD deficiency.

1. Introduction

Microglia plays a crucial role in pathogenic invasion and injury and maintains the physiological function of the CNS, particularly under oxidative stress [1]. Oxidative stress occurs due to the imbalance between reactive oxygen species (ROS) production and the cellular antioxidant defenses. Microglia are deeply involved in both the production and mitigation of oxidative stress, highlighting their dual role in CNS health and disease [2]. The chronic activation of microglia can exacerbate neuronal injury and death. On the contrary, microglia are essential for neurogenesis, myelination, synapse modulation, and vasculogenesis [3]. It also plays a critical role in removing dead cells and pathogenic β-amyloid and tau plaques through phagocytosis [4]. Thus, the function of microglia is key to CNS homeostasis, and its alteration results in the pathogenesis and progression of neurodegenerative diseases, highlighting their importance as potential therapeutic targets for promoting CNS health. Microglia become activated in response to pathogens, injury, or other stress signals and produce ROS as part of the innate immune response. NADPH oxidase and mitochondrial pathways are primarily responsible for ROS production and are essential for defense against pathogens and signaling processes. However, the

* Corresponding author.
** Corresponding author.
E-mail addresses: spati@amity.edu (S. Pati), shailja.jnu@gmail.com, shailjasingh@mail.jnu.ac.in (S. Singh).

https://doi.org/10.1016/j.heliyon.2025.e42735
Received 28 September 2024; Received in revised form 3 February 2025; Accepted 14 February 2025
Available online 15 February 2025

accumulation of ROS can lead to oxidative damage to lipids, proteins, and DNA and further promote neurodegenerative processes [5]. Microglia also produce antioxidant molecules such as NADPH and glutathione to neutralize oxidative stress, thus protecting neurons and other glial cells from oxidative damage.

However, NADPH is the key molecule that allows microglia to mediate pro and anti-oxidative functions depending on the cellular microenvironment [6]. During pathogen invasion, NADPH is being utilized to generate ROS to neutralize foreign particles. On the contrary, NADPH is crucial for detoxifying hydrogen peroxide (H_2O_2) in a glutathione-dependent manner. The G6PD is a key enzyme for pentose phosphate pathways, contributing to the major production of cytoplasmic NADPH. The deficiency of G6PD causes dysregulation of redox homeostasis and accelerated production of ROS, causing damage to the cell. Notably, the decrease in cellular NADPH and dysregulation of the redox microenvironment due to the deficiency of the G6PD enzyme results in hemolytic anemia [7,8]. Several reports suggested that the G6PD gene is highly susceptible to the mutation as it contains many GC-rich sequences [9]. Besides, the mutation in this gene showed diverse pathophysiology ranging from severe pathophysiology like hemolysis to asymptomatic unless there is any oxidative stress (caused by food, drug, or infection). However, several studies indicated that oxidative stress and neuroinflammation are correlated with neurodegenerative disorders [10–13]. Few recent studies in an animal model and postmortem human brain neurodegenerative (Alzheimer's, Parkinson's, and Huntington) tissue showed either reduced G6PD expression or activity is the primary cause of pathophysiology [14–16]. Interestingly, ferroptosis, iron-dependent cellular death pathways, is very common in all neurodegenerative disorders [17–20]. A recent study also showed that cellular NADPH concentration is a key determining factor for ferroptosis [21,22]. A low level of cellular NADPH promotes ferroptosis, which might correlate with G6PD deficiency. Besides, G6PD deficiency was also reported in 2 autistic male children in Saudi Arabia [23]. Recently, a few reports suggested that an altered redox microenvironment is one of the causes of neurodevelopmental disorders such as autism [24–27]. Given the rising number of neurological cases associated with oxidative stress-induced neuroinflammation in various neurological disorders, there may be a potential link to G6PD deficiency. The spectrum of G6PD deficiency disorder and the unavailability of potential therapeutics put millions of individuals at life risk. Notably, the G6PD deficiency decreases cytoplasmic NADPH levels, which can be restored by promoting the enzymatic activities of IDH1 and ME1. The IDH1 catalyzes the conversion of isocitrate to α-ketoglutarate, generating 1 mol of NADPH [28]. Similarly, ME1 facilitates the conversion of malic acid into pyruvic acid, generating 1 mol of NADPH [29]. However, the expression of these enzymes may vary widely across different tissue types. Thus, in this study, we targeted IDH1 and ME1 by enhancing their expression and enzyme activities to restore the cytoplasmic NADPH pool, thereby enabling microglia to regulate oxidative stress and lysosomal functions. Using our previously developed G6PD-deficient microglia cell culture model [30], we evaluated the efficacy of our therapeutic strategies through flow cytometry, biochemical assays, quantitative polymerase chain reaction (qPCR), and microscopy.

2. Materials and methods

2.1. Culturing of human microglia

Human microglia clone 3 (HMC3) cells from ATCC (CRL-3304) were cultured and passaged in complete EMEM media containing EMEM (Sigma #M4655), 10 % FBS (Gibco #10270106), 1 mM Sodium Pyruvate (Gibco #11360070), 1 x NEAA (Gibco #11140050), and 0.1 % Penicillin-streptomycin(Gibco #10378016). Microglia 5 to 16 passages were used for all the experiments. Additionally, previously prepared and characterized G6PDd-deficient microglia were used in this study [30].

2.2. Cytotoxicity assays

HMC3 Cells were seeded at a density of 4000 cells/well in 96-well plates. The following day, varying concentrations (0–100 μg/mL) of Dieckol and (0–100 μM) resveratrol were prepared in complete EMEM (cEMEM) and added to the respective well. DMSO control was also taken to nullify the solvent toxicity. Plates were incubated in a 37 °C incubator for 24 h. Then, the media was removed from the cells, and 0.5 mg/mL MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide) solution in 1x phosphate buffer saline

Table 1
NADPH estimation by WST-8 and 1-mPMS assay.

S. No	Samples	Treatment (6 h) prepared in 1xPBS
1	G6PDwt	200 μM WST-8 + 8 μM 1-mPMS
2	G6PDwt	0–100 μg/mL Citric acid + 200 μM WST-8 + 8 μM 1-mPMS
3	G6PDwt	0–100 μg/mL Malic acid + 200 μM WST-8 + 8 μM 1-mPMS
4	G6PDwt	0–100 μg/mL Citric and 0–100 μg/mL Malic acid + 200 μM WST-8 + 8 μM 1-mPMS
5	G6PDd	200 μM WST-8 + 8 μM 1-mPMS
6	G6PDd	100 μg/mL Citric acid + 200 μM WST-8 + 8 μM 1-mPMS
7	G6PDd	100 μg/mL Malic acid + 200 μM WST-8 + 8 μM 1-mPMS
8	G6PDd	18 μg/mL Dieckol + 200 μM WST-8 + 8 μM 1-mPMS
9	G6PDd	500 nM Resveratrol + 200 μM WST-8 + 8 μM 1-mPMS
10	G6PDd	18 μg/mL Dieckol + 100 μg/mL Citric acid + 200 μM WST-8 + 8 μM 1-mPMS
11	G6PDd	18 μg/mL Dieckol + 100 μg/mL Malic acid + 200 μM WST-8 + 8 μM 1-mPMS
12	G6PDd	500 nM Resveratrol + 100 μg/mL Citric acid + 200 μM WST-8 + 8 μM 1-mPMS
13	G6PDd	500 nM Resveratrol + 100 μg/mL Malic acid + 200 μM WST-8 + 8 μM 1-mPMS

(PBS) was added to each well for 3 h. Then, MTT was removed, and the formazan crystal was dissolved in a 200 μL DMSO (SRL #24075) solution. Then, the plates were incubated in a 37 °C incubator for 30 min. The absorbance at 570 nm was measured in a spectrophotometer (BioTek, Synergy H1).

2.3. NADPH estimation assay

NADPH level was measured by using highly sensitive WST-8 (# HY-D0831, MCE) and 1-mPMS (#HY-D0937, MCE) assay [31], and the following conditions were used (Table 1). Spectrophotometry was performed to measure absorbance at 460 nm (BioTek, Synergy H1). All the experiments and replicas were further used to calculate the standard error mean (SEM). Experimental data was represented as a bar plot generated by GraphPad.

2.4. Immunocytochemistry

HMC3 cells were seeded at a density of 25000 cells/coverslips. The next day, cells were treated with 0.5 μM of Resveratrol and 18 μg/mL Dieckol for 24 h. Then, cells were fixed with 4 % Paraformaldehyde for 10 min and washed using 1x PBS. After that, 0.1 % Triton X-100 was used to permeabilize the membrane, and blocking was performed by 5 % BSA for 1 h. Then, the NRF2 primary antibody (Affinity, Cat No. BF8017) was incubated (1:100 dilution) for 3 h at room temperature in a humid chamber. After washing three times with 1x PBS, the anti-rabbit secondary-FITC (Bio-RAD #STAR34B) secondary antibody (1:200 dilution) was incubated for 90 min. Then, coverslips were washed using 1x PBS and mounted on the slide using Vectashield antifade DAPI (Vector Lab, H-1200) mounting media. Images were captured using a Nikon Ti2 Confocal microscope and analyzed by Fiji ImageJ software.

2.5. Study the ROS by flow cytometer

1.0×10^5 cells/well were seeded in 6 well tissue culture plates. The following day, treatment was given to cells, as shown in Table 2. After the treatment, cells were trypsinized and collected by centrifugation (Eppendorf # 5810R) at 130g for 5 min. The cell pellet was washed with 1x PBS two times to remove the residual cell culture media. Finally, the cell pellet was dissolved in 200 μL of 1x PBS. In one set, 30 μM H2O2 was added to the cell and incubated for 20 min (positive control of our experiments). Then, 10 μM carboxy-H_2DCFDA (Invitrogen #I36007) ROS labelling dye was added to all samples incubated in a 37 °C incubator for 30 min. In one set, cells were kept without adding any dye to check the background noise. Flow cytometry was performed in CytoFLEX-S (Beckman), and 10000 events were recorded for analysis. Flow cytometry data was analyzed in CytExpert and Floreada.io online tool.

2.6. Q-PCR-based gene expression study

The RNA was isolated using a TRIZOL reagent (Thermofisher part#15596206). The RNA concentration was measured in the Nanodrop 2000 instrument (Thermo Scientific). Using a High-Capacity cDNA reverse transcription kit, 1 μg of RNA was used to make complementary DNA (cDNA) (Applied Biosystems #4368814). Then, quantitative PCR (qPCR) was performed using the Powerup SYBR green master mix (Applied Biosystems # A25742). Fold change ($2_t^{-\Delta\Delta C}$) was calculated in Microsoft Excel. The qPCR primers for respective genes are mentioned in Table 3.

Table 2
Experimental conditions for detection of ROS positive cells flowcytometry.

S No.	Sample	Pretreatment (12 h)	Treatment (6 h)
1	G6PDwt		No dye control
2	G6PDwt		Control
3	G6PDwt		500 ng/mL LPS
4	G6PDwt	500 ng/mL LPS	100 μg/mL Citrate
5	G6PDwt	500 ng/mL LPS	100 μg/mL Malate
6	G6PDd		control
7	G6PDd	500 ng/mL LPS	
8	G6PDd	500 ng/mL LPS	100 μg/mL Citrate
9	G6PDd	500 ng/mL LPS	100 μg/mL Malate
10	G6PDd		100 μg/mL Citrate
11	G6PDd		100 μg/mL Malate
12	G6PDd	18 μg/mL Dieckol	
13	G6PDd	500 nM Resveratrol	
14	G6PDd	18 μg/mL Dieckol	100 μg/mL Citrate
15	G6PDd	18 μg/mL Dieckol	100 μg/mL Malate
16	G6PDd	500 nM Resveratrol	100 μg/mL Citrate
17	G6PDd	500 nM Resveratrol	100 μg/mL Malate

Table 3
Q-PCR primer details.

Gene	Primers
IDH1 FP	CTATGATGGTGACGTGCAGTCG
IDH1 RP	CCTCTGCTTCTACTGTCTTGCC
ME1 FP	GGAGTTGCTCTTGGTGTTGTGG
ME1 RP	GGATAAAGCCGACCCTCTTCCA
18S FR	ACCCGTTGAACCCCATTCGTGA
18S RP	GCCTCACTAAACCATCCAATCGG

2.7. Lysosome acidification study by confocal microscope

G6PDd microglia at a 30000 cells/well density were seeded on a 35 mm glass bottom dish. The following day, all metabolites and combination therapeutics were supplemented to cells for 6 h in cEMEM media. Only the NADPH was incubated for 30mins which acts as a positive control for our experiments. Then, cells were labelled with 70 nM LysoTracker Deep Red (Invitrogen #L12492) and NucBlue Live Cell Stain (Hoechst 33342, R37605 for 20 min. Then, the probe was washed, and the fresh cEMEM was added. After that, confocal imaging was performed in a Nikon Ti2 confocal microscope. The acidified lysosomal bright puncta were counted manually on Fiji-image J software.

2.8. Statistical analysis

All the experiments were performed more than thrice with multiple replicates. GraphPad Prism was used to calculate the standard error mean (SEM). Statistical significances were calculated using either a *t*-test or ANOVA based on experimental parameters. P-values and statistical tests were mentioned in the respective figure legends. All results of our experiments were collected from at least 3 independent biological replicates.

3. Results

3.1. Analysis of the expression of NADPH-producing enzymes and formulation of metabolic supplementation strategies

The pentose phosphate pathway produces 2 mol of NADPH from 1 mol of Glucose-6-phosphate, which is a major contributor to cytosolic NADPH. The G6PD is the key rate-limiting enzyme of PPP, which catalyzes the first step of NADPH production, followed by 6-phosphogluconate dehydrogenase (6PGD) (Fig. 1A). Alternatively, IDH1 and ME1 also contribute to 1 mol of NADPH production in a tissue-specific manner (Fig. 1 A). Therefore, understanding the expression of NADPH-producing genes is crucial for developing metabolic supplementation strategies. Here, we analyzed the expression of G6PD, IDH1, and ME1 in the brain using the Brainspan RNA sequencing (RNAseq) database [32]. Our analysis indicated that G6PD is ubiquitously expressed throughout the developmental stages and in adults (Fig. 1B). IDH1 is highly expressed during early development, and the expression decreases and stabilizes 1 year after birth (Fig. 1B). On the contrary, ME1 expression increases at 4 months post-conception week (PCW) and stabilizes thereafter (Fig. 1B). Further, utilizing the human protein atlas (HPA) RNAseq database, we analyzed the expression of these genes in different brain regions, which also suggested that G6PD is highly expressed as compared to IDH1 and ME1 (Fig. 1C) [33]. However, analysis of proteomics data from the human protein atlas (HPA) showed tissue-wise heterogeneity in the expression of these 3 genes (Fig. 1D) [34]. Considering the differential expression of these genes and their basal level of expression across the brain tissues and other tissues indicates supplementation of citric acid and malic acid could increase the production of NAPDH in cells under G6PD deficient (G6PDd) condition. To validate our hypothesis, spectrophotometric NADPH estimation was performed by WST-8 assay, which indicated an increase in NADPH production in the human G6PD wild-type (G6PDwt) microglia cells treated with citric acid and malic acid (Fig. 1E). We also determine the efficacy of citric and malic acid in reducing oxidative stress by flow cytometry. Our data indicated that citric and malic acid reduce the oxidative stress in G6PDwt microglia pre-activated with lipopolysaccharides (LPS) (Fig. 1F). Therefore, both metabolic supplementation strategies can be used as potential therapeutics.

3.2. Citric acid and malic acid increase NADPH production and reduce oxidative stress in G6PD-deficient microglia cells

The supplementation of citric and malic acid should increase the production of NADPH via metabolic pathways catalyzed by IDH1 and ME1 in G6PDd microglia. The production of NADPH allows G6PDd microglia to regulate redox homeostasis. To study the efficacy of the citrate and malic acid supplementation, CRISPR-mediated G6PDd microglia cells were used, which were previously generated by guide RNA targeting exon 10 of G6PD (Fig. 2A) [30]. While characterizing the G6PDd microglia, we found a 50–60 % reduction in NADPH level compared to G6PDwt. Thus, NADPH estimation was again performed in G6PDwt and G6PDd cells to confirm a reduction in level NADPH in G6PDd microglia for further use in therapeutics strategies (Fig. 2B). Notably, G6PDd microglia supplemented with citric and malic acid exhibited an increase in NADPH production (Fig. 2C). However, our previous study showed that G6PD-deficient microglia exhibit increased basal oxidative stress as compared to wild-type [30]. Therefore, to check the efficacy of our metabolites in regulating oxidative stress, we performed flow cytometry analysis to capture the level of ROS in G6PDd microglia supplemented with

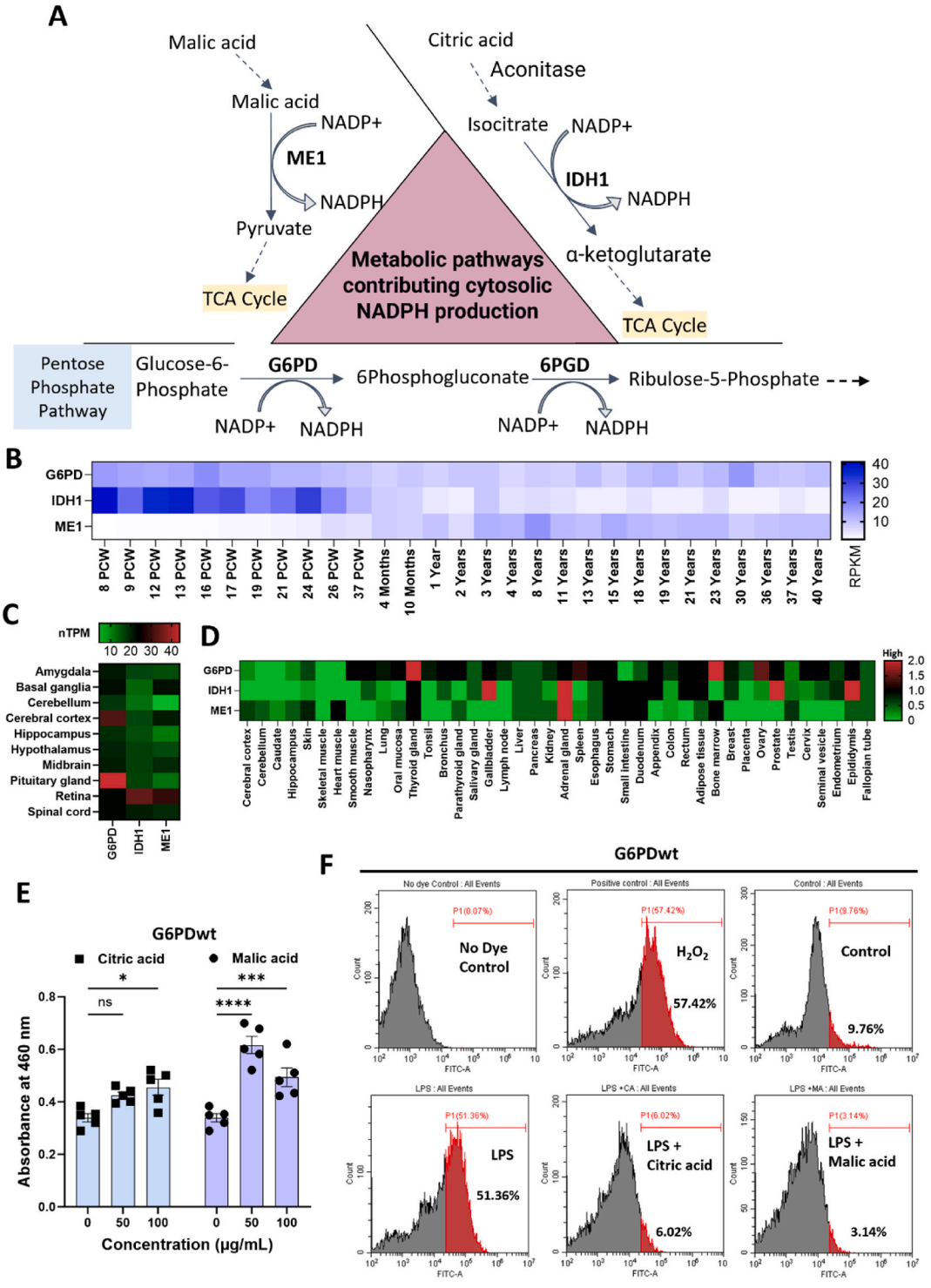


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Fig. 1. Expression analysis of cytosolic NADPH-producing genes for development and validation of therapeutic strategies: **A)** Diagrammatic representation of the pathways contributing to cytosolic NADPH pool; **B)** Heatmap represents FPKM (Fragments Per Kilobase of transcript per Million) value of the expression of cytoplasmic NADPH-producing genes from prenatal to 40 years adult brain RNAseq data from Brainspan database; **C)** Heatmap represents n-TPM (normalized Transcripts per million) values of expression of NADPH-producing genes in different brain regions derived from Human protein atlas database; **D)** Heatmap represents protein expression (low to high) of NADPH producing enzymes across all tissue types in human-generated by human protein atlas database; **E)** Bar graph represents absorbance (at 460 nm) of WST8-NADPH estimation assay in microglia cells incubated with various concentrations of citric acid and malic acid. Data represent the mean ± SEM (n = 5). *Represents significance values compared to control, (**P = 0.0022, ****P < 0.0001). Statistical significance was calculated using ordinary two-way ANOVA; **F)** Histogram represents the percentage of ROS-positive microglia treated with LPS and LPS + metabolites. A gate on the histogram was placed while considering no dye control sample.

metabolites. Our data indicated that supplementation of citric acid and malic acid reduces the percentage of ROS-positive G6PDd microglia from 20 % to 4.5 % (Fig. 2D and E), which suggests their efficacy in regulating oxidative stress *in-vitro*.

3.3. Dieckol and resveratrol increase the expression of IDH1 and ME1 by nuclear translocation of NRF2

The analysis of RNA-seq and proteomics data (Fig. 1B, C, D) suggests that the heterogeneity in the expression of IDH1 and ME1 across different tissues could potentially limit our strategy. To reduce the heterogenic expression of IDH1 and ME1, we thought to target the transcriptional regulator of these genes using small molecules. A previous study indicated that the kelch-like ECH-associated protein 1 (KEAP1) and nuclear factor erythroid 2-related factor 2 (NRF2) pathway is the primary regulator of cytoprotective responses to oxidative stress [35]. KEAP1 is an adapter for cullin3 E3 ligase, which binds to the NRF2 and helps its degradation [35]. The inhibition of KEAP1 by post-translational modification induces the release of NRF2 [36], which can further enter into the nucleus and upregulate genes essential for redox regulation, such as IDH1, ME1, G6PD, glutathione peroxidases (GPXs) and heme oxygenase1 (HO1) [37]. The recent reports also suggested that plant-derived small molecules such as dieckol and resveratrol inhibit KEAP1 and allow nuclear translocation of nuclear factor NRF2 and thereby increasing the expression of NRF2 downstream genes [37–45]. Therefore, we used dieckol and resveratrol to target the KEAP1-NRF2 pathway for increased expression of IDH1 and ME1 in microglia (Fig. 3A). Initially, we determined the percentage of cell viability by MTT assay (Fig. 3B). Dieckol within 20 μg/mL and resveratrol within 10 μM concentration did not affect cell viability. Therefore, 18 μg/mL of dieckol and 0.5 μM of resveratrol were used to develop therapeutics. As expected, we observed that the expression of IDH1 and ME1 was increased 1.5 to 2-fold (Fig. 3C and D) after 24 h of treatment with dieckol and resveratrol in G6PDwt and G6PDd microglia compared to untreated control. However, resveratrol-treated G6PDd microglia showed the highest expression of IDH1 and ME1 compared to G6PDwt (Fig. 3D). Further, our immunofluorescent study confirmed that these phytochemicals increased the nuclear localization of NRF2, thereby enhancing the downstream expression of IDH1 and ME1 (Fig. 3E and F). Thus, our experiment suggested that dieckol and resveratrol can be used to increase the expression of NADPH-producing genes to overcome the differential expression of this gene across all cell types for the development of combination therapeutics.

3.4. Combination therapeutics strategies increase NADPH production and restore redox balance and acidified lysosomes in G6PD deficient microglia

The citric and malic acid alone increased the NADPH production and decreased the oxidative stress in G6PDd microglia. Additionally, the use of dieckol and resveratrol enhanced the expression of IDH1 and ME1 in both G6PDwt and G6PDd microglia. Therefore, we considered using combination strategies to enhance expression and enzyme activity for improved therapeutic efficacy. The combination of small molecules (dieckol and resveratrol) along with metabolite (citric and malic acids) increases the production of NADPH in G6PDd microglia (Fig. 4A). However, G6PDd microglia treated with only dieckol or resveratrol do not increase NADPH production (Fig. 4A). That could be possibly due to the absence of substrates such as citric and malic acid in basal media (cEMEM) used for the experiments. Further, to evaluate the effectiveness of combination therapeutics on oxidative stress, we performed flow cytometry to detect the percentage of ROS-positive G6PDd microglia. Treatment of only citric acid or malic acid is sufficient to reduce the oxidative stress in G6PD deficient microglia *in-vitro* (Fig. 4B). Additionally, the combination of dieckol or resveratrol with citric acid or malic acid showed a maximum reduction in the number of ROS-positive cells (1–3 %) (Fig. 4B). Our experimental data suggests that our phytochemicals and their combination with metabolites can be potential therapeutics for replenishing the NADPH pool and minimizing oxidative stress in the G6PDd microglia. However, previously, we reported that the deficiency of the G6PD-NADPH axis also affects lysosomal acidification in microglia, and supplementation with NADPH restores lysosomal acidification [30]. Therefore, we also studied whether the supplementation of our therapeutics increased the number of acidified lysosomes. Our confocal microscopy data indicated an increase in lysosomal acidification (bright puncta of lysosomal vesicles) in all combinations of therapeutics (Fig. 4C and D) compared to untreated control. Therefore, our *in-vitro* study revealed that the metabolic supplementation and its combination with plant-based small molecule NRF2 activator can effectively increase NADPH production, reduce oxidative stress, and restore lysosomal acidification, indicating their efficacy in treating G6PD deficiency.

4. Discussion

G6PD deficiency is the most common enzymatic disorder affecting more than 500 million individuals worldwide [46]. G6PD

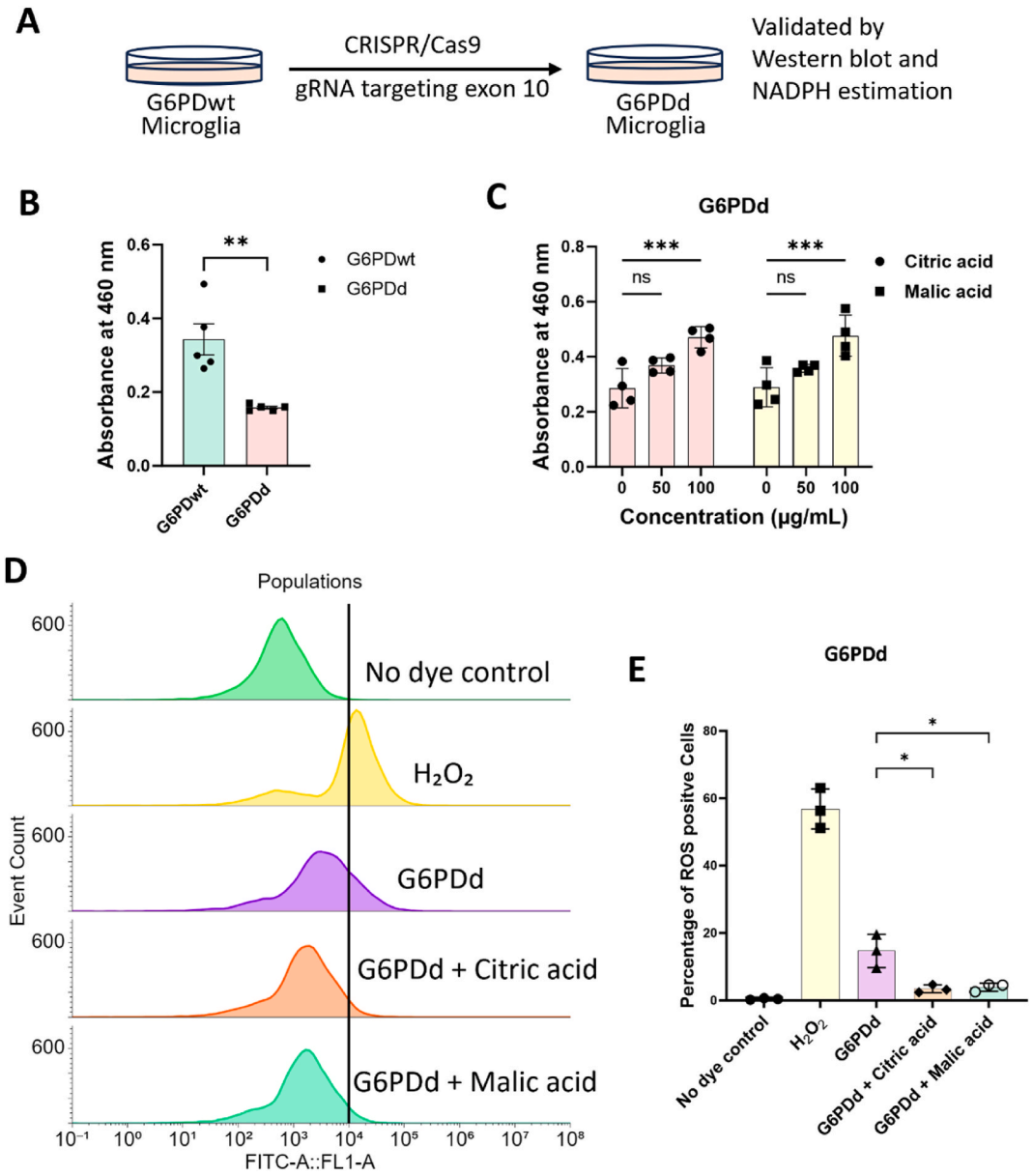

Fig. 2. Metabolic supplementation increases NADPH production and reduces oxidative stress in G6PD-deficient microglia: **A)** Schematic representation of the generation of G6PD-deficient microglia. **B)** Bar plot represents WST-8 -NADPH estimation in G6PDwt versus G6PDd cells. Data represents the mean ± SEM (n = 4). Statistical significance was calculated by unpaired *t*-test (**P = 0.0024); **C)** Bar diagram represents absorbance at 460 nm of NADPH estimation in G6PDd microglia at various citric acid and malic acid concentrations. Significance was calculated by two-way ANOVA (***P = 0.0004; ns = 0.1139 and 0.2069); **D)** and **E)** Histogram and bar plot represent flow cytometry data of the percentage of ROS positive G6PD deficient microglia treated with LPS and LPS + metabolites; Statistical significance was calculated by two-way ANOVA (*P = 0.0117 and *P = 0.0137).

deficiency primarily causes hemolytic anemia, which is often induced by food, medicines, and infections [47]. Recently, G6PD deficiency has been linked with cardiovascular, diabetes, neurological, and autoimmune disorders [48–53]. Although, a large cohort study has yet to elucidate the spectrum of G6PD deficiency disorders. A recent report showed that decreased G6PD enzyme activity is linked to the onset of Parkinson's disease (PD) through altered α-synuclein binding [54]. The study also reported top 6 G6PD missense mutations (p.Asp282His, p.Val309Glu, p.Val400Met p.Arg182Gln, p.Asn126Asp, and p.Asp113Asn) showed greater frequency in PD [54]. Various clinical studies using brain tissues and patient's blood samples of Alzheimer's and amyotrophic lateral sclerosis (ALS) showed decreased activity of G6PD correlated with neurodegeneration [55–57]. Additionally, neonates with G6PD deficiency are more susceptible to sepsis [58,59]. Previously, we found that G6PD deficiency affects the innate immune response by impairing nitric oxide production and lysosomal function in microglia, indicating a link between G6PD deficiency and the severity of infections [30].

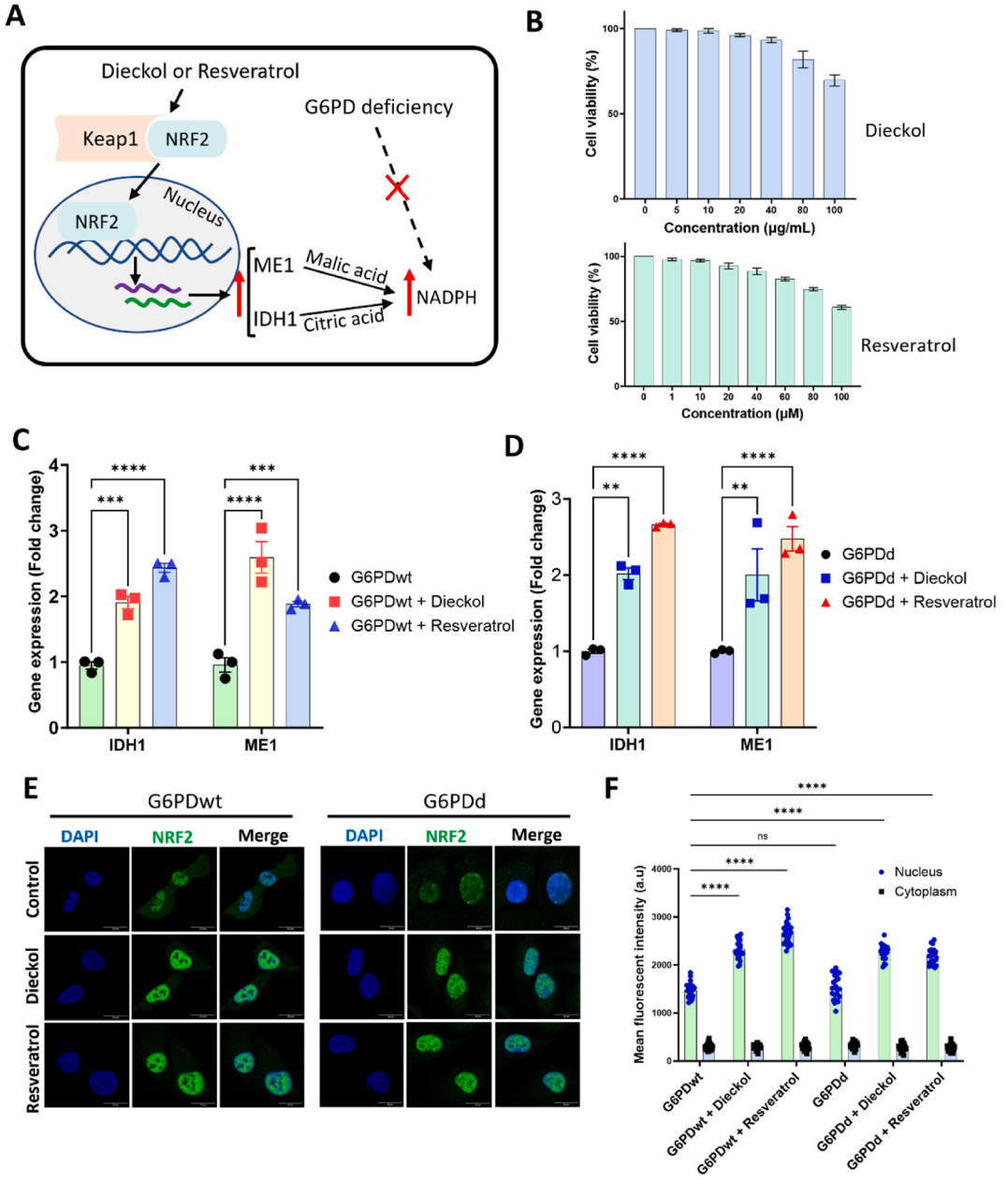

Fig. 3. Phytochemical increases expression of IDH1 and ME1 in G6PDd microglia: **A)** Diagrammatic representation of dieckol and resveratrol-mediated nuclear translocation of NRF2 increases the expression of IDH1 and ME1; **B)** Line diagram showing MTT absorbance (at 570 nm) value and respective IC_{50} of dieckol and resveratrol in microglia; **C)** and **D)** Representing qPCR fold change ($2^{-\Delta\Delta C_t}$) of gene expression of IDH1 and ME1 24 h after treatment of dieckol and resveratrol in G6PDwt and G6PDd microglia (n = 3). Two-way ANOVA was used to calculate the significance (***P = 0.0002, ****P < 0.0001 and **P = 0.0013); **E)** Immunofluorescence image showing nuclear (blue) localization NRF2 (green) in G6PDwt and G6PDd microglia treated with dieckol and resveratrol; **F)** Bar plot representing the mean intensity of nuclear versus cytoplasmic NRF2 level (n = 15) in microglia treated with dieckol and resveratrol. Two-way ANOVA was performed to calculate the significance (****P < 0.0001).

The high susceptibility of mutation in this gene is the major concerning factor for future disease burden globally. Besides, G6PD-deficient patients are sensitive to multiple drugs [7]. Therefore, patients should be screened for G6PD deficiency before prescribing drugs for certain diseases. Our previous study showed that G6PDd microglia cells are insensitive to LPS-mediated ROS generation, which may play beneficial and detrimental roles during foreign pathogen invasion [30]. Besides, no such medication is available to treat the G6PD deficiency. Earlier, clinical case studies using N-acetyl cysteine, α-tocopherol, and α-lipoic acid as therapeutics showed no promising results against G6PD deficiency [60]. Recently, a small-molecule G6PD agonist (AG1) was identified through high-throughput screening, and it increased the activity of G6PD clinical variants *in-vitro* [61]. However, further experiment is

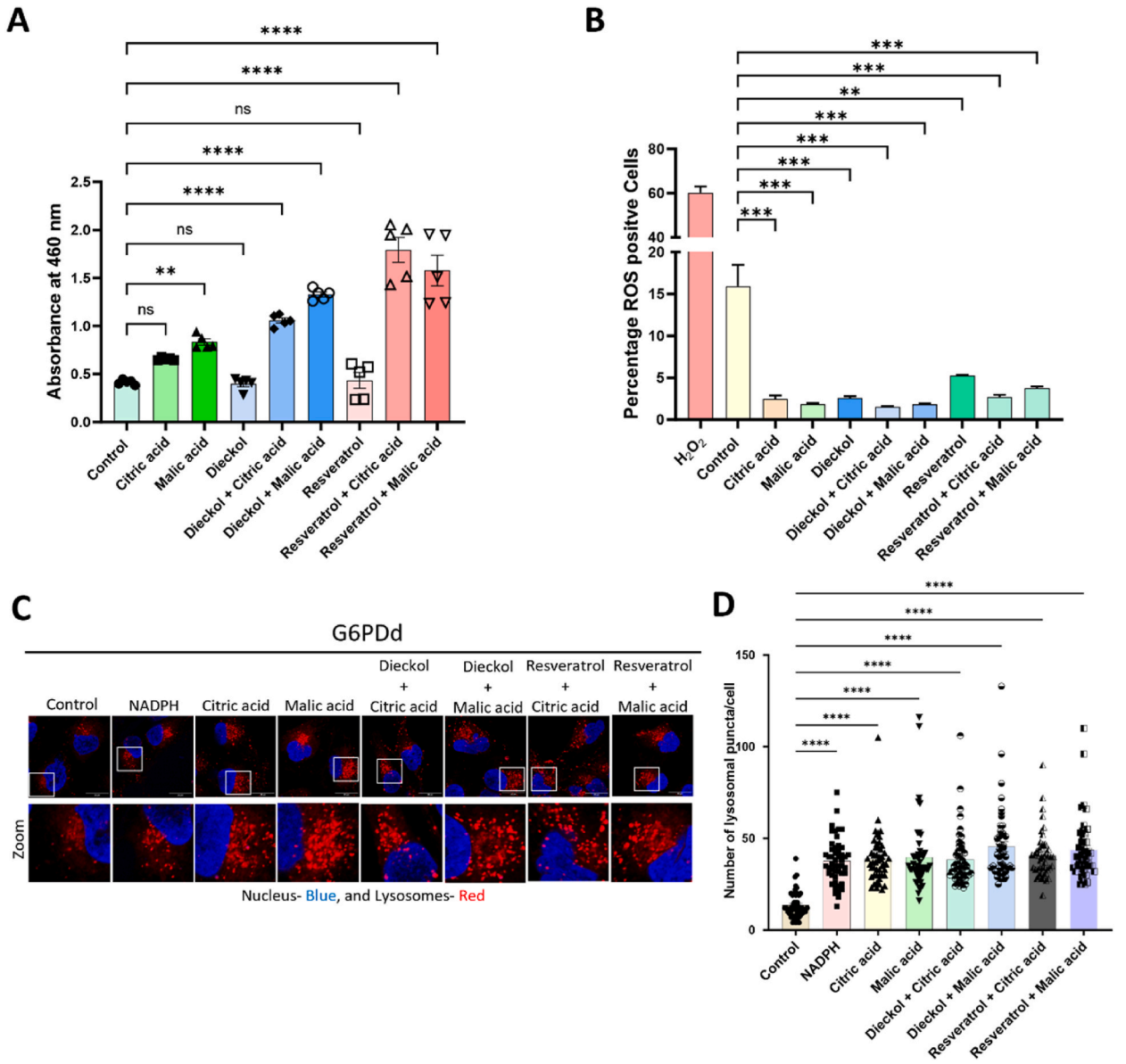

Fig. 4. Combination therapy reduces oxidative stress and increases the number of acidified lysosomes in G6PDd microglia: **A)** Bar plot represents absorbance data of WST-8 -NADPH estimation assay in G6PDd microglia treated with various experimental conditions. Data (n = 5) represented in mean ± SEM. Significance was calculated using one-way ANOVA (**P = 0.0069, ****P < 0.0001, ns = 0.23 and ns > 0.999); **B)** Bar plot representing a population of G6PDd microglia which are positive for ROS while treated with our therapeutics as compared to untreated control. Data represented here as mean ± SEM (n = 3). Statistical significance was calculated by one-way ANOVA (**P = 0.0019, ***P = 0.0003, ****P < 0.0001); **C)** The confocal microscopy images showing the number of acidified lysosomal vesicles in G6PDd microglia with and without treatment of our therapeutics. Corresponding zoom image exhibiting bright lysosomal puncta (red); **D)** Bar graph representing the number of acidified lysosomal puncta per G6PDd microglia (n = 50) in our different experimental conditions. Significance was calculated using one-way ANOVA (****P < 0.0001).

required to prove its efficacy in treating G6PD deficiency-related disorders.

In this study, we found that metabolic supplementation increased the production of NADPH and could reduce oxidative stress in the G6PD-deficient microglia. However, negative feedback mechanisms of metabolic pathways and differential expression of metabolic enzymes across the tissues could be the drawbacks of metabolic supplementation therapy. Here, targeting KEAP1-NRF2 by phytochemicals such as dieckol and resveratrol upregulated the expression of IDH1 and ME1 which may potentially eliminate the limitation associated with expressional heterogeneity. Dieckol and resveratrol were previously reported as potential antioxidants and are being tested clinically for various disorders including Alzheimer's [62–67]. Further, to increase the efficacy, we used a combination of therapeutic approaches to target the KEAP1-NRF2 pathway to increase the expression of IDH1 and ME1, followed by supplementation

of citric and malic acid for enhancing the production of NADPH in G6PD deficient condition. Our *in-vitro* study of combination nutraceuticals using human G6PD deficient microglia showed promising results on NADPH production, regulation of oxidative stress, and lysosomal acidification. However, animal experiments should be conducted to conclude further the efficacy of combination therapeutics in G6PD deficient conditions.

5. Limitations of the study

The negative feedback mechanism of metabolic pathways is the major limitation for developing therapeutics based on metabolites. Notably, we have increased the expression of alternate metabolic enzymes (IDH1 and ME1) by treatment of plant-based small molecule compounds such as dieckol and resveratrol. However, *in-vivo* validation of the efficiency of combination therapeutics is crucial for further applications.

CRediT authorship contribution statement

Abir Mondal: Writing – original draft, Visualization, Validation, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Soumyadeep Mukherjee:** Validation, Methodology. **Prince Upadhyay:** Validation, Methodology. **Isha Saxena:** Validation, Methodology. **Soumya Pati:** Writing – review & editing, Supervision, Project administration, Funding acquisition, Conceptualization. **Shailja Singh:** Writing – review & editing, Supervision, Resources, Project administration, Investigation, Funding acquisition, Conceptualization.

Data availability

Data will be made available on request.

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.

Acknowledgment

Ph.D. students AM and SM are grateful for their research fellowships to the Shiv Nadar Institute of Eminence (SNIoE), Delhi NCR, and the Shiv Nadar Foundation. Ph.D. student PU is grateful to the University Grant Commission (UGC) for the fellowship. Dr Soumya Pati is thankful to the Department of Science and Technology (DST/CSRI/2018/247). Prof. Shailja Singh is acknowledging Drug and Pharmaceuticals Research Programme (DPRP) (Project No. P/569/2016-1/TDT) and DST-SERB (Project No. CRG/2019/002231). We acknowledge the DST-FIST grant [SR/FST/LS-1/2017/59(c)] for the confocal microscopy facility at Shiv Nadar Institute of Eminence, Delhi NCR. We also acknowledge the Shiv Nadar Foundation Core Research grant.

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