



In vitro effects of H₂O₂ on neural stem cell differentiation

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Abstract

The development of the CNS is a complex and well-regulated process, where stem cells differentiate into committed cells depending on the stimuli from the microenvironment. Alterations of oxygen levels were stated to be significant in terms of brain development and neurogenesis during embryonic development, as well as the adult neurogenesis. As a product of oxygen processing, hydrogen peroxide (H₂O₂) has been established as a key regulator, acting as a secondary messenger, of signal transduction and cellular biological functions. H₂O₂ is involved in survival, proliferation, and differentiation of neural stem cells into committed cells of the CNS. Effects of different concentrations of exogenous H₂O₂ on neuronal differentiation and the molecular pathways involved are yet to be clearly understood. Here, we investigated the concentration-dependent effects of H₂O₂ on differentiation of neural stem cells using CGR8 embryonic mouse stem cell line. We have demonstrated that treated doses of H₂O₂ suppress neural differentiation; additionally, our study suggests that relatively high doses of exogenous H₂O₂ suppress the differentiation process of neural stem cells through AKT and p38 pathways.

Keywords Oxidative stress · Neuron differentiation · Neural progenitor cells · Neurogenesis

Introduction

The development of the brain and the CNS (central nervous system) is an elaborated and well-regulated process (Imamura *et al.* 2008). In the early phases of the CNS of vertebrates, a neural tube is formed and then matures into a network containing neural stem cells (NSC) in the

ventricular layer (Bergstrom and Forsberg-Nilsson 2012). NSCs are multipotent cells that undergo self-renewal and also differentiate (Alipour *et al.* 2019). NSCs, incomparably to the rest of the CNS cells, are able to stay inactive for a long time and ensure a pool of cells throughout a lifetime in case of cell replacement within tissues (Andreotti *et al.* 2019). Starting with symmetric division of NSCs to form two daughter cells to provide extensive pool of NSCs required for the brain development, NSCs later perform asymmetric divisions to form a NSC and committed lineages of progenitors and cells consisting of neurons, glia, and astrocytes (Bergstrom and Forsberg-Nilsson 2012). Intermediate progenitor cells reside within the regions to provide source for differentiated cells, while immature neurons migrate and mature into adult neurons (Bergstrom and Forsberg-Nilsson 2012).

Neurogenesis is described as the maturation of committed cells from NSCs (Cavallucci *et al.* 2016). Neurogenesis takes place throughout life, giving rise to new cells within the niche and substitute for the cell loss within the adult CNS. On the other hand, neurogenesis within the adult CNS is relevant with numerous brain functions (Andreotti *et al.* 2019) including cognitive abilities (Bao and Song 2018). This process is known to be regulated by a variety of factors,

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one of which is environmental stress (Varela-Nallar *et al.* 2014). Additionally, proliferation and differentiation of NSCs are able to be triggered via exercise or learning processes (Zhang *et al.* 2011). Given that the deviant neurogenesis in adult brain plays a role in numerous CNS diseases, homeostasis of this significant process is crucial throughout life (Bao and Song 2018) and any inefficacy might lead to impaired learning and memory along with several other malformations within the brain (Bergstrom and Forsberg-Nilsson 2012).

Within the NSC niche, neurotransmitter, cytokine, and factor trafficking have significant roles in behavior and fate of NSCs (Andreotti *et al.* 2019). NSCs of different morphologies that respond to differential external stimuli are thought to coexist within the niche (Bao and Song 2018), where the fate of NSCs is delicately regulated by the discriminating microenvironment including cytokines, hormones, and growth factors and also extrinsically, stress and aging (Cavallucci *et al.* 2016).

The state of cellular redox is important in stem cell homeostasis (Kahroba *et al.* 2021); namely proliferation and differentiation of NSCs are considerably affected by the oxygen concentration (Ortega *et al.* 2017). Dramatic low or high levels are known to be related to impaired functions within the CNS (Ortega *et al.* 2017). When cellular redox balance is dysregulated, it causes oxidative stress within cells which conduces to activation of anti-oxidant pathways (Kahroba *et al.* 2021), and results in damage to cells, lipids, proteins, and DNA (Marx *et al.* 2021). It takes place when the cells are unable to detoxify accumulated reactive oxygen species (ROS), which is the main stressor in terms of oxidative stress (Kahroba *et al.* 2021). Cellular aberrant accumulation of ROS is known to be associated with cell death (Le Belle *et al.* 2011), mitochondrial dysfunction, and aberrant inflammation (Marx *et al.* 2021). Hydrogen peroxide (H₂O₂) is one of the compounds of ROS that is being produced under physiological conditions. The ROS generation process involves membrane-bound NADPH oxidase (NOX) enzymes, endoplasmic reticulum, and mitochondria; modulated levels that are non-toxic levels, categorized as low to moderate, are involved in numerous cellular processes as proliferation, differentiation, and survival conditions (Kahroba *et al.* 2021), whereas, as stated, higher doses of accumulated cellular ROS cause oxidative stress which in turn results in dysfunction of cellular processes and cell death (Kahroba *et al.* 2021). On the contrary of its deleterious effects, ROS has been linked to numerous signaling pathways that increases cell proliferation and survival including PI3K (phosphatidylinositol-3-kinase)/Akt and P38-MAPK (mitogen-activated protein kinase) pathways (Le Belle *et al.* 2011). Also, differentiation capacity of NSCs has been shown to be enhanced upon H₂O₂ treatment (Le Belle *et al.* 2011).

Protein kinases are critical modulators that regulate intracellular and intercellular signal transduction pathways. PI3K/Akt and P38-MAPK are pathways known to be involved in intracellular signaling involved in neuronal regeneration and survival (Liu *et al.* 2020). MAPKs are intracellular enzymes that can be modulated by H₂O₂ and regulate cellular functions by providing phosphorylation of certain proteins (Ferriero 2001). Latest studies have shown that p38 MAPK, associated with the reorganization of neural plasticity and stress responses, acts as a negative organizer of NSC proliferation during early brain development (Kim and Wong 2009). PI3-kinase and the serine/threonine kinase Akt/PKB, which is the one of its main downstream effectors, are known to play a noteworthy role in neuronal survival (Crossthwaite *et al.* 2002). The Akt signaling pathway is also known to participate in the differentiation and proliferation of NSCs. It has been shown in different studies that Akt promotes various cellular functions for instance cell proliferation, transcription regulation, metabolism, and cell survival. Akt has been stated to play a crucial role in inhibiting apoptosis and promoting cell survival. Akt activation possibly is mediated by protein regulatory factors, namely growth factors, cytokines, mitogens, and hormones, or it is possible to be dependent or independent of the PI3K pathway (Liu *et al.* 2020).

This study aims to investigate the effects of redox modulation on differentiation of NSCs and the mechanism behind. The response of NSCs obtained from CGR8 embryonic stem cell line upon incubation with various concentrations of exogenous H₂O₂ and the effects of H₂O₂ on the activation of P38 and AKT signaling pathways were studied.

Materials and methods

Cell culture and treatments In this study, CGR8 mouse embryonic stem cells were used. CGR8 mouse embryonic stem cells were purchased from the European Collection of Authenticated Cell Cultures (ECACC, Salisbury, UK) at passage number 5 (Cat No: 07032901, Lot No: 15D024, Date: 21/05/2015). All experiments were performed with mycoplasma-free cells. Cells were seeded in culture dishes coated with 0.2% gelatin and maintained in DMEM medium supplemented with 10% FBS, 2 mM Glutamax, 2 mM sodium pyruvate, 100 μM non-essential amino acids, 50 μM β-mercaptoethanol, 100 U/ml penicillin, and 100 μg/ml streptomycin at 5% CO₂ at 37°C. Cell culture medium was changed daily with medium containing 10⁴U/ml leukemia inhibitory factor (mLIF) + 3i (2 μM SU5402, 800 nM PD184352, and 3 μM CHIR99021) and passaged 3 times a week.

For differentiation of CGR8 cells into NSCs, 5.6 × 10⁵ cells were seeded in 10-cm petri dishes coated with 0.2%

gelatin in N2B27 medium (1:1 DMEM/F12 and Neurobasal, N2-supplement, B27-supplement, 2 mM Glutamax, 100 μ M β -mercaptoethanol, 7.5 μ g/ml insulin, 50 μ g/ml bovine serum albumin) and incubated for 7 days. On the 7th day of differentiation, the cells were placed in NSC medium (N2B27 medium supplemented with 20 ng/ml endothelial growth factor (endothelial growth factor; EGF) and 20 ng/ml fibroblast growth factor 2 (FGF2)) at 37°C and maintained under 5% CO₂ conditions.

H₂O₂ treatment On the 7th day of differentiation, the cells were passaged and 2.5×10^5 cell seeded into poly-L-ornithine/laminin-coated 10-cm-diameter dishes in N2B27 medium without supplements. At day 2, the cells were exposed to different concentrations of H₂O₂ in range of low dose 10 μ M, medium dose 50 μ M, and high dose 100 μ M. After 24 h of treatment, H₂O₂ was removed and cultured with fresh medium for 5 days (Perez Estrada *et al.* 2014).

Quantitative polymerase chain reaction Total RNA isolation was performed according to the RNeasy mini kit (Qiagen, Hilden, Germany) protocol. RNA concentrations were measured spectrophotometrically and cDNA was synthesized from 1 μ g RNA with the iScript II Reverse Transcription Supermix (Biorad, Hercules, CA) kit. Quantitative PCR was performed on the ABI 7500 Fast quantitative PCR instrument using the SsoFast EvaGreen qPCR Supermix kit (BioRad, Hercules, CA) according to the manufacturer's instructions. Changes in mRNA levels were analyzed according to the $\Delta\Delta$ CT method and GAPDH was used as a housekeeping gene (Livak and Schmittgen 2001). To determine differentiation of stem cell to neural lineage, mRNA levels of Sox2 for stemness, Nestin as neural progenitor cells, Beta-III tubulin for neurons, S100B as astrocyte marker, Olig 1 and Olig 2 as oligodendrocyte markers were examined (Table 1).

Cytotoxicity assay Cells were treated with high dose 100 μ M H₂O₂ and positive control 1000 μ M H₂O₂ for 24 h. After treatments, cell-free supernatant was collected, and the release of lactate dehydrogenase (LDH) was analyzed spectrophotometrically by LDH Cytotoxicity Detection Kit (Roche, Basel, Switzerland) according to the manufacturer's guidelines. Each well's absorbance was measured at 492 nm wavelength with a reference wavelength of 630 nm on a microplate reader. Cell cytotoxicity was expressed as the percentage of the maximum LDH activity.

Western blotting Protein extraction from differentiated neurons was performed with RIPA buffer and protein concentrations were determined by the bicinchoninic acid (BCA) protein determination method. Protein samples were separated by 10%

Table 1. PCR primers

Beta-III tubulin	F	TAGACCCAGCGGCAACTAT
	R	GTCCAGGTTCCAAGTCCACC
Nestin	F	CTGGAAGGTGGGCAGCAACT
	R	ATTAGGCAAGGGGAAGAGAAGGTG
Olig1	F	TCTTCCACCGCATCCCTTCT
	R	CCGAGTAGGGTAGGATAACTTCG
Olig2	F	GGCGGTGGCTTCAAGTCATC
	R	TAGTTTCGCGCCAGCAGCAG
S100B	F	GGTTGCCCTCATTGATGTCTTCCAC
	R	CTTCCTGCTCCTTGATTTCTCCAG
Sox2	F	GCGGAGTGGAAACTTTTGTCC
	R	CGGGAAGCGTGTACTTATCCTT
GAPDH	F	ACCACAGTCCATGCCATCAC
	R	TCCACCCTGTTGCTGTA

sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gel. It was then transferred to a polyvinylidene difluoride (PVDF) membrane and incubated in 5% non-fat milk solution for blocking. After this step, the samples were incubated overnight at 4°C with antibodies to p-p38, p-Akt, p38, and Akt. After the washing steps, the samples were incubated with secondary antibody labeled with horseradish peroxidase (HRP), and after the washing steps, blots were visualized by chemiluminescence method and the images were taken by using a gel documentation system. Band intensities were evaluated densitometrically by ImageJ (Schneider *et al.* 2012) (Table 2).

Statistical Analysis Statistical analyses of the data were performed on GraphPad Prism 9.0.2 (GraphPad Software Inc., San Diego, CA). In order to compare two experimental groups, the Mann–Whitney *U* test was utilized. Data are presented as mean \pm SEM and $p < 0.05$ was considered as statistically significant.

Results

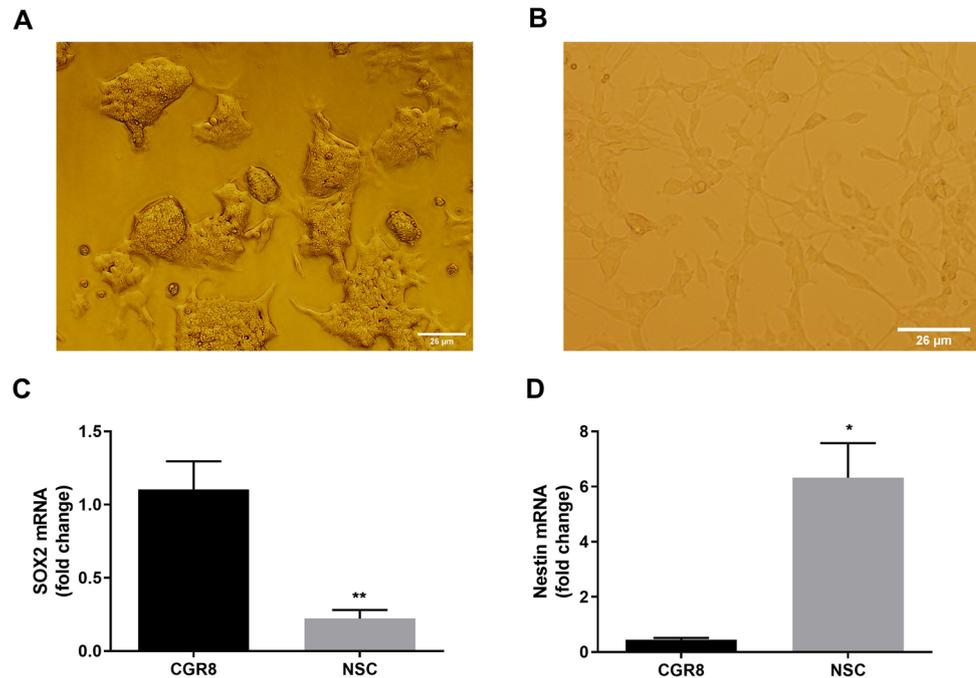
CGR8 cells differentiated to neural stem cells Differentiation of CGR8 cells into NSCs was first observed morphologically on images taken under a phase-contrast microscope. Embryonic bodies of CGR8 cells were seen morphologically (Fig. 1A). When the differentiation was completed, the embryonic bodies were differentiated and the neurites of the cells were formed (Fig. 1B).

Next, differentiation of CGR8 cells into NSCs was confirmed by the quantitative PCR method by controlling SOX2 and nestin mRNA levels. SOX2, an embryonic stem cell marker, was significantly decreased in NSCs compared to CGR8 cells (Fig. 1C). In addition, nestin, a neural stem/progenitor cell marker, was significantly upregulated in NSCs compared to CGR8 cells (Fig. 1D).

Table 2. Primary and secondary antibody list

Antibody	Company	Catalog Number	Application	Dilution
Anti-Phospho Akt (Ser473)	Cell Signaling	4060S	WB	1:1000
Anti-Akt	Cell Signaling	4691S	WB	1:1000
Anti-Phospho-p38 MAPK (Thr180/Tyr182)	Cell Signaling	4511S	WB	1:1000
Anti-p38 MAPK	Cell Signaling	8690S	WB	1:1000
Anti-Rabbit-HRP	Cell Signaling	7074S	WB	1:2000

Figure 1. NSCs were differentiated from CGR8 embryonic stem cell line. The phase-contrast images demonstrate (A) CGR8 embryonic stem cells and (B) NSCs with observed extensions. mRNA expression levels of (C) SOX2 (stemness marker) were decreased in neural stem cells, while (D) nestin (NSC marker) was increased significantly when compared to CGR8 cells. The results are mean \pm SEM * p < 0.05 and ** p < 0.01



H₂O₂ suppressed neural differentiation In order to examine the effect of different doses of H₂O₂ on neural differentiation, Beta-III-tubulin, OLIG1, OLIG2, and S100B mRNA levels in cells treated with 10, 50, and 100 μM H₂O₂ were analyzed by the qPCR method. Our qPCR results demonstrated that H₂O₂ treatment downregulated neuronal marker Beta-III-tubulin, oligodendrocyte markers OLIG1 and OLIG2, and astrocyte marker S100B expression (Fig. 2A, B, C, D). In addition, to demonstrate the suppressive effect of H₂O₂ on neural differentiation is not due to cytotoxicity, we analyzed the high dose of 100 μM H₂O₂ with a positive control dose of 1000 μM H₂O₂ both in CGR8 cells and NSCs by LDH assay. Our results demonstrated that there were no significant differences between control and 100 μM H₂O₂ in CGR8 cells and NSCs. Non-significant toxicity with the cells was due to the differentiation process as we expected (Fig. 2E, F).

H₂O₂-induced phosphorylation of p38 and Akt pathways To evaluate the effects of different doses of H₂O₂ on the activity of PI3K and MAPK signaling pathways, we analyzed the phosphorylation states of p38 and Akt by Western blotting method. Phosphorylated and total p38 and Akt protein levels

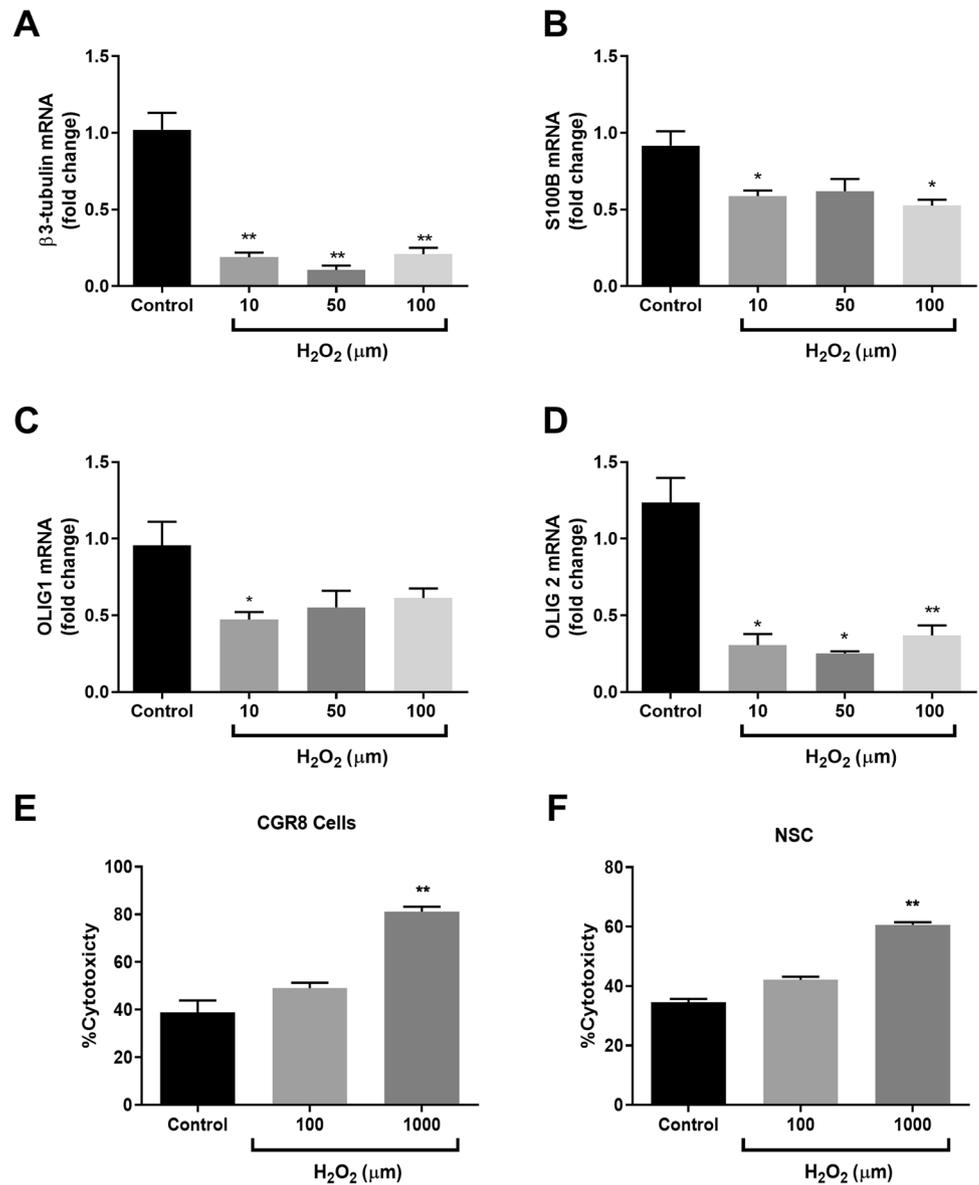
were investigated in the experiment with proteins obtained from differentiated cells.

According to the results we obtained, p38 protein phosphorylation increases significantly in cells treated with low dose (10 μM) of H₂O₂ (Fig. 3A, B). On the other hand, high dose (100 μM) of H₂O₂ increased significantly the band intensity of phospho-Akt protein (Fig. 3C, D).

Discussion

Neurogenesis, that is differentiation of committed cells from NSCs, and self-renewal of NSCs are regulated by immediate microenvironment (Andreotti *et al.* 2019). Both in developing CNS and throughout the adult life, the differentiation and proliferation of NSCs are strictly modulated by intrinsic and extrinsic factors, i.e., cytokines, hormones, and growth factors, as well as aging, stress, and exercise (Cavallucci *et al.* 2016). Given that numerous cellular pathways are involved in these procedures, it is obvious that there is a great number of cellular interactions, cross-interactions of pathways, and molecular cascades. Nevertheless, it is yet to be clarified

Figure 2. H₂O₂ treatment suppressed NSC differentiation on mRNA level. NSCs were treated with 10, 50, and 100 μM doses of H₂O₂ for 24 h. mRNA expression levels of (A) Beta-III-tubulin neural marker, (B) S100B astrocyte marker, (C) OLIG1, and (D) OLIG2 oligodendrocyte markers were down-regulated differentially upon H₂O₂ treatment when compared to control group. (E) The toxicity of H₂O₂ on CGR8 cells was determined. (F) The toxicity of H₂O₂ on NSCs was determined. The results are mean ± SEM **p* < 0.05 and ***p* < 0.01 compared with untreated control



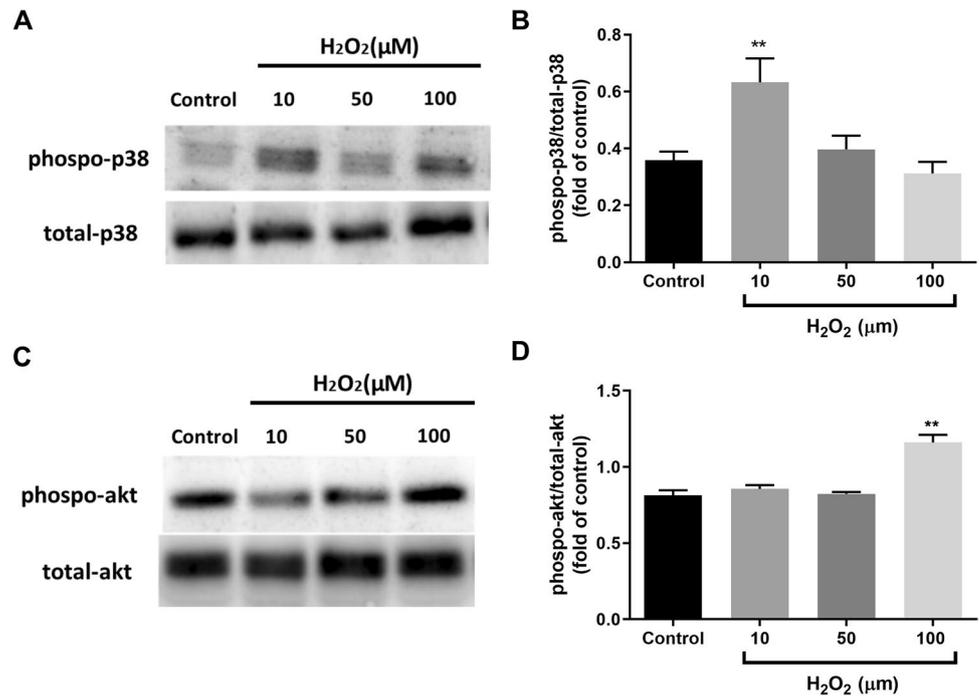
how the NSCs are maintained throughout a lifetime; what are the exact mechanisms, interactions, and the signals modulating the behavior and fate of the NSCs.

The niche accommodating the NSCs is known to retain low O₂ conditions; additionally, O₂ availability is linked to NSC fate determination and functions (Mazumdar *et al.* 2010). Metabolism of oxygen within the cells results in production of ROS including free radicals such as H₂O₂ (Gough and Cotter 2011). The state of cellular redox or oxidative stress is a regulatory factor in terms of the equilibrium between the maintenance, self-renewal, and activation of the stem cells (Adusumilli *et al.* 2021). Cellular levels of H₂O₂ are able to act as secondary messengers in cellular signaling pathways (Adusumilli *et al.* 2021), including proliferation, migration, survival, and differentiation pathways (Gough and Cotter 2011). Generation of H₂O₂

is known to regulate downstream signaling pathways, namely PI3K/Akt pathway (Gough and Cotter 2011) and p38-MAPK pathway (Ito *et al.* 2006). H₂O₂ production has been linked to oxidative damage over the years whereas recently has been categorized as an important molecule for a number of pathways. H₂O₂ is now known to have contradictory roles as a secondary messenger depending on the localization of NOX enzymes producing H₂O₂, the signal received by the cell, the niche accommodating the cell, and the type of the cell (Gough and Cotter 2011).

Even though extremely low or high levels are known to be related to impaired functions within the CNS (Ortega *et al.* 2017), ROS levels are now considered to be necessary for NSC differentiation and self-renewal (Le Belle *et al.* 2011). In their study, Le Belle *et al.* found that the effects of exogenous

Figure 3. H₂O₂-induced phosphorylation of p38 and Akt signaling pathways. NSCs were treated with 10, 50, and 100 μ M doses of H₂O₂ for 24 h. Protein levels of (A, B) Phospho-p38 were increased in 10 μ M H₂O₂-treated cells, while (C, D) Phospho-Akt was increased in 100 μ M H₂O₂-treated cells. The results are mean \pm SEM * p < 0.05 and ** p < 0.01 compared with untreated control



ROS stimulation in lower doses in undifferentiated cells in the presence of growth factors will promote NSC self-renewal in a NOX- and ROS-dependent manner. Their results suggested that a low dose of ROS enhanced clonal neurosphere formation. However, there is more to find out about how exogenous and endogenous ROS levels affect neurogenesis. On the other hand, it is still not known that how exactly alterations in redox state and ROS signaling regulate the aforementioned processes.

In this study, the effect of H₂O₂ on neuronal, astroglial, and oligodendrocytic differentiation was investigated by the quantitative PCR method, and it was shown that all doses of H₂O₂ suppressed neuronal, glial, and oligodendrocytic differentiation. In a study conducted in rat-derived adult neural progenitor cell culture, it was observed when 100 μ M H₂O₂ was added, it positively affected neuronal differentiation and increased the number of neurons and oligodendrocytes (Perez Estrada *et al.* 2014). However, their study model started with adult NSCs, and our study was conducted on early NSCs derived from embryonic stem cells. Hereby, we postulated that H₂O₂ in the studied dose range suppressed neuronal and glial differentiation by creating oxidative stress and affected neuronal fate in NSCs differentiated from embryonic stem cells.

A number of experimental studies indicated that p38 MAPK, associated with the reorganization of neural plasticity and stress responses, acts as a negative organizer of NSC proliferation during early brain development (Yang *et al.* 2006; Sato *et al.* 2008).

In a study, which showed that by increasing ROS levels with H₂O₂ treatment, neurosphere formation and proliferation were significantly reduced, they demonstrated that p38 phosphorylation

increased at higher doses of H₂O₂ 100 μ M and above (Kim and Wong 2009). Accordingly, we found that 10 μ M H₂O₂ treatment activated the MAPK pathway and increased phosphorylated p38 remarkably in protein level. In our study, we found that higher dose of H₂O₂ 100 μ M caused phosphorylation of Akt. Consistent with our results, Kim *et al.* also demonstrated Akt phosphorylation at a dose of 100 μ M. In another study conducted in cultured NSCs detached from embryonic day 13 (E13) rat cerebral cortexes to characterize H₂O₂-stimulated NSC apoptosis and involved cell signaling pathways, they found that H₂O₂ transiently activates PI3K-Akt in a dose-dependent manner (Lin *et al.* 2004). Similarly, Lin *et al.* showed in this study a significant increase in p-Akt protein levels starting from 100 μ M of H₂O₂ and also demonstrated that the increase enhanced depending on the dose. On the other hand, they declared that they could not distinguish the notable effect on p38 phosphorylation at any dose of H₂O₂.

The aim of this study was to explore the effect of H₂O₂ exposure on the development of NSCs and the MAPK and PI3K/Akt pathways known to be important in neuronal survival. Here we reported that exogenous H₂O₂ in the dose range of 10 to 100 μ M suppressed NSC differentiation by phosphorylating p38 and Akt pathways.

Conclusions

Embryonic neurogenesis requires a consecutive intriguing cascade of events that necessitates a delicate interplay among cellular elements and the extracellular environment

of developing brain tissue, and even any minor changes in the redox state can interfere with neural differentiation. The fate of neural stem cells is affected by cellular ROS level and H₂O₂ has a pivotal role in the differentiation of neural stem cells into neurons.

Author contribution All authors contributed to the study. Material preparation, data collection, and analysis were performed by Bedir Irem Eltutan and Kemal Ugur Tufekci. Bedir Irem Eltutan and Cagla Kiser wrote the first draft of the manuscript. All authors read and confirmed the final manuscript.

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Declarations

Conflict of interest The authors declare no competing interests.

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