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Research article

Oxygen exposure in early life activates NLRP3 inflammasome in mouse brain

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ABSTRACT

Despite widely known detrimental effects on the developing brain, supplemental oxygen is still irreplaceable in the management of newborn infants with respiratory distress. Identifying downstream mechanisms underlying oxygen toxicity is a key step for development of new neuroprotective strategies. Main purpose of this study is to investigate whether NLRP3 inflammasome activation has a role in the pathogenesis of hyperoxia-induced preterm brain injury. C57BL6 pups were randomly divided into either a hyperoxia group (exposed to 90 % oxygen from birth until postnatal day 7) or control group (maintained in room air; 21 % O_2). At postnatal day 7, all animals were sacrificed. Immunohistochemical examination revealed that hyperoxic exposure for seven days resulted in a global increase in NLRP3 and IL-1β immunopositive cells in neonatal mouse brain ($p \leq 0.001$). There was a significant rise in Caspase-1 positive cell count in prefrontal and parietal area in the hyperoxia group when compared with controls ($p \leq 0.001$). Western blot analysis of brain tissues showed elevated NLRP3, IL-1β and Caspase-1 protein levels in the hyperoxia group when compared with controls ($p \leq 0.001$). To the best of our knowledge, this is the first study that investigates an association between hyperoxia and establishment of NLRP3 inflammasome in preterm brain.

1. Introduction

Supplemental oxygen plays a critical role in the management of both term and preterm infants with respiratory distress [1,2]. However, with our increased understanding regarding adverse effects of this molecule, there has been a renewed interest for better oxygen management in neonatal intensive care units. During fetal life, brain is primed to maintain its development in an environment with relative low levels of oxygen. Mean PaO_2 in the intrauterine life is 3.2 kPa, that is equivalent to arterial oxygen saturations of about 70 % [3]. Following birth, even in the absence of supplemental oxygen, preterm infants are subjected to relative high levels of oxygen that results in generation of reactive oxygen species (ROS). Free radical attack is one of the principal downstream mechanisms leading to dysmaturational events in preterm brain by triggering maturational arrest of premyelinating oligodendrocytes, neuronal loss and subsequent gliosis [4].

As being one of the most powerful and commonly used drug in neonatal medicine, supplemental oxygen should be carefully titrated in order to prevent hyperoxia induced brain injury [3]. Although several well designed randomized controlled trials for target oxygen saturations of preterm infants were published in past years [5], in current practice, we still have scarce evidence regarding the optimum dose of oxygen [1]. Moreover, those infants who require oxygen support are usually the ones with more limited availability of antioxidant defense systems for proper

Abbreviations: CNS, central nerve systems; NLRP3, nod-like receptor pyrin domain-containing 3; IL-1β, interleukin-1 beta; IL-18, interleukin-1; PFC, prefrontal cortex; CA1, cornu ammonis; DG, dentate gyrus; PC, parietal cortex; Neu-N, neuron-specific nuclear protein; MBP, myelin basic protein; TuJ1, neuron specific class III beta-tubulin; PBS, phosphate buffer saline; DAB, diaminobenzidine; SDS, sodium dodecyl sulfate; RIPA, radioimmunoprecipitation assay buffer; BCA, bicinchoninic acid; PVDF, polyvinylidene fluoride; TBST, tris-buffered saline with tween; SPSS, statistical package for the social sciences.

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handling the burden of oxygen (*e.g.*, infants born preterm or undergone perinatal asphyxia) [1]. Brain injury and permanent neurological deficits continue to impair the well-being of many children, and protective strategies are particularly required in cases requiring oxygen support [6]. Gaining a clear understanding of the mechanisms of oxygen toxicity is essential to develop new therapeutic avenues for neuroprotection of babies on supplemental oxygen [7].

NLRP3 (NOD-, LRR- and pyrin domain-containing protein 3) is an intracellular multimeric protein complex resulting in Caspase 1-dependent release of the pro-inflammatory cytokines and finally cell death upon activation [8]. NLRP3 is shown to be involved in the pathogenesis of brain injury in mouse model of hypoxic ischemic encephalopathy [9,10] however the role in hyperoxia- induced-brain injury remains to be investigated. Assembly of the NLRP3 inflammasome can occur by diverse cellular insults one of which is increased production of ROS [11]. Oxidative stress can promote various inflammatory cascades in the preterm brain, such as upregulation of certain cytokines and increased expression of redox sensitive transcription factors (*i.e.*, NF-κB) [12,13] which may trigger the two critical steps in the cellular process of NLRP3 inflammasome establishment: priming and activation [14]. Priming process is associated with inflammatory stimuli that promote NF-κB-mediated NLRP3 and pro-IL-1β expression and the activation process results in inflammasome assembly with Caspase-1-mediated IL-1β and IL-18 secretion. Moreover, modulation of ROS generation has been reported to interfere with NLRP3 inflammasome activation [14].

Main purpose of this study is to determine the role of NLRP3 activation in a mouse model of hyperoxia-induced preterm brain injury and if any, investigate the downstream mechanisms associated.

2. Material and methods

2.1. Animals

This study was performed in accordance with the guidelines provided by the Experimental Animal Laboratory and approved by the Animal Care and Use Committee of the Dokuz Eylül University School of Medicine. C57BL6 mice were maintained on standard 12 h light-dark cycles, at 21 °C temperature with 45 % humidity and with *ad libitum* access to standard food and water. All experiments were performed during the light phase of the 12 h day/night cycle.

2.2. Neonatal hyperoxia model

After giving birth, dams and their newborns were transferred into an experimental room composed 38 L of isobaric plexiglass chambers equipped with oxygen. Mice were randomly divided into either a hyperoxia group (n = 12) (exposed to 90 % oxygen from birth until day postnatal day (PN) 7 or control group (n = 12) (maintained in room air; 21 % O_2). The hyperoxic environment was created by a continuous flow of oxygen at 3 L/hours rate. The oxygen concentration in the chamber was monitored twice daily and measured as 90 ± 2 % [15,16]. Both groups were housed with their dams, the mothers were removed and then put back in the chamber in every 24 h to avoid oxygen toxicity. During experimental procedure, none of the animals were lost or suffered from conditions associated with hyperoxia (*e.g*, fever, convulsions or paralysis). After exposure to 90 % oxygen from PN0 through PN7 (that is comparable to 23–36 gestational weeks in human [17]), the mice in the hyperoxia group were returned to room air until sacrifice. All animals were sacrified under ether anesthesia without sex-dependent randomization at PN7. Following measurement of brain and body weights, brain tissues were fixed at 10 % formalin for histology (n = 6) and other tissues were stored at -80 °C for western blot assay (n = 6).

Table 1
Antibodies for Immunohistochemical Examination.

Antibody	Company	Catalogue Number	Application	Dilution
Anti-NLRP3	Adipogen	AG-20B-0014-C100	IHC WB	1:200 1:1000
Anti -Caspase-1	Adipogen	AG-20B-0042	IHC	1:200
Anti- Neu-N	Neuromics	MO15002	IHC	1:200
Anti-MBP	Santa Cruz	SC-71546	IHC	1:200
Active Caspase-3	Bioss	Bs-0081R	IHC	1:200
Anti-IL1β	Santa Cruz Abcam	Sc-12742 ab9722	IHC WB	1:200 1:1000
TuJ1 (Anti-Tubulin Antibody, beta III isoform)	Merck	MAB1637	WB	1:1000
Anti-β-actin	Cell Signaling	4970	WB	1:1000
Anti-Rabbit	Cell Signaling	Cst-7074	WB	1:2000
Anti-Mouse	Cell Signaling	Cst-7076	WB	1:2000

2.3. Histomorphological evaluation

Brain tissues were processed and embedded in paraffin blocks. The blocks were cut into 5 μm sections at multiple levels and stained with cresyl violet and neuronal count was obtained by the numbers of Nissl positive cells. Each sample was subjected to the estimation of prefrontal cortex (PFC), hippocampus (CA1 and DG) and parietal cortex (PC). The boundary of these brain areas was defined in accordance with Mouse Brain Atlas [18].

2.4. Immunohistochemical examination

Sections were mounted on poly-L-lysine-coated slides. The streptavidin-biotin-peroxidase assay was performed using the primary antibodies against anti-NLRP3, anti-Caspase-1, anti-Neu-N, anti-MBP, activated Caspase-3 and anti- IL-1β.The antibodies were listed at Table 1. Following deparaffinization and rehydration, the sections were treated with trypsin (TA-125-TR, Thermo Fisher) for 15 min and endogenous peroxidase activity was blocked using a 0.3 % solution of hydrogen peroxidase in PBS at room temperature for 10 min. Sections were incubated with primary antibodies sequentially for 2 h at 4 °C temperature and washed in PBS. After washing, secondary antibodies (85–9043, Invitrogen) were applied for 30 min, followed by washes in PBS. The peroxidase activity was visualized with DAB (diaminobenzidine) (Roche, Penzberg, Germany). Slides were counterstained by Mayer's hematoxylin, dehydrated, cleared, and were visualized by light microscopy.

2.5. Scoring for NLRP3, Caspase-1, NeuN, activated Caspase-3 and IL-1β

For quantitative measurement of immunopositive cells, 100 cells were randomly counted by Image J software in different areas for each group and percentages were calculated [19].

2.6. Semiquantification of immunohistochemical data for MBP

In order to quantify immunostaining, following score was used: 0: no immunoreactivity; 1: very little positive staining, 2: moderate positive staining, 3: strong positive staining. The average of the scores was used for grading [20].

2.7. Western blot

Protein from brain tissue was extracted with using RIPA lysis buffer including the protease and phosphatase inhibitor (Thermo Scientific,

Table 2
Brain and body weights of pups at PN7.

Group	Brain weight (g)	Body weight (g)
Control	8.00±0.89	11.90±0.48
Hypeoxia	6.83±0.75	10.06±0.67
p value	0.04	0.004

USA) with the help of homogenizer. Protein amounts were detected with BCA assay kit (Takara Bio; Japan) and 50 μ g protein was resolved in 15 % sodium dodecyl sulfate (SDS) polyacrylamide gel and separated by electrophoresis. Gels were blotted onto polyvinylidene fluoride (PVDF) membranes (Sigma-Aldrich, USA). After blocking with non-fat dry milk or BSA in PBS-T (0.2 % Tween-20 in 1X PBS, pH 7.2) for 1 h, the membranes were probed with primary antibodies overnight at 4 °C according to manufacturer's protocol. Later, probed membranes were incubated with HRP-conjugated secondary antibody for 1 h at room temperature. The antibodies were given in Table 1. After four times wash with TBST, Supersignal West Dura ECL reagent was used for detection of antigen–antibody complex with a densitometer (UVP Gel Imager System, CA). Band density was analyzed by Image Studio LiteVer 5.2 software and normalized to β-actin band density as a loading control [19].

2.8. Neuronal density

For the estimation of neuronal densities, images were analyzed by using a computer assisted Image-J analyze program. The counting frame was randomly placed on the image analyzer system monitor for three times, the number of neurons stained by cresyl violet were counted in a square area of ~1.875 μm^2 [15].

2.9. Statistical analysis

Data were analyzed by SPSS version 22.0 (SPSS, Chicago, IL, USA). Values were presented as mean ± SD. Since the data were inconsistent with normal distribution and due to small sample size, non-parametric tests were applied. Differences between groups were examined with Kruskal Wallis test and Mann–Whitney *U*-test, $p < 0.05$ was considered significant.

3. Results

3.1. Hyperoxia leads brain and body weight loss

Brain and body weights were measured at PN7, and both brain and body weights were found to be lower in the hyperoxia group compared to control (p = 0.04, p = 0.004 respectively) (Table 2).

3.2. Hyperoxia increases NLRP3, Caspase-1 and IL1β protein expression

In order to understand the relation between the hyperoxia-induced preterm brain injury and inflammasome activation, NLRP3, Caspase-1 and IL1β levels in brain tissue were determined by

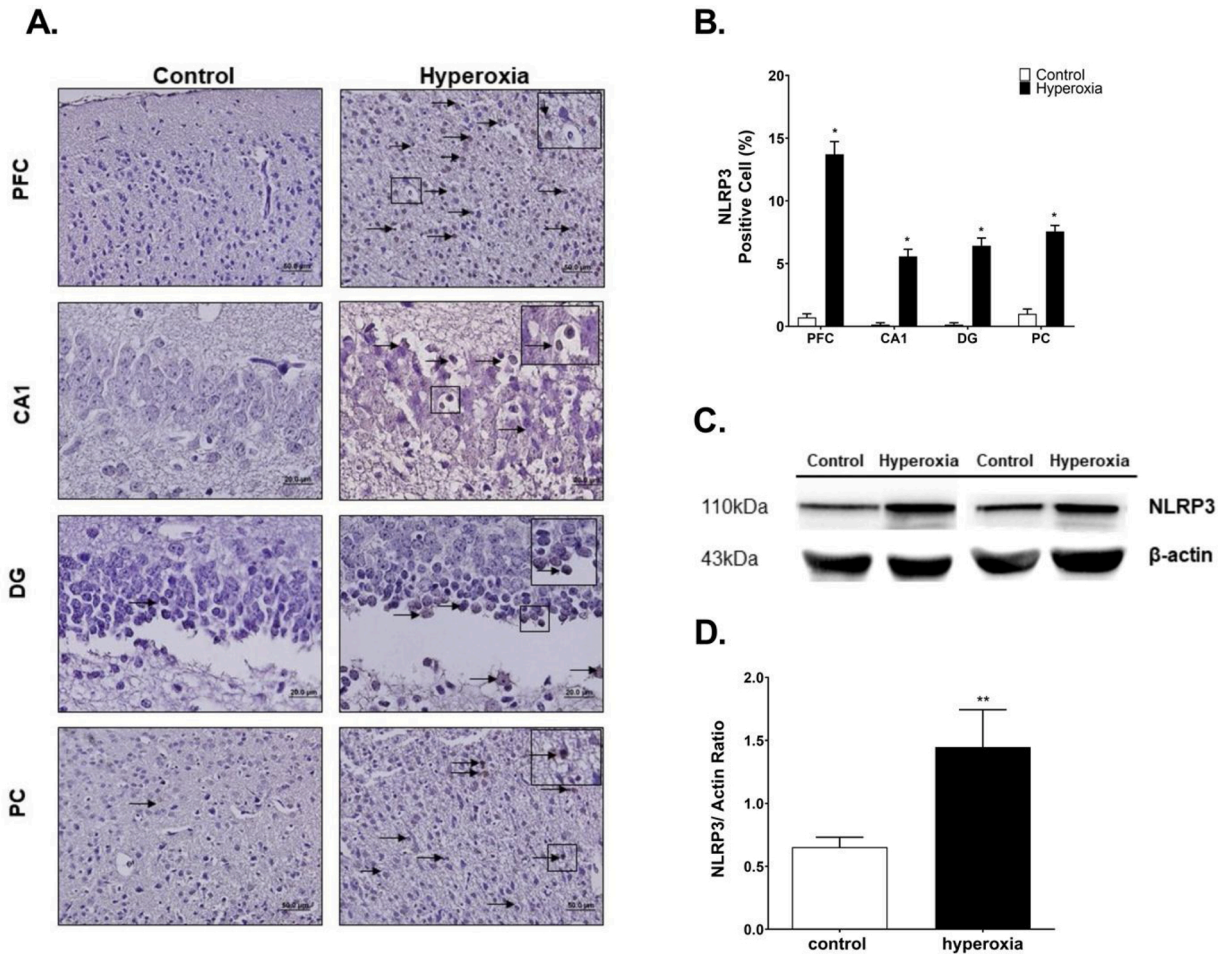

Fig. 1. A: NLRP3 immunohistochemistry. NLRP3 signaling was activated in PFC, CA1, DG, and PC regions of newborn mice following hyperoxia. Black arrows show immunopositive cells. **B**: NLRP3 positive cells were significantly increased in hyperoxia group *vs* control group in all regions of brain ($p \leq 0.05$) **C, D:** NLRP3 protein levels were significantly increased in hyperoxia group *vs* control group of the brain ($p \leq 0.05$). Immunopositive cells (X100 magnification) are shown in square.

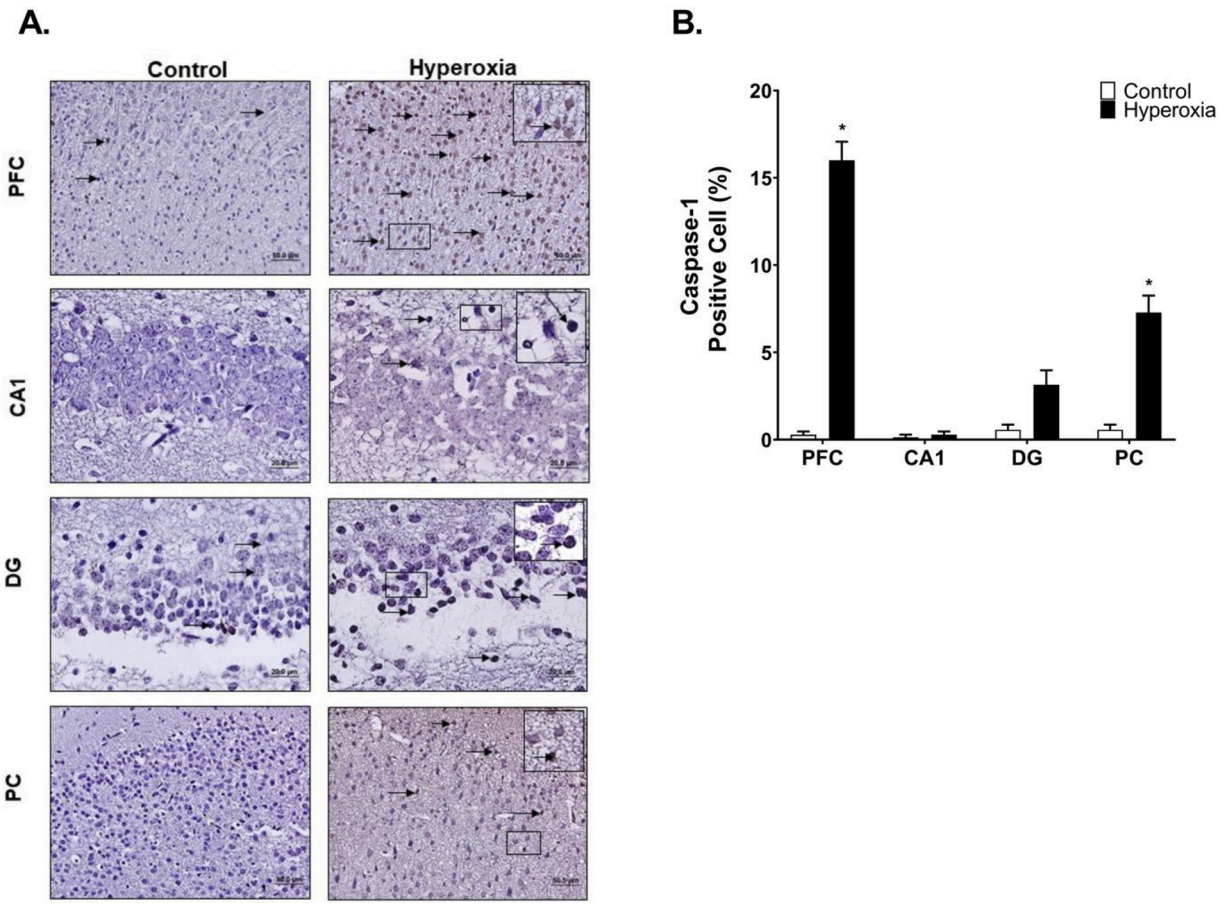

Fig. 2. **A:** Caspase-1 immunohistochemistry. Caspase-1 signaling was activated in PC, CA1, DG and PC regions of newborn mice following hyperoxia. Black arrows show immunopositive cells. **B:** Caspase-1 positive cells were significantly increased in hyperoxia *vs* control group in PFC and PC regions ($p \leq 0.05$). Immunopositive cells (X100 magnification) are shown in square.

immunohistochemical assay. There were a global increase in NLRP3 immunopositive cells in brain tissues of hyperoxia group when compared with control ($p \leq 0.001$) (Fig. 1**A**, **B**). Caspase-1 positive cells were significantly increased in hyperoxia group in PFC and PC regions when compared with control ($p \leq 0.001$) (Fig. 2**A**, **B**). IL1β positive cells were globally increased in the hyperoxia group when compared with control ($p \leq 0.001$) (Fig. 3**A**, **B**). NLRP3 protein level was also determined by Western Blot analysis. According to band intensities, NLRP3, Caspase-1 and IL1β protein levels were significantly increased in hyperoxic brain tissue when compared with control (Figs. 1**C**, **D**; 2 **B**, 3 **C**, **D**)

3.3. Hyperoxia leads neuronal loss

Next, we examined the question whether inflammasome activation promotes neuronal loss with exposure to hyperoxia. Neurons in the PFC, CA1, DG and PC regions of control group had regular shape and cell morphology with large and round nuclei and rare presence of inflammatory cells and cell edema. In the hyperoxia group, partial nuclear pyknosis and few inflammatory cells were observed, with cellular edema and rupture. The number of neurons in the PFC, CA1, DG and PC regions in hyperoxia group were found to be significantly lower than control group ($p \leq 0.001$) (Fig. 4). NeuN immunostaining was performed to determine neuronal damage caused by hyperoxia. NeuN immunopositive cells were globally decreased in the hyperoxia group when compared with control ($p \leq 0.001$) (Fig. 5**A**, **B**). Western Blot analysis indicated that protein level of neuronal marker TuJ1 (class III beta-tubulin) were significantly diminished in hyperoxic brain tissue when compared with control pups (Fig. 5**C**, **D**). Activated Caspase-3 immunostaining was performed in order to determine apoptotic damage caused by hyperoxia. Global increase in activated Caspase-3 immunopositive cells were noted in the hyperoxia group when compared with control ($p \leq 0.001$) (Fig. 6**A**, **B**).

3.4. Hyperoxia leads oligodendrocyte loss

MBP staining was performed to determine whether hyperoxia results in white matter injury. There was a global decrease in MBP immunopositive cells in the hyperoxia group when compared with control ($p \leq 0.001$) (Fig. 7).

4. Discussion

In the present study, we succesfully demonstrated that seven days of exposure to hyperoxic environment leads to NLRP3 inflammasome activation in the brain tissue of neonatal mice. To the best of our knowledge, this is the first study to investigate the association between hyperoxia and establishment of NLRP3 inflammasome in brain.

Inflammasomes are known to be involved in the pathogenesis of acute hyperoxic lung injury in adults for several years [23,24]. The inflammasome protein complex is shown to activate Caspase-1, which results in secretion of proinflammatory cytokines that are responsible for the increase in permeability of the alveolar epithelium. This process ends up with epithelial barrier dysfunction and ultimately cellular death [23].

Regarding newborns, Liao J. et al. [25] showed that NLRP3 inflammasome has a key role in the development of bronchopulmonary dysplasia. This study group reported that NLRP3−/− mice are protected

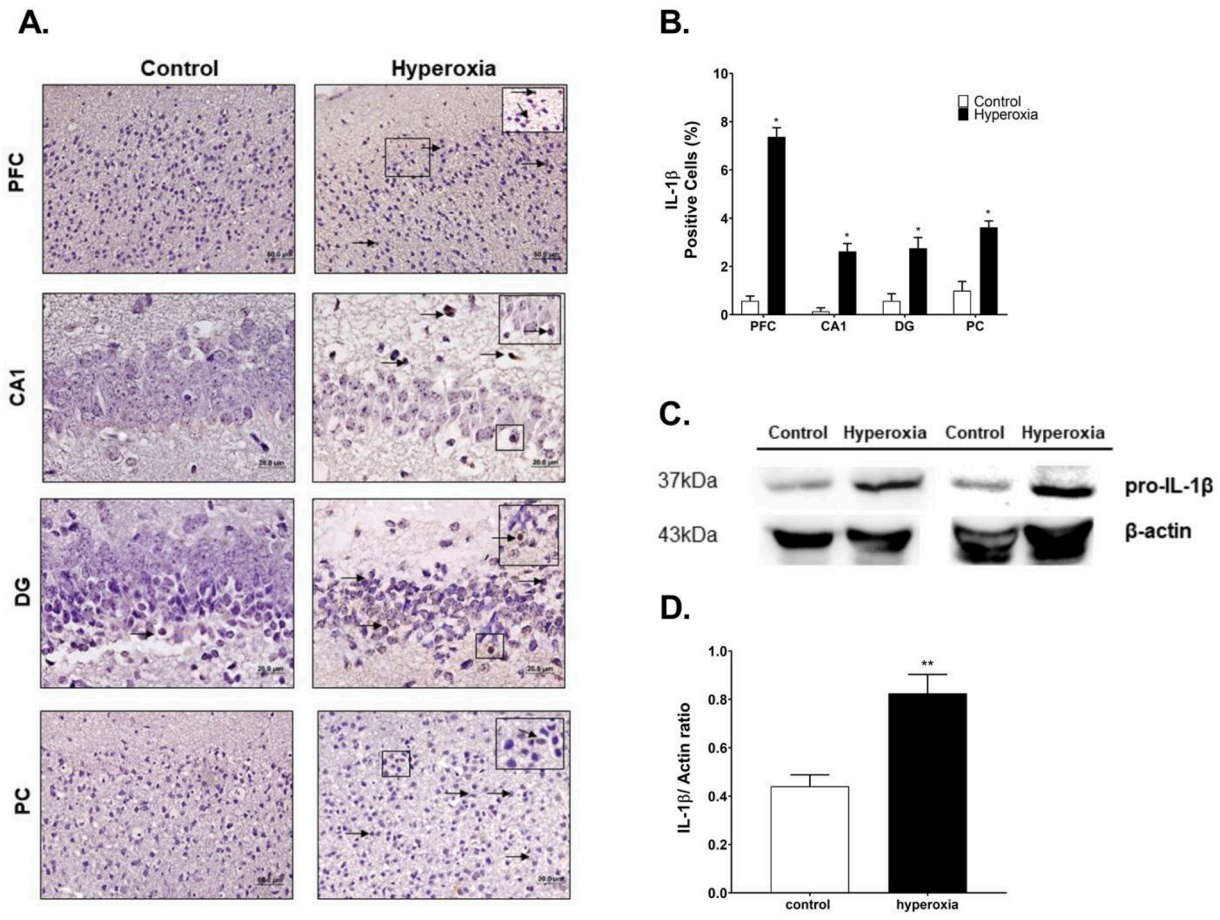

Fig. 3. A: IL1β immunohistochemistry. IL1β signaling was activated in PC, CA1, DG and PC regions of newborn mice following hyperoxia. Black arrows show immunopositive cells. **B:** IL1β positive cells were significantly increased in hyperoxia *vs* control in PFC and PC regions ($p \leq 0.05$). **C, D:** IL1β protein levels were significantly increased in hyperoxia group *vs* control ($p \leq 0.05$). Immunopositive cells (X100 magnification) are shown in square.

from adverse effects of neonatal hyperoxia on lungs. According to this study, Caspase-1 activity in whole-lung tissue was increased twofold in wild type mice in 85 % O2, whereas was undetectable in NLRP3−/− mice. Additionally, although NLRP3−/− mice were able to generate pro-IL1β and pro-IL18, deficiency of NLRP3 prevented the procession of these molecules into mature IL1β and IL18.

Recently, Ystgaard and collegues reported that NLRP3 was upregulated 24 h after hypoxia-ischemia in the hippocampus, striatum and thalamus in mouse model [10]. Their study also showed that, NLRP3 expression occurred in astrocytes located in the hippocampus and habenular nucleus of the thalamus after 24 h of injury. Increased NLRP3 expression was also reported in microglial cells in the next 3 days. Interestingly, NLRP3 deficiency did not provide early protection of neonatal hypoxic ischemic brain damage in this study. This finding was interpreted by the authors that the astrocytes expressing increased NLRP3 activity, might not be the same cells that lead to progression of injury by an overstimulation. They further investigated role of NLRP3 together with its adaptor protein ASC and subjected both NLRP3-/- and ASC-/- mice to hypoxia at P9 following unilateral common carotid artery ligation. They still found NLRP3 deficiency was associated with an increase in brain damage, whereas ASC deficiency provided neuroprotection at some degree.

The role of NLRP3 inflammasome on hyperoxia induced brain injury has not been studied yet. However, Dapaah-Siakwan and collegues recently reported activation of NLRP1 inflammasome in brain tissues of mice exposed to 85 % O2 from PN1 through PN10. Hyperoxia lead to upregulated expression of NLRP1, ASC, as well as active Caspase-1 in this study, whereas treatment with Caspase-1 inhibitor down-regulated expression of these proteins in brain tissue. They state that hyperoxia resulted in a marked decrease in the subgranular zone (SGZ) and subventricular zone (SVZ) of brain, which can be alleviated by Caspase-1 inhibition [16]. Increased apoptosis in the SVZ was demonstrated in hyperoxia group which is lessened by Caspase-1 inhibition. Proliferation index (% of Ki67+ nuclei/total nuclei) was reported to be decreased in SVZ and SGZ by hyperoxia exposure which can also be reversed by Caspase-1 inhibition [16].

In humans brain maturation during third trimester involves several dynamic processes such as cellular maturation, outgrowth, and the formation of connectivity. During this critical phase of maturation with highest vulnerability, exposure to hyperoxia leads to neuronal and glial cell death, as well as alteration of synaptogenesis and myelination in prematurely born infants [6]. In rodents, this rapid growth period of brain is seen in the first postnatal week and oxygen exposure during this period resulted in increased apoptosis, decreased neuronal density, impaired neuronal networks and hypomyelination in experimental models [6,21,22]. In the present study, we succesfully demonstrated that hyperoxia resulted in an increase in NLRP3 signal in PFC, hippocampus and PC regions of newborn mice shown by immunohistochemistry. This finding was also confirmed by Western Blot analysis. Hyperoxia resulted in increased Caspase1 in PFC and PC regions. There was a significant neuronal loss in the PFC, CA1, DG and PC regions and global oligodendrocyte injury in the hyperoxic group.

IL-1β is a well-recognized mediator in hyperoxic brain injury and plays a pivotal role in cerebral pro-inflammatory cascade [35,36]. Upon activation, microglia process and release IL-1β in an inflammasome driven manner which induces an innate immune response in the brain

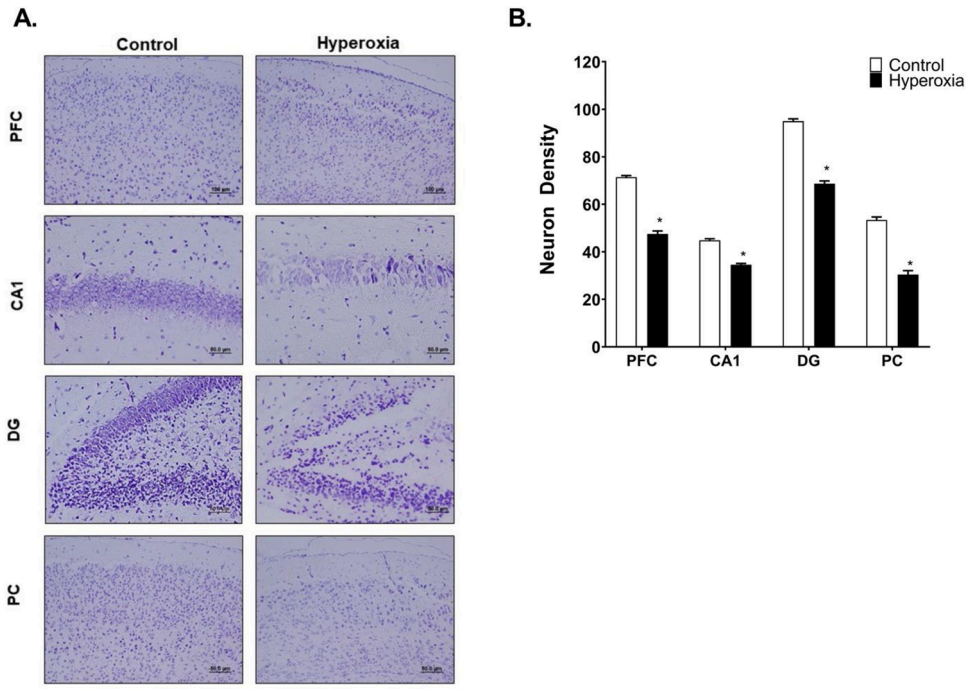

Fig. 4. **A:** Cresyl violet stain with Nissle positive cells. Neuronal density in the PC, CA1, DG and PC regions of were lower in hyperoxia group when compared to control. **B:** Neuronal density in hyperoxia group was significantly lower than control group ($p \leq 0.05$). Immunopositive cells (X100 magnification) are shown in square.

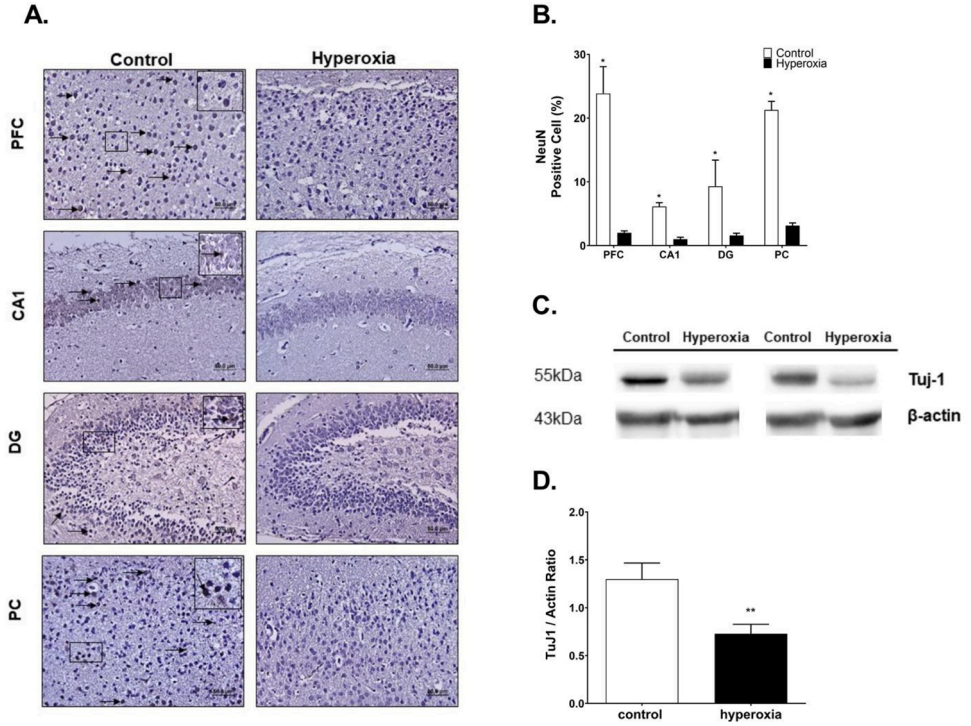

Fig. 5. **A:** NeuN immunohistochemistry. NeuN signaling was decreased in in PC, CA1, DG and PC regions of newborn mice following hyperoxia. Black arrows show immunopositve cells. **B**: NeuN positive cells were significantly decreased in hyperoxia group compared to control. **C, D;** Tuj-1 protein levels was significantly decreased in hyperoxia group compared to control group ($p \leq 0.05$). Immunopositive cells (X100 magnification) are shown in square.

tissue. ROS accumulation is one of the factors that couples microglial secretion of IL-1β. Molecular evidence for inflammasome activation in the brain tissue mostly relies on demonstration of increased levels of IL-1β along with increased expression of inflammasome components [37] and our results clearly indicate elevated levels of this cytokine.

Current practice in care of preterm infants involves targeting of oxygen saturations between 91 % and 95 % which is associated with lowered risk of mortality when compared with saturations between 85 % and 89 % [26,27,28]. Oxygen saturation targeting is important in means of lowered risk of mortality and mortality [28], however

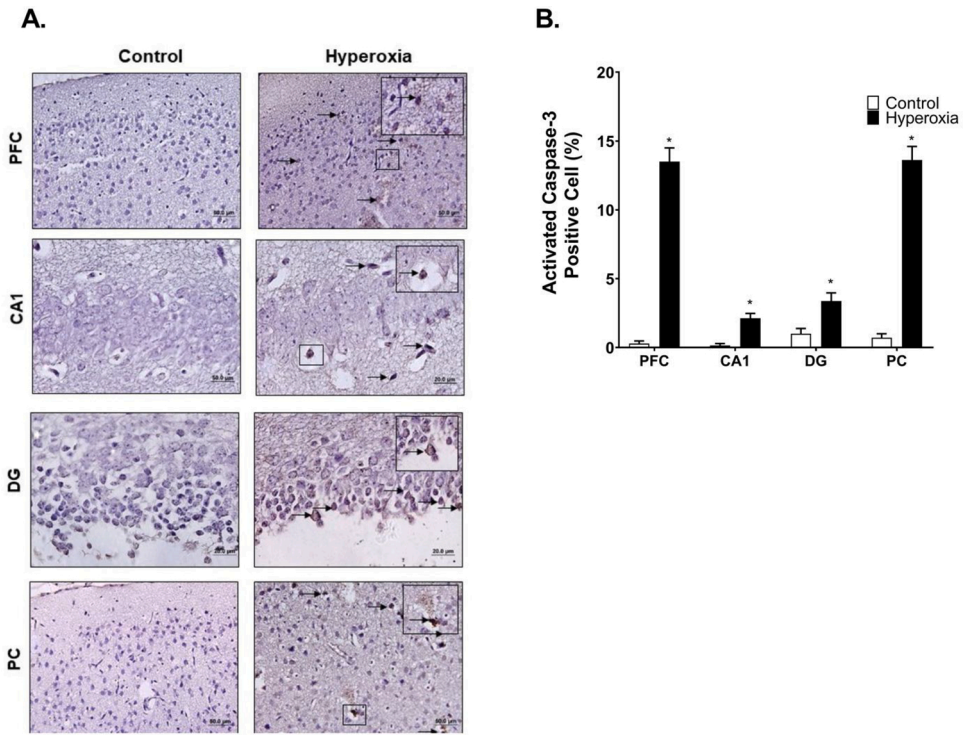

Fig. 6. A: Activated Caspase-3 immunohistochemistry. Activated Caspase-3 signaling was activated in PC, CA1, DG and PC regions of newborn mice following hyperoxia. Black arrows show immunopositive cells. **B:** Activated Caspase-3 positive cells were significantly increased in hyperoxia *vs* control group in PFC and PC regions ($p \leq 0.05$). Immunopositive cells (X100 magnification) are shown in square.

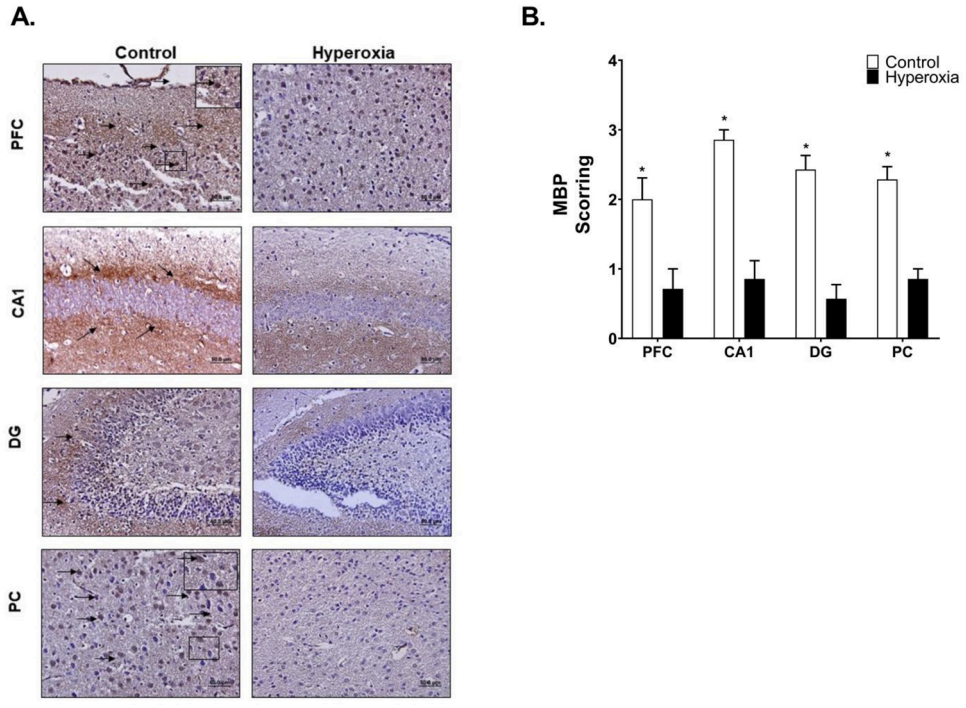

Fig. 7. A: MBP immunohistochemistry. MBP signaling was inhibited in PFC, hippocampus (CA1, GD) and PC regions of newborn mice following hyperoxia. Black arrows show immunopositive cells. **B:** MBP positive cells were significantly decreased in hyperoxia group when compared with control group ($p \leq 0.05$) Immunopositive cells (X100 magnification) are shown in square.

maintenance of this target may not be easy in preterm infants. Owing to their respiratory instability and tendency for apnea, preterm infants usually experience spontaneous episodes of intermittent hypoxemia and these episodes are usually associated with subsequent periods of hyperoxemia that results from excessive oxygen supplementation in order to correct the hypoxemia [29,30]. Thus, preterm infants receiving supplemental oxygen therapy usually experience hyperoxemia, despite close monitoring. Considering the significant potential of preterm

infants for hyperoxic exposure [31], unveiling the mechanism of hyperoxic brain injury is of vital importance for development of a future protective strategy. The next step in our research will be focused on whether establishment of NLRP3 inflammasome is the main pathway in the pathogenesis of cellular death or not. In order to enlighten this point, we planned to work with NLRP3 knock out mice and functional inhibitors.

It has been shown that hyperoxia results in permanent impairment of mitochondrial function in the hippocampal area of rodents [32,33]. Moreover, recent studies have revealed a key association between mitochondrial damage and establishment of the NLRP3 inflammasomes [34]. Downstream mechanisms of hyperoxia, regarding this association inherits a valuable opportunity for future research.

In conclusion, this study shows that in early life, high levels of oxygen exposure results in NLRP3 inflammasome formation in mouse model of preterm brain injury. Future studies focusing on this pathway are warranted for providing clinicians not only a deepened understanding of the hyperoxia induced brain injury but also new insights for developing effective neuroprotective strategies in preterm infants that require prolonged support of oxygen.

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CRediT authorship contribution statement

Serap Cilaker Micili: Conceptualization, Methodology, Writing - review & editing. **Defne Engür:** Conceptualization, Methodology, Writing - review & editing. **Sermin Genc:** Project administration. **Ilkcan Ercan:** Methodology, Resources. **Sıla Soy:** Methodology, Resources. **Bora Baysal:** Investigation. **Abdullah Kumral:** Supervision, Project administration.

Declaration of Competing Interest

None.

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