European Review for Medical and Pharmacological Sciences 2023; 27: 5083-5096

Hydrogen peroxide signaling modulates neuronal differentiation *via* microglial polarization and Wnt/β-catenin pathway

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Abstract. – OBJECTIVE: Reactive oxygen species (ROS) are generated within the cell and serve as second messengers in fundamental cellular processes under physiologic conditions. Although the deleterious effects of high-level ROS associated with oxidative stress are well established, it is unclear how the developing brain reacts to redox changes. Our aim is to investigate how redox alteration affects neurogenesis and the mechanism that underlies it.

MATERIALS AND METHODS: We investigated in vivo microglial polarization and neurogenesis in zebrafish after hydrogen peroxide (H_2O_2) incubation. To quantify intracellular H_2O_2 levels *in vivo*, a transgenic zebrafish line that expresses Hyper and termed Tg(actb2:hyper3)ka8 was used. Then, *in vitro* studies with N9 microglial cells, 3-dimensional neural stem cell (NSC)-microglia coculture, and conditioned medium experiments are carried out to comprehend the mechanism underlying the changes in neurogenesis upon redox modulation.

RESULTS: In zebrafish, exposure to H_2O_2 altered embryonic neurogenesis, induced M1 polarization in microglia, and triggered the Wnt/β-catenin pathway. N9 microglial cell culture experiments revealed that exposure to H_2O_2 resulted in M1 polarization in microglial cells, and this polarization was mediated by the Wnt/β-catenin pathway. Redox modulation of microglia interfered with NSC differentiation in coculture experiments. Neuronal differentiation was significantly higher in NSCs cocultured with H_2O_2-treated microglia when compared to control microglia. Wnt inhibition prevented the effects of H_2O_2-treated microglia on NSCs. No significant alterations were observed in conditioned medium experiments.

CONCLUSIONS: Our findings point to a robust interplay between microglia and neural progenitors influenced by the redox state. Intracellular H_2O_2 levels can interfere with neurogenesis by altering the phenotypic state of the microglia *via* the Wnt/β-catenin system.

Key Words:
Neurogenesis, Hydrogen peroxide (H_2O_2), Reactive oxygen species (ROS), Redox signaling, Microglia polarization, Wnt/β-catenin, Newborn, Premature.

Introduction

H_2O_2 is a signaling molecule that is essential for the developing brain. Changes in intracellular H_2O_2 levels can impact how neural precursors and microglia communicate. A phenotypic transition is induced by H_2O_2 in microglia, which is controlled by the Wnt/β-catenin pathway and interferes with continuing neurogenesis. Brain development continues in preterm infants in the neonatal intensive care unit. Since supplemental oxygen is one of the most frequently used therapeutic agents for preterm infants and can

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potentially change intracellular H_2O_2 levels, this study may offer insight into clinical practices in neonatology.

Reactive oxygen species (ROS) are oxygen-derived molecules generally associated with oxidative damage. However, scholars[1] suggest that ROS are essential for propagating various cellular processes, including embryonic neurogenesis. Hydrogen peroxide (H_2O_2) is one of the most significant ROS molecules that regulates numerous cellular events *via* different post-translational modifications[2]. It is recognized as a critical modulator in the redox-dependent control of biological functions[3]. Evidence[4] suggests that H_2O_2 signaling is implicated in the embryonic period and brain development. Redox signaling is known to contribute to embryonic neurogenesis and neuronal maturation as well as the maintenance of neural stem cells (NSC)[5,6]. However, the mechanism behind the redox alterations and their effects on embryonic neurogenesis are not well studied.

Microglial cells are resident macrophages of the central nervous system, and beyond orchestrating the brain's innate immune response, they tightly control neurogenesis by regulating the differentiation of NSCs[7]. Through their constant surveillance of the developing brain and response to signals derived from the extracellular environment, they interact with neural progenitors and drive the maturation of neurons according to these signals[8]. Under different stimuli, microglia can switch from their quiescent state to an activation state with changes in the cellular phenotype and function. This characteristic feature of microglial cells is called polarization, and activated microglia can be polarized into M1 and M2 phenotypes[9]. M1 phenotype is characterized by the expression of specific proinflammatory cytokines, such as IL-1α, IL-1β, IL-6, IL-12, IL-23, and TNF-α, whereas M2 polarized microglia express anti-inflammatory cytokines (IL4, IL10, IL13, and TGF-β) as well as, arginase-1 (Arg1), CD206 and Fizz-1[10]. The alteration in the redox state of the brain tissue can be a triggering factor eliciting polarization states. However, there are few studies[11] regarding the role of redox modulation on microglial polarization, the underlying mechanism, and the ultimate consequences on neuronal differentiation.

Several molecular pathways are implicated in microglial polarization, one of which is Wnt/β-catenin signaling. It is a redox-dependent mechanism[12,13] that coordinates many cellular and physiological processes, including the proliferation and differentiation of NSCs[14]. Recent findings[15] suggest that the Wnt signaling pathway modulates microglial activation in neurodegenerative diseases. We hypothesize that Wnt/β-catenin signaling could have a role in microglial polarization during embryonic neurogenesis and could be a potential link between redox signaling, microglial polarization, and neurogenesis crosstalk. We have tested our hypothesis utilizing *in vivo* and *in vitro* models.

Materials and Methods

Fish Care

Regular care and maintenance of zebrafish (*Danio rerio*) were performed under standard conditions at Izmir Biomedicine and Genome Center (IBG) Zebrafish Facility. All experiments were performed in compliance with local ethics regulations and EU Directive 2006 and approved by IBG Animal Ethics Committee (2019/24). Water temperature was kept at 28°C, and animals were exposed to a 14 h light to 10 h dark cycle. The fish were fed with live artemia twice a day and flake food once a day. Fertilized eggs were obtained through pairwise breeding and, after collection, kept in embryo medium E3 (5 mM NaCl, 0.17 mM KCl, 0.33 mM $CaCl_2$, 0.33 mM $MgSO_4$) in an incubator at 28.5°C for 96 h during the experimental procedure[16].

H_2O_2 Detection with the HyPer Probe

HyPer is a protein specifically sensitive to H_2O_2 and allows for quantifying H_2O_2 concentration within a living cell. To quantify intracellular H_2O_2 levels *in vivo*, a transgenic zebrafish line that expresses Hyper and termed Tg(actb2:hyper3)ka8 was used[17,18]. Transgenic embryos were purchased from the European Zebrafish Resource Center (EZRC; Eggenstein-Leopoldshafen, Germany) and raised at IBG Zebrafish Core Facility.

Determination of Optimal H_2O_2 Concentration For In Vivo Experiments

Zebrafish larvae were previously reported to survive for 12h without morphological abnormalities at 3 mM H_2O_2 or less[19,20]. We selected a lower concentration (1 mM) to maintain optimal viability for 24h incubation. This concentration was also chosen as a working concentration previously by other authors utilizing Tg(actb2:hyper3)ka8 line[17].

In Vivo Imaging

Tg(actb2:hyper3)ka8 larvae were anesthetized with 0.04% Tricaine in E3 and mounted in 0.8% low melting agarose (Sigma-Aldrich, St. Louis, MO, USA). Live imaging was performed 20 min after incubation of 72 hpf transgenic larvae either in 1 mM H_2O_2 or a control medium under confocal microscopy. For each group, five larvae were visualized. Images were acquired at IBG Optic Imaging Facility using a Zeiss LSM880 confocal microscope (Carl Zeiss AG, Jena, Germany). HyPer fluorescence was excited with 501/16 and 420/40 bandpass excitation filters, and YFP emission was acquired using a 530/35 bandpass emission filter. HyPer ratios were calculated by division of smoothed and background-subtracted images of YFP500 and YFP420[17,18]. Image analysis was performed with the ImageJ program[21]. The total intensity was measured in three similarly positioned ROIs in every image, and an average of five images was determined in each experimental group.

Cell Culture and Treatment

N9 microglial cell line

N9 mouse microglial cell line developed by Dr. Paola Ricciardi-Castagnoli was cultured in Roswell Park Memorial Institute-1640 (RPMI-1640) supplemented with 2 mM Glutamax, 10% fetal bovine serum (FBS), 100 U/mL penicillin, and 100 µg/mL streptomycin. Cell culture operations were performed within the laminar airflow cabinet (Thermo Fisher Scientific, Waltham, MA, USA), and the cells were cultured at 37°C in the incubator (Thermo Fisher Scientific, Waltham, MA, USA) containing 5% CO_2. Microglial cells were treated with 5 µM H_2O_2 (H1009, Sigma-Aldrich, St. Louis, MO, USA) for 6 h. Inhibition of Wnt/β-catenin signaling was performed with 10 µM IWR-1 (I0161, Sigma-Aldrich, St. Louis, MO, USA) incubation for 24 h.

CGR8 embryonic stem cell lines

The murine embryonic stem cell line CGR8 was gifted by Prof. Marcel Leist (University of Konstanz, Germany). They were maintained in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% FBS, 2 mM Glutamax, 2 mM sodium pyruvate, 100 mM MEM nonessential amino acids, and 50 µM β-mercaptoethanol. The medium was changed daily with freshly with 1,000 U/mL murine leukemia inhibitory factor (mLIF) and 3i (2 µM SU5402, 800 nM PD184352, and 3 µM CHIR99021). CGR8 cells were passaged once in two days, cultured on 0.2% gelatin-coated T25 sterile flasks, and incubated at 37°C in a humidified 5% CO_2 incubator.

Differentiation of CGR8 cells

Differentiation of CGR8 cells was performed according to a previously published study[22]. Briefly, CGR8 cells were harvested with 0.05% trypsin when they reached 80% confluency, $5x10^6$ cells in CGR8 medium with LIF were replated on 0.2% gelatin-coated T25 flask and incubated for 24 h in a humidified 5% CO_2 incubator at 37°C. The following day, 1.2 x 10^6 cells/cm^2 were plated on gelatin-coated dishes in N2B27 medium containing equal amounts of DMEM-F12 and Neurobasal-medium, with N2 and B27 supplements, Glutamax, 100 µM β-mercaptoethanol, 7.5 µg/mL insulin, 50 µg/mL bovine serum albumin (BSA), PenStrep; and incubated seven days to obtain NSCs. During seven days of differentiation, cell culture media were replaced on the second, fourth, and sixth days. At the end of differentiation, NSCs were detached with trypsinization and filtered through a 70 µm cell strainer to obtain single cells.

Coculture of NSCs with N9 Microglia in Three-Dimensional Fibrin Hydrogels

The Fibrinogen solution was prepared by dissolving plasminogen-free fibrinogen from bovine plasma containing factor XIII (Sigma-Aldrich, St. Louis, MO, USA) in sterile 1X TBS, and the fibrinogen solution was filtered. The fibrinogen solution's concentration was determined by UV spectroscopy at 280 nm wavelength and diluted to 10 mg/mL with sterile 1X TBS. To obtain proper polymerization of fibrin solution, 2 U/mL thrombin (Sigma-Aldrich, St. Louis, MO, USA), 25 ***µg***/mL aprotinin (Sigma-Aldrich, St. Louis, MO, USA), and 2.5 mM $CaCl_2$ in TBS were mixed to obtain fibrin gel. A basement of 100 µL of fibrin gel solution was polymerized in wells of a 24-well plate for 1 h at 37°C in a humidified 5% CO_2 incubator.

N9 microglial cells were treated with 5 µM H_2O_2 for 6h once; polarized cells were harvested with fresh medium and utilized immediately for the coculture procedure. Control microglia and H_2O_2-treated N9 cells ($2x10^5$ cells/well) were cocultured with NSCs ($8x10^5$ cells/well), suspended within fibrin gel, and immediately transferred to the wells. The mixture of NSC, N9 (4:1), and fibrin gel was incubated at 37°C in a humidified 5% CO_2 incubator for 1 h to

polymerize. After polymerization, N2B27 cell culture media was added to the wells (1 mL/well). The gel was cultured for 12 days by refreshing the culture media daily with 5 µg/mL aprotinin to prevent the early degradation of the fibrin gel construct.

Conditioned-Medium Experiment

To investigate the effect of the conditioned medium on neurogenesis, N9 cells were treated with 5 µM H_2O_2 for 6 h. The medium was discarded, and then polarized cells were incubated with N2B27 medium for 24 h. Finally, NSCs were treated with this conditioned medium in 3D culture for 12 days.

PI Staining for Cytotoxicity Detection

N9 microglial cells growth on the 48-well plaque was incubated with 1 µM, 5 µM, and 50 µM H_2O_2 (H1009, Sigma-Aldrich, St. Louis, MO, USA) for 24 h and later incubated with propidium iodide (PI) stain for 15 min. Then, stained cells were detected with a fluorescent microscope. PI-positive cells were counted *via* the ImageJ program through the photos obtained from each well. The percentage of cytotoxicity was calculated by the number of PI-positive cells compared to the total number of cells.

Real-time PCR Analysis of mRNAs

For *in vivo* experiments, larval heads were transected above the yolk sac, and brain tissues were collected. Pools of 40 or more larval heads for each sample were chilled on ice and sonicated. In both *in vivo* and *in vitro* experiments, total RNA isolation was achieved by the Nucleospin RNA isolation kit (Macherey-Nagel, Germany). The purity and concentration of the collected RNA samples were evaluated by spectrophotometric measurement. High-Capacity cDNA Reverse Transcription Kit (Thermo Scientific Scientific, Waltham, MA, USA) was used for complemental DNA (cDNA) synthesis. After obtaining cDNA, ABI 7500 Fast (Applied Biosystems, Waltham, MA, USA) quantitative PCR device was used with GoTaq® qPCR Master Mix (Promega, Madison, WI, USA) according to the manufacturer's instructions. GAPDH has been used as a housekeeping gene. Gene expression changes were calculated quantitatively with the ΔΔCt method using the Ct values obtained for each sample[23]. The primers used in the qPCR reactions are listed in Table I.

Table I. Primer sequences.

TNF-α	F	TCACAGACGAATGACTCCAA
	R	GTGCCACTTCATACCAGGAGAA
IL-1β	F	CACAGCAGCACATCAACAAG
	R	GTGCTCATGTCCTCATCCTG
IL-6	F	GTGGTATCCTCTGTGAAGTCT
	R	AAGAGCTTCCAGCCAGTTGCC
Arg1	F	CAGAAGAATGGAAGAGTC
	R	CAGATATGCAGGGAGTCA
CD206	F	TGTGGTGAGCTGAAAGGTGA
	R	CAGGTGTGGGCTCAGGTAGT
Fizz-1	F	AGACTTGCGTGACTATGAAGCATTG
	R	GGCCCATCTGTTCATAGTCTTGA
MAP2	F	GCCAGCCTCGGAACAAACA
	R	GCTCAGCGAATGAGGAAGGA
βIII-tubulin	F	TAGACCCCAGCGGCAACTAT
	R	GTTCCAGGTTCCAAGTCCACC
GFAP	F	GCCCGGCTCGAGGTCGAG
	R	GTCTATACGCAGCCAGGTTGTTCTCT
Olig1	F	TCTTCCACCGCATCCCTTCT
	R	CCGAGTAGGGTAGGATAACTTCG
Sox2	F	GCGGAGTGGAAACTTTTGTCC
	R	CGGGAAGCGTGTACTTATCCTT
GAPDH	F	ACCACAGTCCATGCCATCAC
	R	TCCACCCTGTTGCTGTA
Rpl13a	F	TCTGGAGGACTGTAAGAGGTATGC
	R	AGACGCACAATCTTGAGAGCAG
Huc	F	AGA CAA GAT CAC AGG CCA GAG CTT
	R	TGG TCT GCA GTT TGA GAC CGT TGA

TNF-α: Tumor necrosis factor α; IL-1β: Interleukin 1β; IL-6: Interleukin 6; Arg1: Arginase 1; Fizz-1: Found in inflammatory zone 1; MAP2: Microtubule associated protein 2; GFAP: Glial fibrillary acidic protein; Olig1: Oligodendrocyte transcription factor 1; GAPDH: Glyceraldehyde-3-phosphate dehydrogenase; Rpl13a: Ribosomal protein l13a.

Immunofluorescent Staining

For immunofluorescence (IF) imaging, NSC-N9 coculture was fixed with 4% paraformaldehyde for 30 min, followed by washing with PBS for 5 min twice. Next, permeabilization was performed with 0.2% Triton-X-100 for 45 min at room temperature (RT). After washing twice, blocking was achieved with 2% donkey serum blocking solution for 30 min at RT. Cells were stained overnight with Neun antibody at +4°C and with Alexa Fluor-488 conjugated secondary antibody for 2h at RT. Images were obtained *via* fluorescence microscopy at 40 X magnification (Olympus IX-71, Tokyo, Japan).

Western Blot Analysis

For *in vivo* experiments, larval heads were transected above the yolk sac, and brain tissues were collected. Pools of 120 larval heads for each sample were chilled on ice and sonicated. RIPA solution buffer (50 mM Tris-HCl, pH 7.4, 150

mM NaCl, 0.25% deoxycholic acid, 1% Nonidet P-40, 1 mM EDTA), including protease inhibitor (Thermo Scientific Scientific, Waltham, MA, USA) was used for total protein isolation.

For *in vitro* experiments, the cells were kept on ice for 30 min, and then, RIPA solution buffer was applied. The protein was obtained by centrifugation of samples for 15 min at 12000 rpm; the concentration was determined by the BCA protein determination assay. Equal amounts of protein samples were separated with 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a polyvinylidene difluoride (PVDF) membrane. Membranes were blocked by 5% non-fat dry milk or BSA in PBS-T (0.2% Tween-20 in 1X PBS, pH 7.2) for 1h and probed overnight at 4ºC with specific primary antibodies (Table II). The following day, membranes were incubated with Horseradish peroxidase (HRP) conjugated secondary antibody for 1h, and washing was performed. Protein bands were detected by chemiluminescence system (Thermo Fisher Scientific, MA, USA) with Supersignal West Pico ECL reagent (Thermo Fisher Scientific, MA, USA). Protein band density was analyzed using the Image Studio Lite software, version 3.1 (LI-COR Biosciences, Lincoln, NE, USA). Protein band levels were normalized to the evolutionarily conserved β-actin protein.

Statistical Analysis

Data were analyzed by SPSS version 22.0 (IBM Corp., Armonk, NY, USA). Values were presented as mean ± SD. Since the data were inconsistent with normal distribution and due to the small sample size, non-parametric tests were applied. Mann-Whitney U-test was used to compare the difference between two groups, and the Kruskal Wallis test with Dunn's correction was used to determine the difference between three or more independent groups. $p<0.05$ was considered significant.

Results

Exposure To H_2O_2 Elicits M1 Polarization in Microglia, Alters Embryonic Neurogenesis, and Activates the Wnt/β-Catenin Pathway in Zebrafish

To quantify intracellular levels with exogenous H_2O_2, live imaging was performed 20 min after incubation of 72 hpf Tg(actb2:hyper3)ka8 zebrafish larvae in 1 mM H_2O_2 under confocal microscopy. The Hyper signal from the brain area was significantly increased with 1 mM H_2O_2 compared to the control (Figure 1A, B).

Then, wild-type zebrafish larvae were incubated in 1 mM of H_2O_2 through 72-96 hpf. Larval heads were transected above the yolk sac, and brain tissues were investigated for microglial polarization and neurogenesis by qPCR at 96 hpf. mRNA level of M1 polarization marker TNF-α was higher in the H_2O_2 group when compared to the control (Figure 1C). Likewise, the mRNA level of neuronal marker Huc was increased upon incubation in 1 mM of H_2O_2 compared to the control (Figure 1D).

We also investigated whether H_2O_2 treatment results in the activation of the Wnt/β-catenin pathway in zebrafish. Western blot analysis revealed decreased Axin 1 in the H_2O_2 group (Figure 1E, F).

Table II. Primary and secondary antibody list.

Antibody	Company	Catalog number	Application	Dilution
Wnt 5a/b	Cell Signalling	2530S	WB	1:1000
LRP6	Cell Signalling	3395S	WB	1:1000
Phospho-LRP6	Cell Signalling	2568S	WB	1:1000
Dvl2	Cell Signalling	3224S	WB	1:1000
Dvl3	Cell Signalling	3218S	WB	1:1000
Axin1	Cell Signalling	2087S	WB	1:1000
β-catenin	Santa Cruz	SC-59737	WB	1:500
β-actin	Cell Signaling	4970S	WB	1:1000
Anti-Rabbit-HRP	Cell Signaling	7074S	WB	1:2000
Neun	Proteintech	26975-1-AP	IF	1:100
Anti-Rabbit IgG, Alexa Fluor 488	Thermo Scientific	A21206	IF	1:1000

LRP6: Low-density lipoprotein receptor-related protein 6; Dvl: Dishevelled; HRP: Horseradish peroxidase; Neun: Neuronal nuclei. WB: Western Blot; IF: Immunofluorescence.

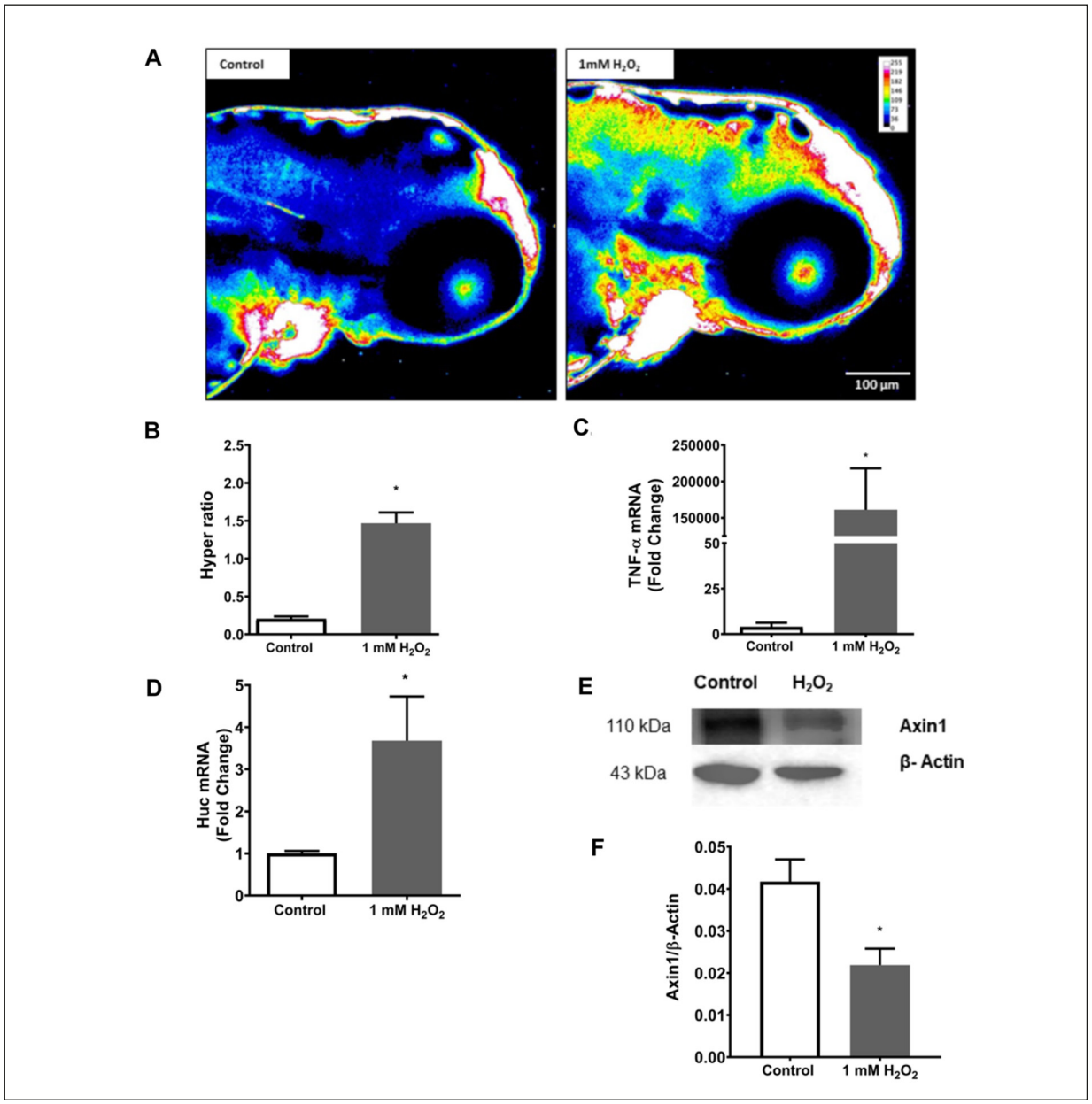

Figure 1. *In vivo* effects of H_2O_2 exposure on microglial polarization, neurogenesis, and the Wnt/β-catenin pathway. Live imaging 20 min after incubation of 72 hpf Tg(actb2:hyper3)ka8 zebrafish larvae in 1 mM of H_2O_2, under confocal microscopy Zeiss LSM 880 (20×) and respective Hyper ratios (**A, B**). Wild-type zebrafish larvae were incubated in 1 mM H_2O_2 through 72-96 hpf. mRNA levels of M1 polarization marker TNF-α (**C**) and neuronal marker Huc (**D**) were detected by qPCR. Axin 1 levels detected by Western blot analysis (**E, F**). The results are mean ± SEM of three independent experiments, n = 5. **p*<0.05 compared with untreated control.

Exposure To H_2O_2 Leads to M1 Polarization in Microglial Cells

The highest non-toxic concentration of H_2O_2 was determined by PI staining to determine whether redox signaling affected microglia polarization in cell culture conditions. Microglial cells are incubated with various doses (1-100 µM) of H_2O_2; the number of PI-positive cells was 16% with 1 µM H_2O_2, 19% with 5 µM H_2O_2, whereas approximately 100% with 50 µM H_2O_2 (Figure 2A, B).

qPCR revealed that mRNA expression of M1 polarization markers, i.e., IL-6, TNF-α, and IL-1β were significantly increased upon incubation of N9 microglia with 1 and 5 µM H_2O_2 for 6h. The most notable difference was detected with 5 µM H_2O_2; therefore, this dose was chosen for

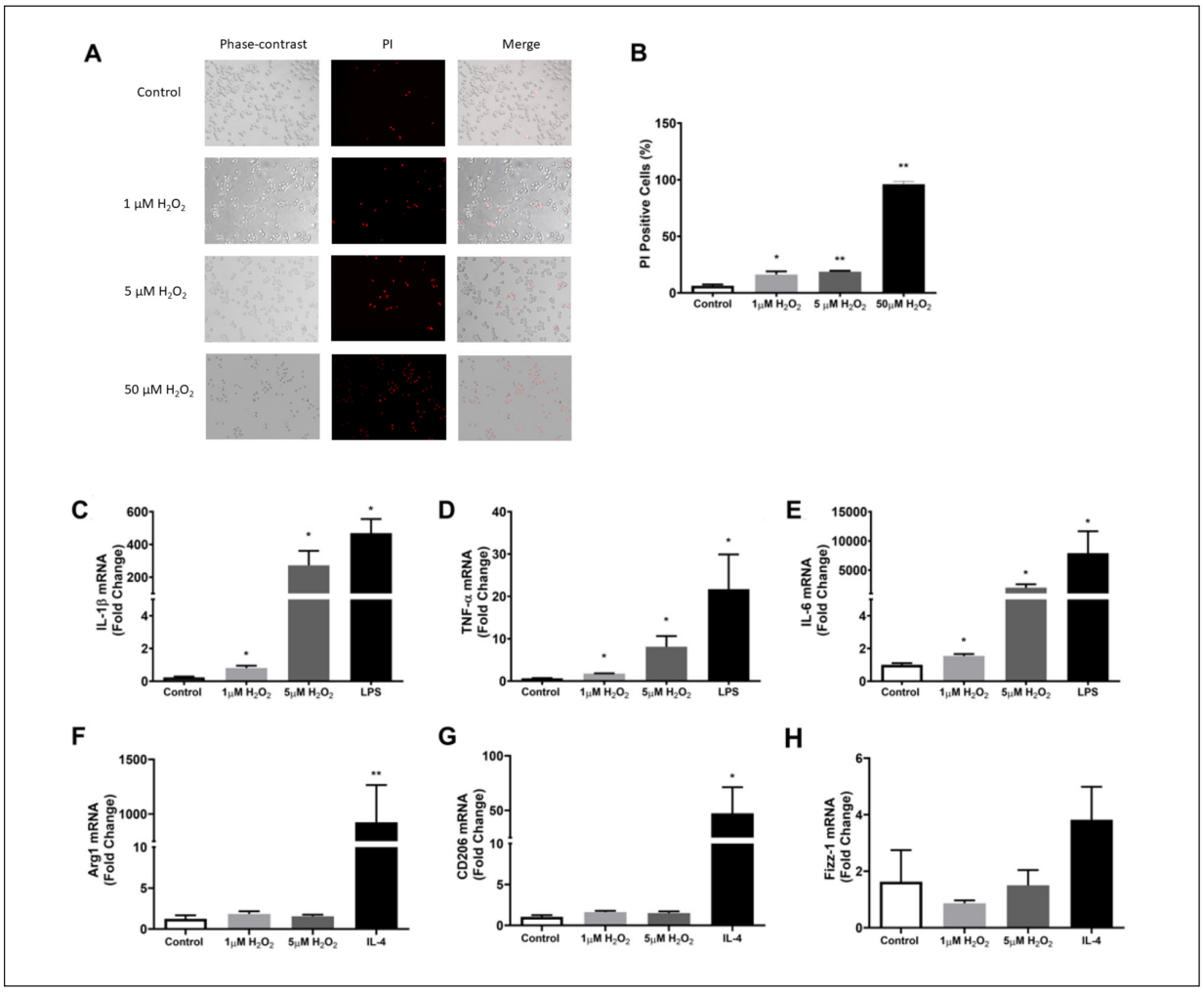

Figure 2. Effects of H_2O_2 exposure on microglia polarization. N9 microglial cells were treated with 1 μM, 5 μM, and 50 μM H_2O_2 for 6 h. Usable non-toxic concentrations were determined by PI staining (**A, B**). mRNA levels of M1 polarization markers upon 1 μM and 5 μM H_2O_2 treatment were detected by qPCR (**C-E**). LPS was used as a positive control for M1 polarization. mRNA levels of M2 polarization markers upon treatment with 1 μM and 5 μM H_2O_2 were detected by qPCR (**F-H**). IL-4 was used as a positive control for M2 polarization. The results are mean ± SEM of three independent experiments, n = 5. *p<0.05 and **p<0.01 compared with untreated control.

the rest of the experiments. LPS was used as a positive control for M1 polarization (Figure 2C-E). In addition, mRNA expression levels of M2 polarization markers, i.e., Arg1, CD206, and Fizz-1, were checked to determine whether the determined concentration of H_2O_2 has a role in M2 polarization. No significant changes were observed for M2 polarization (Figure 2F-G-H).

M1 Polarization of Microglial Cells by H2O2 Exposure Occurs Through the Wnt/β-Catenin Pathway

To examine the impact of the Wnt/β-catenin pathway in M1 polarization of N9 microglia in response to H_2O_2, protein levels of Wnt5a/b, Phospho-LRP6, LRP6, Dvl2, Dvl3, Axin1, and β-catenin were determined by Western blot method. Wnt5a/b, phospho-LRP6, LRP6, Dvl-3, and β-catenin protein levels were significantly increased, while Axin1 amounts were significantly decreased in the H_2O_2 treated cells. (Figure 3A, B).

In order to elucidate the interaction between the Wnt pathway and H_2O_2 mediated M1 polarization, N9 cells were incubated with a Wnt inhibitor (10 μM IWR-1) for 24 h and then treated with 5 μM H_2O_2. mRNA expression levels of IL-6, TNF-α, and IL-1β were significantly lower in IWR-1+H_2O_2 treated cells when compared with cells treated only with H_2O_2 (Figure 3C-E).

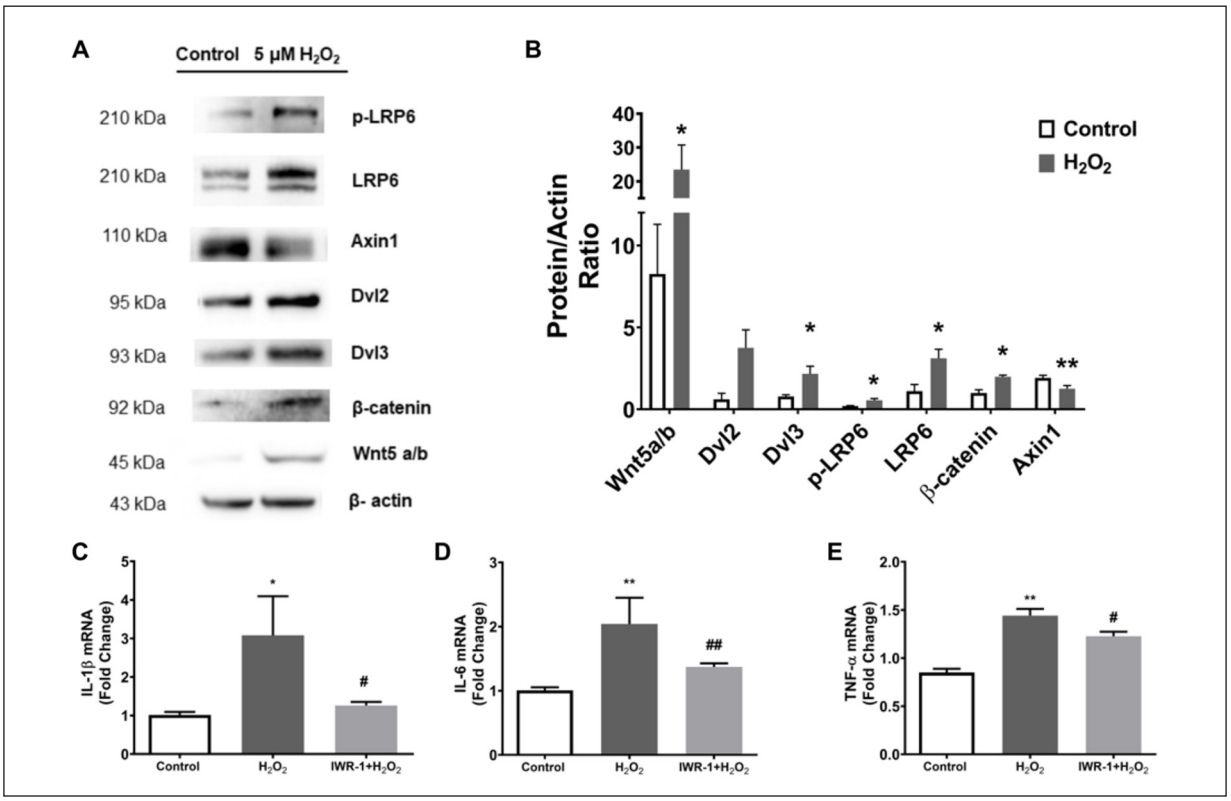

Figure 3. The role of the Wnt/β-catenin pathway in microglia polarization through H_2O_2 exposure. N9 microglial cells were treated with 5 μM H_2O_2 for 6 h. Wnt/β-catenin pathway protein levels were detected by Western blot analysis (**A, B**). Wnt/β-catenin pathway was inhibited by 10 μM IWR-1 incubation for 24 h, microglia were then treated with 5 μM H_2O_2 for 6 h. M1 polarization markers were assessed with qPCR (**C-E**). The results are mean ± SEM of three independent experiments, n = 5. *p<0.05 and **p<0.01 compared with untreated control. $^{\#}p$<0.05 and $^{\#\#}p$<0.01 compared with H_2O_2 treatment.

Redox Modulation of Microglia Interferes with NSC Differentiation on mRNA Level in 3D Culture in Vitro

To investigate the effects of H_2O_2 treated N9 microglia on neurogenesis, NSCs were cocultured with either control microglia or 5 μM H_2O_2 treated N9 microglia within a 3D fibrin hydrogel matrix for 14 days (Figure 4A). mRNA expression levels of neurons, stem cells, astrocytes, and oligodendrocyte markers were investigated *via* qPCR. Upon coculture of NSCs with H_2O_2-treated microglia, the mRNA levels of immature neuronal marker bIII-tubulin and mature neuronal marker MAP2 were significantly increased compared to NSCs cocultured with control microglia. However, no significant alterations were observed in mRNA levels of stem cell, oligodendrocyte, and astrocyte markers; (i.e., Sox2, Olig1, and GFAP, respectively) (Figure 4B). We also performed conditioned media experiments to determine whether H_2O_2 treated microglia exert effects on NSC *via* secreted molecules. NSCs were incubated with the conditioned medium, and no significant alterations were observed regarding the mRNA expression of neural and glial cell markers (Figure 4C).

To determine whether the effects of H_2O_2 treated microglia on NSCs rely on Wnt signaling, N9 cells were incubated with Wnt inhibitor (10 μM IWR-1) for 24 h and then treated with 5 μM H_2O_2. The fluorescence intensity of neuron marker Neun was significantly higher in NSCs cocultured with H_2O_2 treated microglia when compared to control microglia. However, Wnt inhibitor treatment prevented this response (Figure 4D, E). Additionally, NSCs cocultured with Wnt inhibitor-treated microglia displayed neurite outgrowth differences compared to H_2O_2 treatment. The average neurite length (mm) per cell and the average number of neurites per cell were increased in NSCs cocultured with H_2O_2, whereas the Wnt inhibitor abolished this effect (Figure 4F, G).

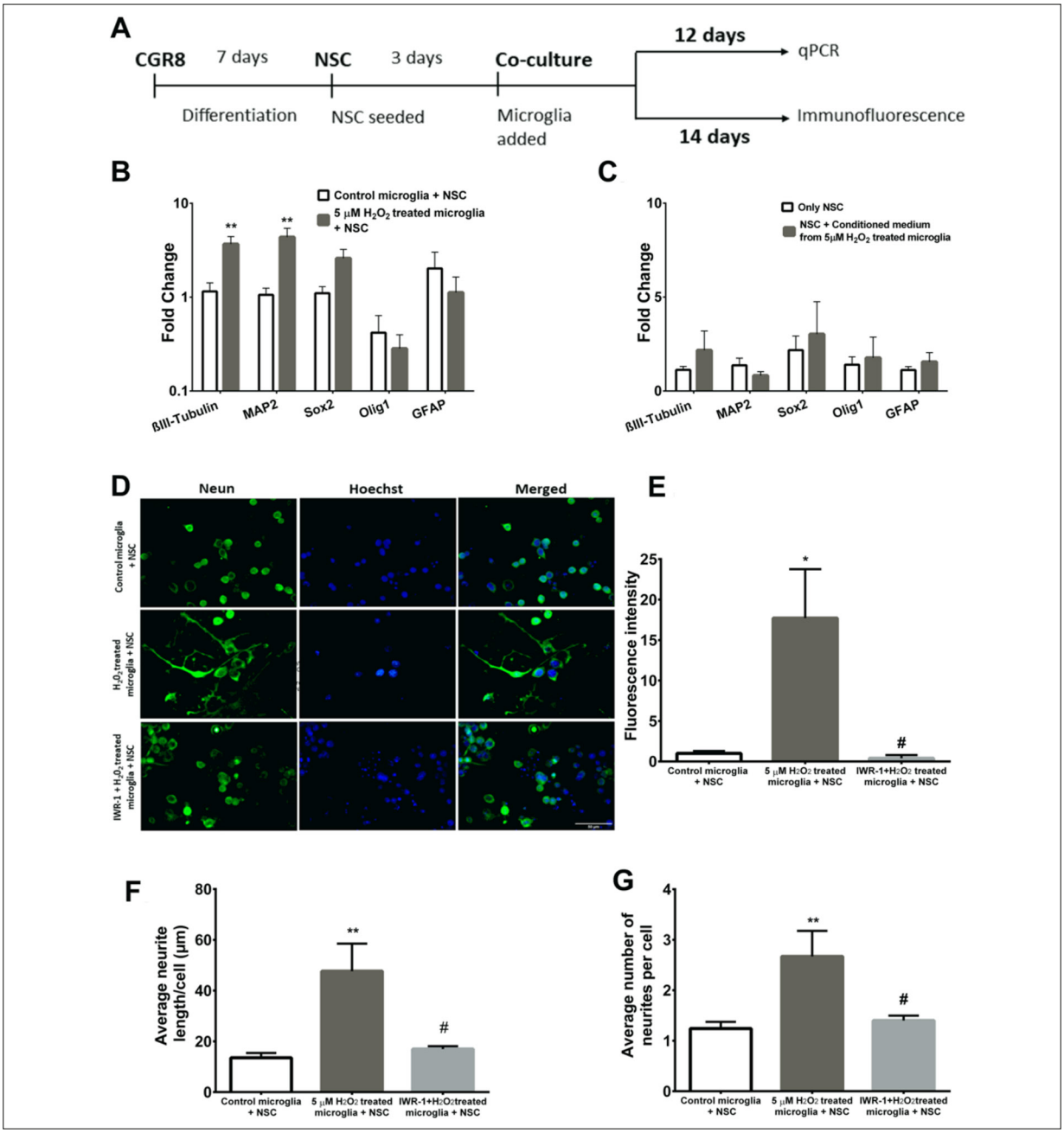

Figure 4. Effects of microglia on NSCs within 3D fibrin hydrogel. Workflow of 3D culture from the differentiation of CGR8 embryonic stem cells to NSCs, NSC-microglia coculture, and investigation of the markers (**A**). Microglial cells were treated with 5 μM H_2O_2 and incubated with NSCs for 12 days. mRNA levels of neural and glial markers were assessed *via* qPCR (**B**). NSCs were incubated with a conditioned medium harvested from H_2O_2 treated microglia, and no significant alterations were observed in mRNA levels (**C**). The wnt/β-catenin pathway was inhibited by 10 μM IWR-1, followed by 5 μM H_2O_2 treatment, and then microglia were incubated with NSCs for 14 days. Neun levels were determined by IF staining *via* fluorescence microscopy (Olympus IX-71, 40×) (**D, E**). Neurite outgrowth analysis of NSCs upon microglia coculture (**F**, **G**). The results are mean ± SEM of three independent experiments, n = 5. *p<0.05 and **p<0.01 compared with untreated control. $^{\#}p$<0.05 and $^{\#\#}p$<0.01 compared with H_2O_2 treatment.

Discussion

There is growing evidence that physiologic levels of ROS are essential for successful neurogenesis and neural differentiation, while low levels or overload of ROS impair brain development. H_2O_2 is one of the non-radical species and a significant ROS compound[24]. Moreover, the fate

of NSC is affected by intracellular redox homeostasis, and H_2O_2 is thought to have a pivotal role in the differentiation of NSCs into neurons[25,26]. In the present study, we aimed to investigate the impact of H_2O_2 signaling on microglial polarization and neuronal differentiation and the molecular mechanism behind it.

Here, in the first part of the present study, we explored the consequences of redox modulation on neurogenesis in an *in vivo* model. Our results imply that exposure of zebrafish larvae to 1 mM of H_2O_2 during 72-96 hpf promoted neurogenesis. Previously, a transient increase in H_2O_2 has been reported in zebrafish embryos during the first days of life, and depletion of H_2O_2 resulted in impairment of brain development[27]. According to Gauron et al[17], H_2O_2 levels are highest during organogenesis in zebrafish embryos, whereas mature tissues display gradually decreased levels. Short-term exposure to H_2O_2 during oocyte maturation was also shown to improve embryonic development[28]. Accumulating evidence indicates that intrinsic ROS functions as an intracellular signaling molecule in the cell, especially for cell cycle-related events[29]. The concentration of ROS in the cell has been implicated in determining the balance between quiescence, self-renewal, and differentiation states of the stem cells, and the primary stimulus for differentiation during the embryonic stage is thought to be the primary stimulus for differentiation during the embryonic stage increased amount of ROS in stem cells[30].

Generation of neurons from NSCs involves symmetric and asymmetric cell division of neural precursors, which is tightly controlled by microglia[31]. Microglial cells are engaged in an intense interplay between neurons during embryogenesis and support the survival and differentiation of neuronal progenitors by secreting a variety of neurotrophic factors[32,33]. In response to environmental cues, microglial cells display different phenotypes with diverse behavior. In the adult brain, M1 is the classical activation that exerts proinflammatory responses, whereas the M2 phenotype mediates resolution and repair[34,35].

In the present study, we found that exposure of zebrafish larvae to 1 mM of H_2O_2 during 72-96 hpf resulted in an increase in TNF-α (i.e., M1 polarization marker) in the brain tissue. Our *in vitro* experiments also are along with these results, i.e., exposure to 5 μM H_2O_2 resulted in the polarization of N9 microglial cells towards the M1 phenotype.

According to Vay et al[36], regardless of their polarization, microglia accelerate differentiation of NSCs; however, with differential effects, neurogenesis is supported by M2 phenotype while M1 microglia results in differentiation through astrocytes. Contrary to their results, in the present study, neural differentiation seems to be enhanced by H_2O_2 treated microglial cells, which display the M1 phenotype.

M1 microglia are designated as proinflammatory and generally known to cause neurogenesis failure in adults. However, categorizing the microglial cells may not be that much simple. According to Crain et al[37], P3 mouse brain microglia express increased levels of both M1 and M2 genes compared with adult microglia, and expression of M1 genes is higher at P3 compared to P21. In addition, the authors indicate that TNF-α was highly expressed at P3 but significantly lower between P21 and 4 months of age. Besides their well-established role in neuroinflammation, the expression of M1 genes during early central nervous system development may suggest a need for their vital roles in embryonic neurogenesis.

According to Takeda et al[38], incubating microglial cells with 1-100 μM H_2O_2 enhanced the ruffling process of the lamellipodia and formation of phagosomes in a concentration-dependent fashion, and microglial cells are transformed into a typical phagocytotic form of ameboid microglia. Authors underscore free radicals in the tissue as critical substances in manipulating microglia into various forms[38]. According to another study[39], 15-50 μM bolus dose of H_2O_2 elicited M2 polarization at 24h with CD206 and Arg1 positivity. However, authors also report positivity for M1 markers at 4h along with M2 markers. Moreover, increased expression of proinflammatory cytokines was reported upon continuous exposure to 1-5 μM H_2O_2, whereas anti-inflammatory cytokine levels did not differ significantly. Interestingly, exposure to sustained low levels of H_2O_2 resulted in higher LDH release when compared to bolus doses. Authors interpret these results as pleiotropic effects of H_2O_2 exposure based on the mode of application. They suggest that low but sustained levels of H_2O_2 elicit a preconditioning mechanism priming microglia for future insults by eliminating the M1 phenotype and promoting the M2 phenotype. There is not much information in the literature about how exposure to H_2O_2 affects microglial cells, and inconsistent findings may

be related to the origin of the microglial cells as well as variations in the dose, length, and method of exposure.

During the embryonic period, especially between the second and third trimester, brain tissue is heavily infiltrated with microglial cells, which can display phenotypic switches in response to environmental stimuli[40]. Microglia polarization is a dynamic process, and various molecular signals mediate the transition of the microglial cells through the M1 state. For example, in the presence of inflammation, phosphorylation of p38 mitogen-activated protein kinase (p38/MAPK) and extracellular signal-regulated kinases (ERKs) are the main pathways that trigger M1 phenotype in order to enhance immune functions, PI3K/Akt/mTOR pathway is implicated in M1 polarization in neurodegenerative disorders[41]. However, the mechanism underlying the shift towards the M1 phenotype through redox alteration during early brain development is unclear. Recently it was shown that H_2O_2 activates the Wnt/β-catenin signaling pathway[42]. Wnt/β-catenin signaling has an established role in regulating the asymmetric terminal division of neuronal progenitor cells and is one of the key players in orchestrating embryonic neurogenesis[43]. Thus, we have proposed that the Wnt/β-catenin pathway could have a role in the molecular machinery of M1 polarization under H_2O_2 exposure, and our results were in line with our hypothesis. Our *in vitro* results indicate that polarization of microglial cells upon H_2O_2 treatment depends mainly on the involvement of the Wnt/β-catenin signaling since the exposure of Wnt inhibitor-treated microglia to H_2O_2 failed to elicit the M1 phenotype. We have successfully demonstrated that Wnt5a/b, phospho-LRP6, LRP6, Dvl-3, and β-catenin protein levels were significantly increased while Axin1 amounts were significantly decreased in the H_2O_2 treated microglial cells. We have also shown decreased Axin 1 upon incubation with H_2O_2 in zebrafish. In addition, our *in vitro* results imply that cell-to-cell contact rather than a conditioned medium is required to promote neurogenesis *via* polarized microglia. Conditioned medium experiments also suggest that beyond the direct effects of H_2O_2 on NSCs, Wnt signaling acts on NSCs through the polarization of microglia. The function of Wnt/β-catenin signaling has been implicated in microglial polarization in neuroinflammatory processes in adults[15]. However, the present study, for the first time, reveals the vital role of this pathway during embryonic neurogenesis in response to redox modulation.

Limitations

This study has certain limitations; first of all, being an important vertebrate model for embryonic neurogenesis, zebrafish may not reflect *in utero* brain development in humans. The embryonic development of the zebrafish brain is relatively accelerated compared to the human fetus. The central nervous system development consists of a primary and a secondary phase in zebrafish. During the primary neurogenesis phase, which begins at 16 hpf, motor, sensory, and interneurons are generated within the spinal cord, hindbrain, midbrain, and forebrain creating a simple neural network, which is completed by 24 hpf. The secondary wave of neurogenesis occurs in zebrafish during the second to third days of life, contributing significantly to the growth of the larval brain[44,45]. Due to the rapid growth of the zebrafish model, we assessed the impacts of redox modulation 24 hours after the introduction of H_2O_2. On the other hand, we used cell lines for *in vitro* studies, where NSC differentiation needs to be monitored for about two weeks.

Cell lines constitute another considerable limitation. We used mouse N9 immortalized microglial cells in the present study. Despite the clear benefits of cell line cultures, such as their simplicity, uniformity, high yield, and affordability, extrapolating *in vitro* findings to *in vivo* results remains challenging[35]. Although N9 microglial cell lines display comparable cellular responses to primary microglia upon activation, there are still documented variations in gene and protein patterns[35]. In addition, our results need to be confirmed with human microglia. Finally, ROS molecules other than H_2O_2 are also generated due to oxidative stress. Other molecules, including superoxide and NO, contribute to the ROS burden. Nevertheless, this research may significantly advance our comprehension of the processes underlying embryonic neurogenesis and the critical function of H_2O_2.

Conclusions

In conclusion, our results suggest a strong interaction between neural progenitors and microglia, which can be affected by the changes in the redox state. H_2O_2 signaling contributes to embryonic neurogenesis and neuronal maturation, governed by microglial cells. There is an intricate interplay between cellular components and the extracellular environment during embryonic

neurogenesis, and even small changes in the redox state can interfere with this delicate balance through the phenotypic switch of the microglia and the Wnt/β-catenin pathway.

Conflict of Interest
The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Acknowledgements
Authors would like to thank Dr. Gulcin Cakan Akdogan for her technical support in zebrafish experiments.

Informed Consent
Not applicable.

Ethics Approval
All experiments were performed in compliance with local ethics regulations and EU Directive 2006 and approved by IBG Animal Ethics Committee (2019/24).

Authors' Contribution
Conceptualization, A.K., and Ş.G.; Methodology, D.E., S.C.M., I.E., C.K., S.S., and K.U.T.; Software, K.U.T.; Validation, A.K., and Ş.G.; Formal Analysis, D.E., S.C.M., I.E., C.K., and K.U.T.; Investigation, D.E., S.C.M., I.E., C.K., and S.S.; Resources, G.O., and S.G.; Data Curation, D.E., S.C.M., I.E., C.K., and S.S; Writing – Original Draft Preparation, D.E., I.E., and C.K., Writing – Review & Editing, Ş.G., K.U.T, G.O., and S.G.; Visualization, D.E., S.C.M., I.E., C.K., S.S., and K.U.T; Supervision, A.K., and Ş.G.; Project Administration, A.K. and Ş.G.; Funding Acquisition, Ş.G.

Funding
This study received financial support from The Scientific and Technological Research Council of Turkey (TUBITAK) (Project number: 118S406).

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Availability of Data and Materials
The Authors declare that they have no conflict of interests.

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