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# MicroRNA Expression Profiles in the Neonatal Rat Hippocampus Exposed to Normobaric Hyperoxia

**Yuewei Xia[1,2], Tong Liu[1], Ruolin Zhang[1], Tao Bo[1*]**

[1]Department of Pediatrics, The Third Xiangya Hospital, Central South University, Changsha, China
[2]Department of Neonatology, The Changsha Maternal and Child Health Hospital, Changsha, China
Email: *boily@yeah.net

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## Abstract

**Objective:** This study aimed to identify differentially expressed microRNAs (miRNAs) using microarray and to predict special target genes using bioinformatics methods in the neonatal rat hippocampus after normobaric hyperoxia exposure, and to unravel the molecular mechanisms of developing brain injury induced by normobaric hyperoxia. **Methods:** Eight neonatal Sprague-Dawley rats at postnatal 1 day were divided equally between a control group and an experimental group, followed by 24-hour exposure to 21% oxygen and (95 ± 5) % oxygen, respectively. Total RNAs were extracted from the rat hippocampus. Three samples were randomly selected from each group to detect differentially expressed mRNA profiles using the affymetrix GeneChip Rat Genome 230 2.0 Array. Differentially expressed miRNA profiles were determined by miRNA enrichment analysis. The starBase software was applied to predict target genes abundantly expressed in the hippocampus, and Kyoto Encyclopedia of Genes and Genomes pathway enrichment analyses were conducted for bioinformatics analysis. **Results:** Microarray analysis revealed 681 differentially expressed miRNAs in the neonatal rat hippocampus after normobaric hyperoxia exposure. Only one miRNA, miR-489-5p, was significantly upregulated ($P < 0.05$), and the predicted gene was *Mdfic*. The other 680 miRNAs were significantly downregulated ($P < 0.05$). Among these downregulated miRNAs, six miRNAs, miR-342-5p, miR-449a-3p, miR-200b-5p, miR-200a-5p, miR-125a-3p, and miR-324-5p, were extremely significantly downregulated ($P < 0.01$), and the predicted target genes included *Gjb6* and *Bnc2*. KEGG analysis indicated that differentially expressed miRNAs were closely related to multiple signaling pathways. **Conclusions:** Differentially expressed miRNA profiles in the neonatal rat hippocampus after normobaric hyperoxia exposure may be involved in the physiopathological processes of developmental

midbrain injury induced by normobaric hyperoxia.

**Keywords**

Normobaric Hyperoxia, MicroRNA, Neonate, Brain Injury, Hippocampus

## 1. Introduction

A neonate is a developing individual with immature organs. In neonates, improper treatment with normobaric hyperoxia can cause serious organ damage, leading to poor prognosis. Previous studies have focused on the relationship between retinopathy of prematurity, or chronic lung disease, and normobaric hyperoxia [1] [2]. Normobaric hyperoxia can also cause irreversible damage to the developing brain, closely related to periventricular leukomalacia and intracranial hemorrhage in premature infants [3] [4]. The mechanism by which normobaric hyperoxia causes developmental brain damage is a complex process involving various factors [5]-[7].

MicroRNAs (miRNAs) are a set of non-coding single-stranded RNAs of approximately 22 nucleotides in length that post-transcriptionally regulate gene expression. Alteration in miRNA expression results in changes in the profile of genes involving a range of biological processes, contributing to numerous human disorders [8] [9]. MiRNAs have been found to be expressed in high levels in the brain, and they play important regulatory roles in the plasticity and survival of nerve cells [10] [11]. Differentially expressed miRNAs have been found in numerous cases of brain damage due to various causes [12] [13].

It is still unclear whether differentially expressed miRNAs are observed in normobaric hyperoxia-induced developmental brain damage; if so, discovering the significance of differentially expressed miRNAs is of interest. In this study, an animal model of normobaric hyperoxia exposure was established. Using this animal model, we detected differentially expressed mRNAs in the neonatal rat hippocampus exposed to normobaric hyperoxia by mRNA profile analysis and predicted the variation of miRNA expression profiles and related target genes by bioinformatics methods. Furthermore, we attempted to elucidate the mechanism of developmental brain damage caused by normobaric hyperoxia.

## 2. Materials and Methods

### 2.1 Animal Grouping and Models of Normobaric Hyperoxia Exposure [14]

Eight newborn Sprague-Dawley rats from the same mother rat at postnatal day 1, irrespective of gender, were purchased from Hunan SJA Laboratory Animal Co., Ltd. (license No. SCXK [Xiang 2014-0012]) and randomly divided into two groups (n = 4/group). All animal studies were performed in strict accordance with Chinese legislation on the use and care of laboratory animals and also with the guidelines

established by the Institute for Experimental Animals at Central South University. This study was approved by the IRB of The Third Xiangya Hospital of Central South University and it received expedited approval (No: 2021-XMSB-0045).

All neonatal rats were fed by foster mothers. In the experimental group, neonatal rats and their foster mothers were placed in an experimental oxygen box under exposure to (95 ± 5) % oxygen, simulating normobaric hyperoxia. In the control group, the feeding conditions were the same as those in the experimental group, except that the animals inhaled air. After 24 hours of normobaric hyperoxia exposure, the neonatal rats were decapitated to separate the left and right hippocampi, which were then placed in 1.5 mL centrifuge tubes and stored in liquid nitrogen. Three rat hippocampal specimens from each group were randomly selected for microarray examination, and the other hippocampal specimens in each group were reserved for use.

## 2.2. RNA Isolation, Purification and Amplification

Total RNA was extracted using a Trizol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instruction and purified using a RNeasy Mini Kit (QIAGEN, Inc, Valencia, CA, USA). In vitro, amplification and biotin labeling were performed with the total RNA sample to be tested as a template. The labeling process was performed using a MessageAmp™ Premier RNA Amplification Kit (Amibion, Austin, TX, USA), and 100 ng of RNAs were used for synthesis and amplification of the first strand cDNA, double-stranded cDNA, and biotinylated antisense RNA. mRNA and cDNA were quantitatively analyzed by Nanodrop 2000 (Thermo, Waltham, MA, USA).

## 2.3. Gene Chip Analysis

Biotinylated cDNA (15 μg) samples were hybridized with Rat GeneChip Transcriptome 2.0 arrays (Affymetrix Inc., Santa Clara, CA, USA). Microarray hybridization was performed in a GeneChip® Hybridization Oven 645 (Affymetrix Inc.) for 16 hours at 45˚C and 60 rpm. Hybridized samples were automatically washed and stained using a GeneChip Hybridization Wash and Stain Kit (Affymetrix Inc.) on an Affymetrix fluidics station 450 (Affymetrix Inc.) and scanned using an Affymetrix Scanner 3000 7G (Affymetrix Inc.).

With the .CEL file as the source file, data were preprocessed using the Range Migration Algorithm (RMA) [15]. Unusable data, background noise, and other interference signals were removed, and the original data were analyzed. Cluster analysis was then executed for sample correlation, sample similarity, and grouping. Differentially expressed genes were analyzed to screen for differentially expressed mRNAs. miRNA enrichment analysis was conducted to determine $P$ values of miRNA enrichment by a hypergeometric test to predict the most likely affected miRNAs of differentially expressed mRNAs. The public gene prediction software starBase was used to predict target genes abundantly expressed in the neonatal rat hippocampus, and the Kyoto Encyclopedia of Genes and Genomes (KEGG) database was used for bioinformatics analysis.

### 2.4. Statistical Analysis

Rat pups' body weight data were analyzed using SPSS v 13.0 software (IBM, Armonk, NY, USA). Sample sizes in microarray were determined using R statistical software v4.0.2 and the statistical power was 0.75. The Student *t* test was used for group comparison. Normally distributed data are presented as mean ± SEM. $P < 0.05$ was considered statistically significant.

## 3. Results

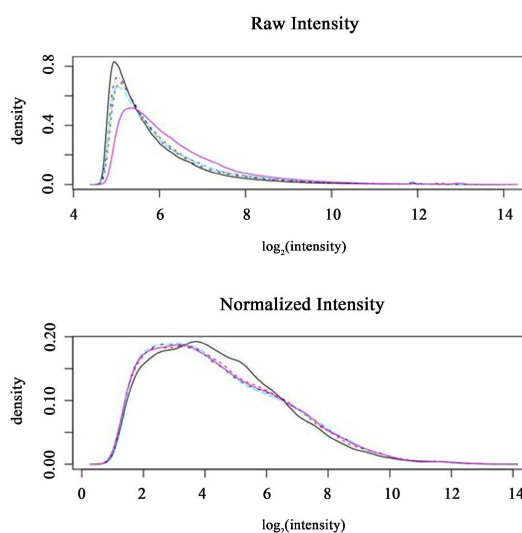
### 3.1. Neonatal Rat Models of Normobaric Hyperoxia Exposure

During the 24 hours of normobaric hyperoxia exposure, there were no deaths in the experimental or control groups, and no significant differences in rat body mass were observed between the two groups [(7.33 ± 0.44) g vs. (6.96 ± 0.69) g, $P = 0.401$, $t = 0.904$, n = 4]. There were also no significant differences in body mass of the newborn rats selected for genechip analysis [(7.14 ± 0.27) g vs. (7.12 ± 0.75) g, $P = 0.973$, $t = 0.036$, n = 3].

### 3.2. mRNA Array Analysis

Raw and normalized probe data indicated no bimodal distribution on the intensity curve with log (base2) intensities, suggesting that there was no artifact defect.

We preliminarily assessed data distribution based on a box plot. Original and normalized mRNA data were distributed symmetrically and dispersed consistently on the box plots composed of the minimum, first quartile, median, third quartile and maximum values of mRNA array data from each hippocampal sample (Figure 1). The degradation curve of each hippocampal specimen showed a smooth increasing trend, with higher fluorescence intensities in the 3'-UTR region than in the 5'-UTR region, suggesting that the quality of the chip was acceptable.

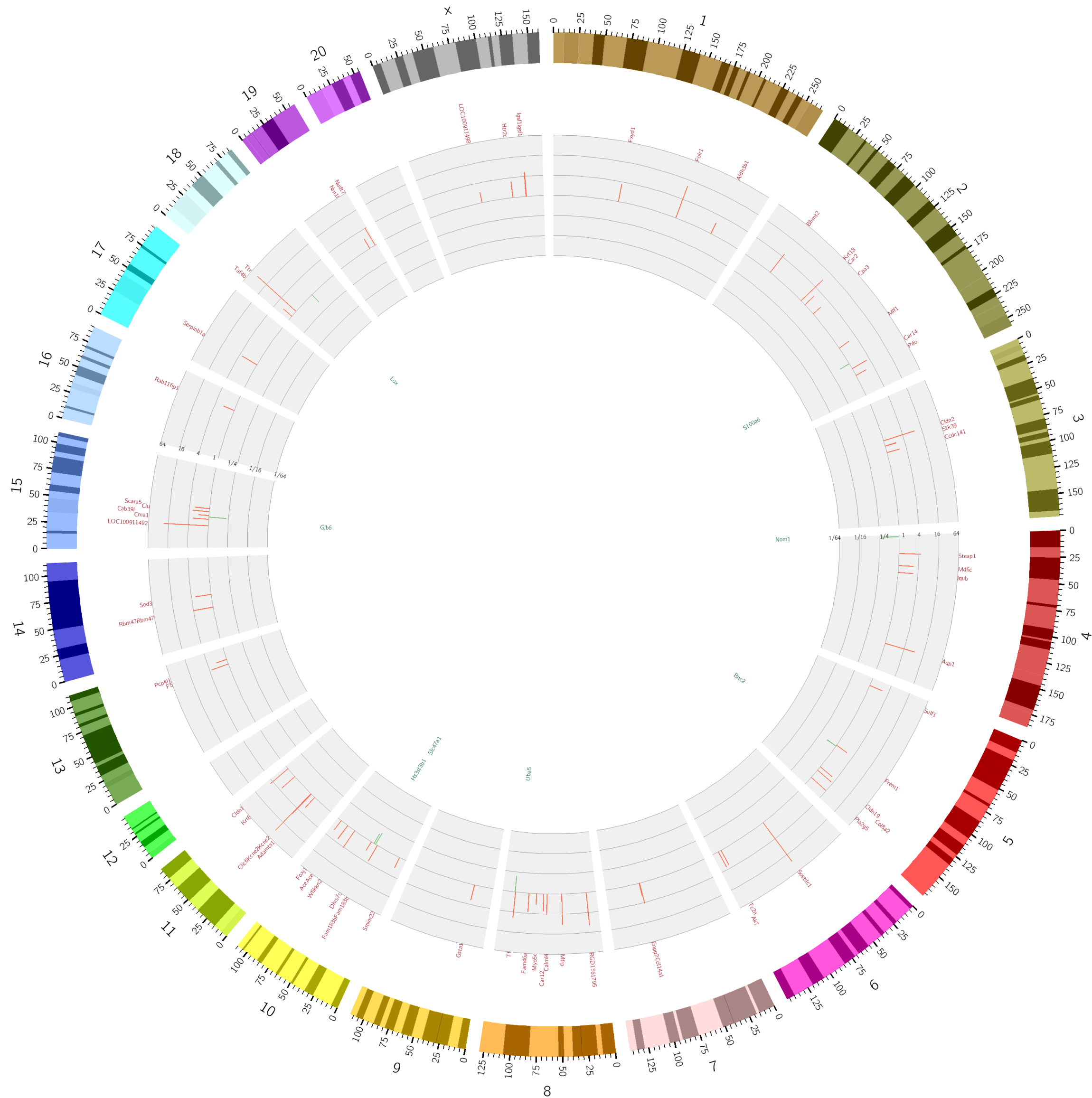



Note: The left indicates the non-normalized box plots, and the right indicates the normalized box plots. The data are distributed symmetrically and dispersed consistently.

Figure 1. Density plots of mRNA array data.

### 3.3. Screening of Differentially Expressed Genes

The SAM R package was used to identify differentially expressed mRNAs. There were 31,100 mRNAs, of which 83 were significantly up-regulated and 12 were down-regulated (**Figure 2**).



Note: Red indicates up-regulated genes, and green indicates down-regulated genes. The column length indicates the fold change, and a longer length of the column indicates a larger fold change. There were 31,100 mRNAs, of which 83 were significantly up-regulated and 12 were down-regulated

**Figure 2.** Signal ratio ring charts for HO group vs control group.

### 3.4. Prediction of miRNA Target Genes

As per starBase software analysis, there were 681 differentially expressed miRNAs in the hippocampus of the experimental group. Only miR-489-5p was significantly up-regulated ($P = 0.03$), and the predicted target gene was the MyoD family

inhibitor domain-containing (*Mdfic*) gene. The other 680 miRNAs were significantly down-regulated, among which miR-342-5p, miR-449a-3p, miR-200b-5p, miR-200a-5p, miR-125a-3p, and miR-324-5p were extremely down-regulated, and the predicted target genes were gap junction protein beta 6 (*Gjb6*) and basonuclin 2 (*Bnc2*), as shown in **Figure 3**.

As per KEGG database analysis, these differentially expressed miRNAs are involved in multiple signaling pathways in the body, including nitrogen metabolism, the renin-angiotensin system, leukocyte transendothelial migration, cell adhesion molecules, tight junctions, and metabolism of amino acids (methionine, tyrosine, cysteine), as shown in **Figure 4**.

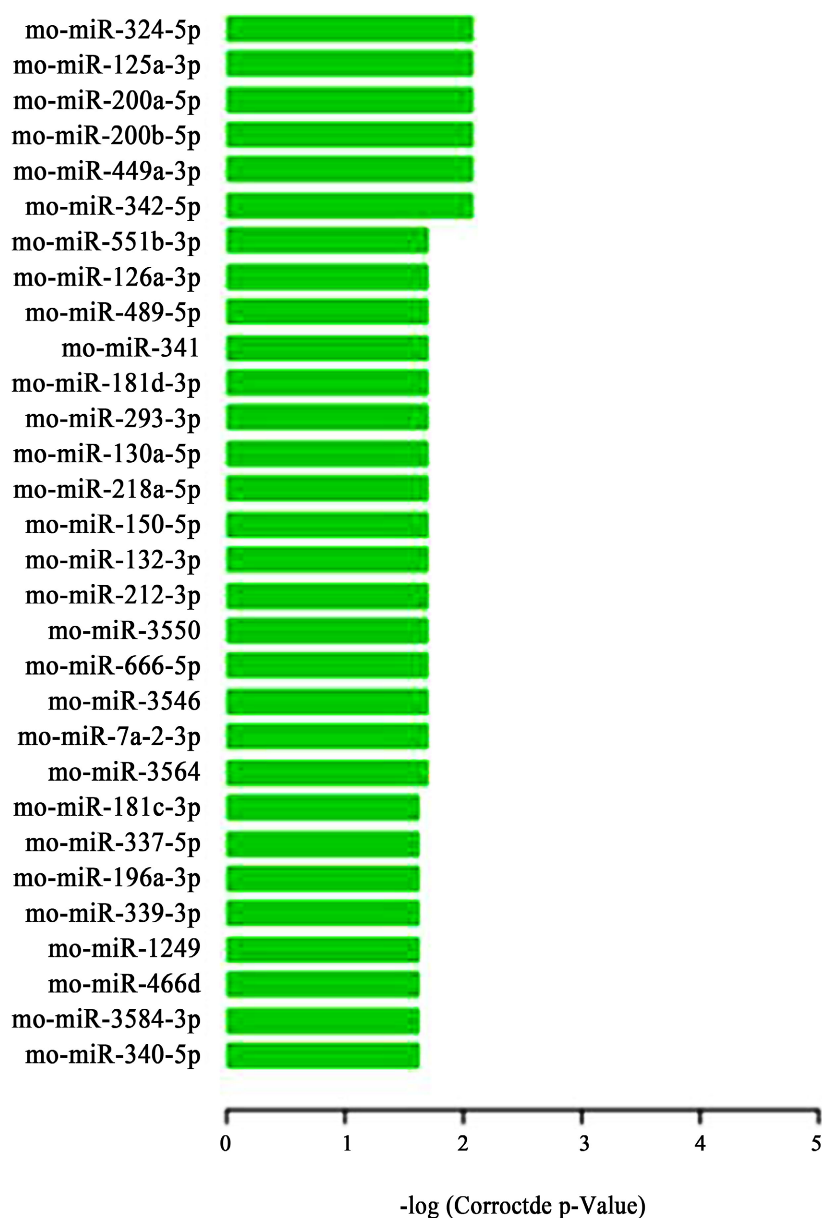



**Figure 3.** Top 30 down-regulated miRNAs in the hippocampus exposed to normobaric hyperoxia.

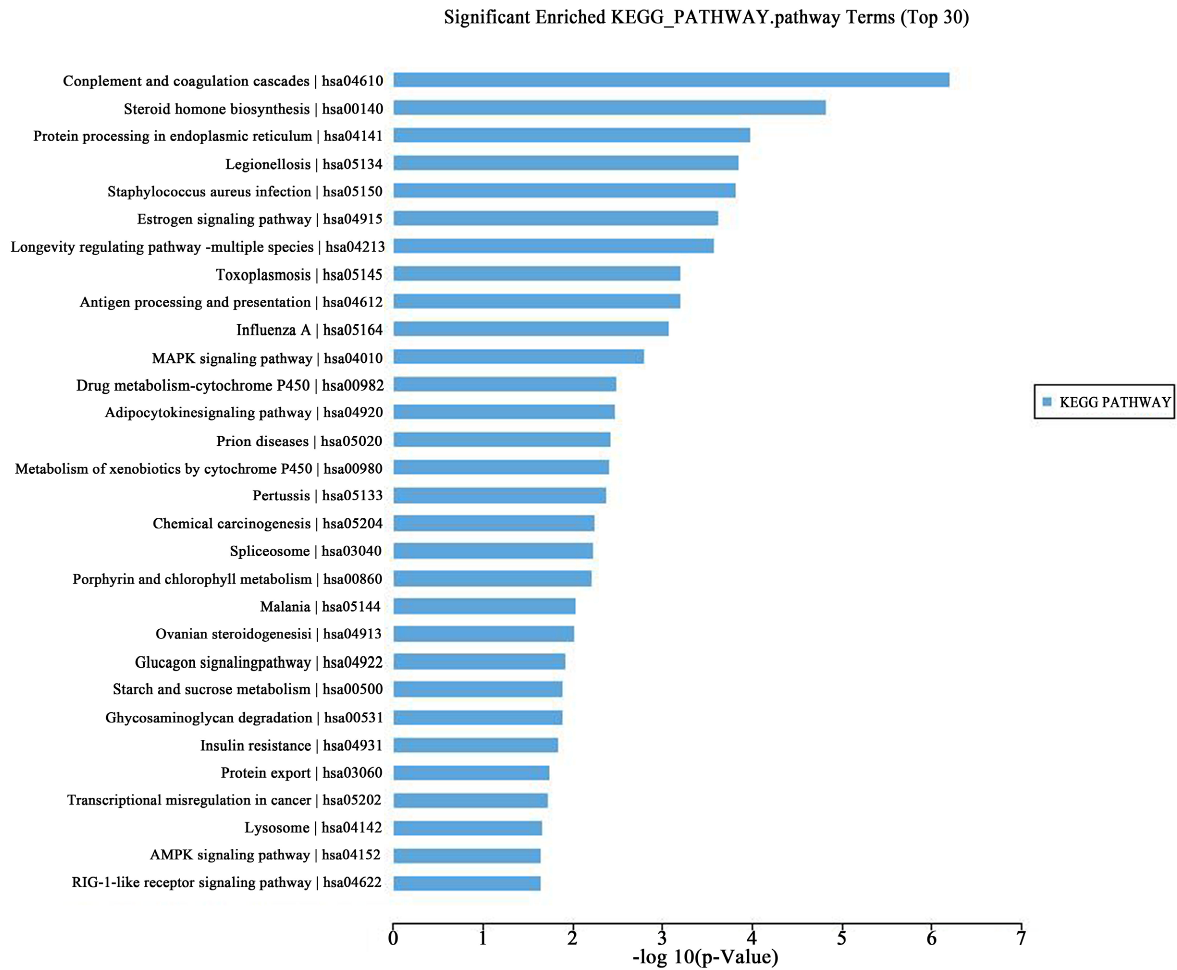



**Figure 4.** Significant enriched KEGG pathways (top 30) of differentially expressed miRNAs in the hippocampus exposed to normobaric hyperoxia.

## 4. Discussion

To date, many differentially expressed genes that are responsive to normobaric hyperoxia exposure have been identified. Normobaric hyperoxia exposure can cause up-regulation of toll-like receptor (TLR)-4 mRNA in microglia and increases in levels of tumor necrosis factor α and oxygen free radicals, initiating the inflammatory process [16]. In neonatal C57Bl/6 mice, normobaric hyperoxia can down-regulate the expression of brain-derived neurotrophic factor mRNA in the frontal cortex and up-regulate the expression of glial cell-derived neurotrophic factor mRNA. These factors are closely related to neuronal migration, differentiation, and survival, and deregulated gene expression leads to variations in neurodevelopmental programming [17] [18]. Furthermore, hyperoxia can lead to variations in transcription factors associated with the regulation of gene expression. In neonatal rats at postnatal day 6, exposure to normobaric hyperoxia can cause down-regulation of genes responsible for cell growth, differentiation, migration, and vesicle transport in neural precursor cells and mature neurons in a short period, while exerting a long-term influence on the genes involved in cytoskeleton

formation, intracellular transport, synapse formation, and energy metabolism [7].

Now, it has been demonstrated that cytoplasmic mature miRNA binds to Ago protein to regulate the expression of related downstream target genes. The post-transcriptional complex can partially base-pair to complementary sequences of target mRNA 3'UTR regions, reducing the stability of the target mRNA and inducing its degradation or directly inhibiting translation, thus regulating gene expression [19] [20]. As an important gene expression regulator, miRNA is widely involved in the occurrence and development of various diseases [21]-[23]. In this study, gene chip and bioinformatics techniques were used to analyze the differential expression of miRNAs in the hippocampus of neonatal rats at postnatal day 1 exposed to 24-hour normobaric hyperoxia. In the experimental group, there were 681 differentially expressed miRNAs in the hippocampus, including one up-regulated miRNA and 680 down-regulated miRNAs. Thus, short-term normobaric hyperoxia exposure can lead to significant changes in hippocampal miRNAs in neonatal rats at postnatal day 1, mainly presenting with down-regulated miRNAs, indicating that miRNAs are involved in the effects of normobaric hyperoxia in the hippocampus of neonatal rats.

A majority of differentially expressed miRNAs have been reported to be involved in developmental brain damage. In vitro transfection of miRNA-342-5p promotes differentiation of neural stem cells into intermediate neural precursor cells and inhibits differentiation of neural stem cells/intermediate neural precursor cells into astrocytes [24] [25]. MiR-342-5p is also involved in macrophage activation and inflammatory cytokine secretion, which directly inhibits Aktl through its 3'UTR region, up-regulates miR-155-induced proinflammatory factors, such as nitric oxide synthase, and is highly correlated with serum interleukin 6 and tumor necrosis factor α levels [26] [27] miR-125a-3p is responsible for regulating myelination-related genes in mammals and is recruited in the maturation of oligodendrocyte precursor cells (OPCs). Overexpression of miR-125a-3p in OPCs cultured in vitro can cause maturation of OPCs [28]. miR-324-5p inhibits the expression of voltage-gated potassium channel Kv4.2 protein in neurons, and down-regulated miR-324-5p is neuroprotective and seizure suppressive [29].

In this study, *Gjb*6 and *Bnc*2 are two target genes mainly predicted in normobaric hyperoxia-induced down-regulation of miRNAs in the hippocampus of neonatal rats. *Gjb*6 encodes a connexin protein that forms a gap junction, which is associated with the transfer of ions and metabolites between adjacent cells. *Gjb*6 is widely expressed in the brain, and its mutations are closely related to deafness and some familial ectodermal dysplasia [30] [31]. *Gjb*6 is also confirmed to be differentially expressed in hippocampal astrocytes in certain neurological diseases, including epilepsy and acute hypoxia [30]. *Bnc2* encodes a zinc finger protein that is tumor suppressive and is involved in lung cancer, ovarian cancer, and neurogliocytoma [32]-[34]. For miR-489-5p, the only significantly up-regulated miRNA found in this study, bioinformatics analysis showed that the predicted target gene was *Mdfic*, which is a member of a family of proteins characterized by

a specific cysteine-rich C-terminal domain. *Mdfic* is involved in transcriptional regulation of genomic expression and is also closely associated with tumor proliferation and metastasis [35]. Further investigations are warranted to illuminate whether these target genes alter the cell cycle of nerve cells and impact the development of the nervous system. Further verification of the significance of these changes in developmental brain damage caused by normobaric hyperoxia is also required.

According to the KEGG database, differentially expressed miRNAs are involved in multiple signaling pathways that are associated with leukocyte transendothelial migration, cell adhesion molecules, nitrogen metabolism, and the renin-angiotensin system. Nitrogen metabolism is related to the production of oxygen free radicals, and in this process, the production of nitric oxide, a gas signaling molecule, may have an effect on local vasoconstriction and vasodilation and intervene with the blood supply in the brain whether this hemodynamic change is closely related to the changes in related signaling pathways warrants further studies. The clinical significance of other signaling pathway variations also necessitates further investigation.

## 5. Conclusion

Based on the findings of the present study, 24-hour normobaric hyperoxia exposure may trigger significant changes in the expression profiles of miRNAs in the neonatal rat hippocampus. These differentially expressed miRNAs, their predicted target genes, and the involved signaling pathways are associated with inflammation, nerve cell proliferation and differentiation, and the production of oxygen free radicals. This study provides suggestions for further research on the mechanism by which normobaric hyperoxia causes developing brain injury, as well as disease diagnosis and treatment.

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## Conflicts of Interest

The authors have no potential conflicts of interest to disclose.

All authors approved the final manuscript as submitted and agreed to be accountable for all aspects of the work.

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