# Neuroprotective effect of dexamethasone and its pathway identified by NGS in neonatal hypoxic ischemic brain injury mouse model

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# Neuroprotective effect of dexamethasone and its pathway identified by NGS in neonatal hypoxic ischemic brain injury mouse model

Directed by Professor Kook In Park

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This certifies that the Doctoral Dissertation of Joohee Lim is approved.

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Above all, I am deeply grateful for my husband, Minsu Cho and two lovely sons, Yoonu and Hyunoo.

Finally, I dedicate this dissertation to my dad who I deeply look up to as a neonatologist and pediatrician, and my mom who gave loving support.

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ABSTRACT

**Neuroprotective effect of dexamethasone and its pathway identified by NGS in neonatal hypoxic ischemic brain injury mouse model**

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Perinatal hypoxic ischemic (HI) brain injury is the major cause of neonatal mortality and severe long-term neurological morbidity. HI brain injury occurs in 1-8 cases per 1000 births. Pathophysiological features of HI brain injury are complex, the multiple steps leading to cellular damage provide many opportunities for therapeutic intervention.

We aim to identify the effect of dexamethasone pretreatment in in neonatal hypoxic brain injury animal model by neuropathology and identify signaling pathway underlying the neuroprotective effects of dexamethasone pretreatment by performing NGS. We analyzed and validated DEGs related to neuroprotective effect of dexamethasone. Total 962 DEGs were confirmed. 407 DEGs were upregulated, and 555 DEGs were down regulated. Upregulated genes were related to central nervous system development, ionic homeostasis and synapse . Down regulated DEGS were related to cell metabolism. By performing the protein-protein interaction analysis among core genes in enriched pathway, DLG2, Calm1, Grin1 seemed to have high interaction.

DLG4, CAlm1, Grin1 might mediate neuroprotective effect of dexamethasone.

This study can lead to new insights on the pathogenesis of HI brain injury and offer promising interventional molecular targets leading to well-being of HI brain injured newborns.

Key words: hypoxic ischemic brain injury, newborn, dexamethasone, ngs

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## I. INTRODUCTION

Perinatal hypoxic ischemic (HI) brain injury is the major cause of neonatal mortality and severe long-term neurological morbidity. HI brain injury occurs in 1-8 cases per 1000 births.[1] Interruption of blood flow and gas exchange to the fetus in the perinatal period can trigger a cascade of neuronal injury leading to cerebral palsy, mental retardation and epilepsy.[2,3] Unfortunately, there are no definite therapeutic interventions available other than hypothermia in moderate to severe HI brain injury.[4] The advent of therapeutic hypothermia as a neuroprotective treatment has improved short-term prognosis but still affects long-term motor, sensory, cognitive, and behavioral outcome of the child.[5] Therefore, new supportive options are needed to enhance neuroprotective effects of the hypothermia. Research is currently underway to identify other agents that may be synergistic with therapeutic hypothermia. Potential agents include xenon, erythropoietin, melatonin, cannabinoids and stem cell therapy.[6-8] Additional therapeutic agents have been investigated in animal models throughout years including glucocorticoids, such as dexamethasone.[9]

The pathogenesis of HI brain injury is complex, involving short-term neuronal damage that evolves into long-term chronic inflammation.[10] Neonatal HI brain injury induces cell death, which is exacerbated by abnormal expression/activation of the ATP-dependent Na+/K+ pump, oxidative stress, and aberrant excitatory neurotransmission due to insufficient oxygen and blood flow.

Furthermore, loss of highly vulnerable axons, oligodendrocyte progenitors and neurons disrupt maturation of the neural network, with a destructive impact on the structure and connectivity of the brain.[11-13]

Recent studies reported that glucocorticoids have a potential role at neuro-inflammatory response to acute cerebral HI injury which is a major contributor to the pathophysiology of perinatal brain injury.[14] Glucocorticoids are steroid hormones secreted by the adrenal gland and have an important role in the regulation of metabolism and immune response. Dexamethasone is a synthetic, non-hydrolyzable glucocorticoid that selectively acts on glucocorticoid receptors, but not mineralocorticoid receptors.[14] Currently, glucocorticoids, especially dexamethasone are used worldwide in neonates with high efficacy, both antenatally and postnatally.[15] Glucocorticoids prevent respiratory distress syndrome antenatally by promoting pulmonary surfactant production during the fetal period and reduce bronchopulmonary dysplasia postnatally in infants requiring long term mechanical ventilation. [16,17] However, Dexamethasone use in neonates has been reported to adversely affect neurodevelopment, neurocognitive, and behavioral development and associated with higher rates of cerebral palsy.[18,19]

Corresponding with these findings, there is experimental evidence for a deleterious impact of neonatal dexamethasone treatment on hypoxic ischemic white matter injury in the developing brain due to reductions in myelin thickness and axon caliber.[20] However, glucocorticoids demonstrate both neurodegenerative and neuroprotective effects in various pathologies.[21] It is thought to be depended on the concentration of glucocorticoids on neuronal viability, anatomical diversity of glucocorticoid-mediated mechanisms in the brain, and species and strain differences. The ability of glucocorticoid to act as a suppressor of neuro-inflammation is dependent on timing, dosing, and duration of exposure after or before injury. Direct glucocorticoid administration to the

neonatal brain via intracerebral ventricular injection and intranasal administration provided neuroprotection and reduced brain damage in neonatal HI injury.[22,23] In many animal studies, pretreatment of dexamethasone has shown a neuroprotective effect.[24-26] Dexamethasone given before the insult has shown to reduce infarct volume after focal cerebral ischemia in mice brain.[27] A study suggested that dexamethasone pre-treatment contributes to apoptotic and neuro-regeneration processes via influencing the inflammatory cascade.[28] Dexamethasone also prevented long-term learning impairment compared to non-treated group in mice experiment.[29]

The mechanism by which dexamethasone exerts this protective effect is not clearly known. Interaction of glucocorticoid-glucocorticoid receptor signaling and L-PGDS-PGD2-DP1-pERK mediated pathway, up-regulation of vascular endothelial growth factor A, phosphatidylinositol-3-kinase/Akt (PI3K/Akt) pathway, down-regulation of chemokine receptor CXCR4 are suggested to play an important role in neuroprotection.[22,26,28,30]

Technological advances, including advances in next-generation sequencing (NGS), offer the vision to identify new signaling pathway. [31] Seq-based methods are now replacing microarray which can identify and quantify rare transcripts without prior knowledge of a particular gene and can provide information regarding alternative splicing and sequence variation in identified genes.[32,33] In this study, high-throughput RNA sequencing (RNA-seq) has been performed to identify the effect of dexamethasone. The comprehensive transcriptome profiling of gene expression between two conditions : dexamethasone treatment before HI and dexamethasone treatment after HI, has been conducted to understand the pathological mechanism of dexamethasone. Differently expressed genes (DEGs) and their potential functions, as well as protein-protein interaction (PPI) between them were identified. In order to validate our result, real-time quantitative reverse transcription polymerase chain reaction(PCR)

(qRT-PCR) has been done. Our study identified the potential signaling pathways such as DLG4, Grin1, Calm1, which are responsible for neuroprotective effects of dexamethasone and revealed the potential therapeutic targets for reducing HI brain injury

Pathophysiological features of HI brain injury are complex, and the multiple steps leading to cellular damage provide many opportunities for therapeutic intervention. It is well recognized that glucocorticoids are critically implicated in various pathological processes as well as the physiological regulation of growth and development in neonatal HI brain injury. However, the underlying mechanisms of neuroprotective effect and dose-depending toxicities are not well clarified. This study tries to identify the pivotal signaling pathway underlying the neuroprotective effects of dexamethasone in neonatal HI brain injury by transcriptome analysis using next-generation sequencing. We aim to provide new insights to effect of dexamethasone in potential therapeutic strategies that can benefit for preventing HI brain injury.

## II. MATERIALS AND METHODS

### 1. Establish of neonatal HI brain injury animal model

At 7 days after birth, ICR mice were subjected to permanent right common carotid artery occlusion under isoflurane anesthesia. Unilateral HI brain injury was induced, followed by exposure to 8% oxygen balanced with nitrogen gas for 90 minutes at 37°C on a hot plate in a closed plastic chamber. All animals are under animal protection regulations and maintained at 12 hours light/dark cycles. All procedures were approved by the Institutional Animal Care and Use Committee at Yonsei University College of Medicine, Seoul, Korea (Permit Number: 2015-0378 the date of approval is February 2, 2016).

**2.** Pre-treatment of dexamethasone/Post-treatment of dexamethasone

Pups are randomly assigned into three experimental groups. (1) Group A : control group (n=6); (2) Group B; dexamethasone pre-treatment group (n=8); (3) Group C: dexamethasone post-treatment group (n=7). In dexamethasone pre-treated animals, dexamethasone is given via intraperitoneal injection at 6 hours prior to HI brain injury with 0.5 mg/kg dose of dexamethasone and the equal volume of phosphate buffered saline (PBS) is given at 6 hours after HI brain injury. In dexamethasone post-treated animals, PBS is given via IP injection at 6 hours prior to HI brain injury and 0.5 mg/kg dose of dexamethasone are given at 6 hours after HI brain injury. In the control group, the same volume of PBS is injected intraperitoneally at 6 hours before and after HI brain injury, respectively.

**3.** Confirming the neuroprotective effect of dexamethasone by assessment of neuropathology

Firstly, we confirmed the potential neuroprotective effects of dexamethasone pretreatment by assessing presence of HI brain injury by gross inspection, measuring the diameter of cerebral hemisphere and cortical thickness of under microscope. Mice from each group were sacrificed at 72hours after HI brain injury. Presence of absence was noted in inspection of brains at the time of removal. The mice were perfused and post-fixed with 4% paraformaldehyde. Their brains were cryoprotected in 30% sucrose, frozen with O.C.T. compound, and cut into a series of 16 μm thick coronal sections using a cryostat. Eight serial coronal sections per mouse (spaced 160 μm apart) were stained with hematoxylin (Vector) and eosin-Y (Sigma), photographed with the Olympus BX51 microscope, and then analyzed with Image J (Broken symmetry software,

NIH) by two different persons blind to the experimental groupings. Degree of atrophy was calculated as ([Diameter or cortical thickness of the left contralateral hemisphere—Diameter or cortical thickness of right ipsilateral hemisphere]/ Diameter or cortical thickness of the left contralateral hemisphere) × 100%.

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**4.** RNA extraction and RNA-seq library construction

The injured mice are sacrificed at 72 hours after HI brain injury. Mice are deeply anesthetized and transcardially perfused with cold PBS. Brains are dissected and homogenized with Precellys Lysing kits (Bertin technologies, Cat # CK14) and Precellys 24 tissue homogenizer (Bertin technologies) in Tri Reagent (Molecular Research Center) to extract total RNA. Preparation of an RNA library using a TruSeq RNA kit (Illumina, San Diego, CA, USA) and sequencing using a HiSeq 2000 (Illumina) according to the manufacturer's instructions are carried out by Macrogen Co. (Seoul, Korea).

**5.** Data preprocessing and DEGs screening

All primary analysis of sequence data is performed with the STAR[34], HTSeq, and DESeq2 software packages. We mapped RNA-seq reads to mm10 genome assembly with STAR pipeline to quantify annotated genes and transcripts. Once the sequence reads are successfully mapped and aligns to the reference genome, HTSeq is used to count the reads mapped to each gene, and DESeq is used for analyses of differentially expressed genes (DEGs). DEGs are identified using rigorous negative binomial distribution with variance and mean linked by local regression. The transcriptome alignment output file is used by RSEM to quantify the levels of expression of genes and transcripts. Pairwise comparisons were

performed between the control group and the three treatment groups to identify significant DEGs ($P < 0.05$). In addition, principal component analysis (PCA) was conducted using R software.

**6.** Gene ontology enrichment analysis and PPI network analysis

Using DAVID database (http://www.david.niaid.nih.gov), enriched Gene ontology(GO) terms for common DEGs in all treatment groups were identified using hypergeometric tests between DEGs and GO-annotated gene sets with a cut-off $P = 0.05$. The STRING 9.1 network (http:// string-db.org/), database 36 is the one of the largest databases of direct (i.e., physical) and indirect (i.e., functional) protein-protein interactions and contains data from various sources including genomic context predictions, high-throughput experiments, co-expression analyses, and known databases. The database covers 9.6 million proteins from more than 2,031 organisms. We used this database to identify protein-protein interaction networks of the identified DEGs. Then, Cytoscape software (http://www. cytoscape.org) was used to visualize the PPI network. Proteins in the PPI network were defined as network nodes, and the number of interactions involved them was taken as their degrees. In the PPI network, the hub nodes were identified as nodes with relative higher degrees

**7.** Validation of the expression changes of key gene by Quantitative reverse-transcription polymerase chain reaction (qRT-PCR)

Quantitative RT-PCR is performed to confirm the expression levels of main upregulated genes( DLG4, Grin1, Calm1). RNA is quantified with a NanoDrop, and 1 μg of isolated RNA is reverse-transcribed into cDNA using a first-strand

cDNA synthesis kit (Roche) following the manufacturer's protocol. Synthesized cDNA is stored at −20 ℃ until use as a template in PCR. Quantitative real-time polymerase chain reaction (qRT-PCR) is performed in 384-well plates, with 0.5 μl of cDNA in a 10 μl reaction volume per well, using LightCycler 480 SYBR Green I Master mix (Roche) on a LightCycler 480
System (Roche) as follows: 95 ℃ for 5 min and 45 cycles of 95 ℃ for 10 sec, 60 ℃ for 20 sec, and 72 ℃ for 15 sec, followed by a melting curve program. Relative gene expression was normalized to that of the housekeeping gene GAPDH (sense, 5′-CCATGAGAAGTATGACAACAGCC-3′, and antisense, 5′-GGGTGCTAAGCAGTTGGTG-3′) and analyzed using advanced relative quantification based on the E-method provided by Roche Applied Science.

| *Primer name* | *PriPrimers (DNA oligomer) used for PCR experiments primer sequence (5'-3')* |
|---|---|
| GAPDH_ F | AGATGGTGATGGGCTTCCC |
| DLG4_F | CAACGACAGCATCCTGTTGTC |
| Calm1_F | ACAGATAGCGAAGAAGAGATCCGC |
| Grin1_F | TCTTCATGCTGGTGGCTGGA |
| GAPDH-R | GGCAAATTCAACGGCACAGT |
| DLG4_R | TCCACTGCAGCTGAATGGGT |
| Calm1_R | TCTGCCGCACTGATGTAACCATTCC |
| Grin1_R | TTGTGTCGCTTGTAGGCGAT |

**Table 1**. Primers (DNA oligomer) used for PCR experiments

**8.** Statistical analysis

All statistical analyses were performed using SPSS version 25 (IBM Corp., Armonk, NY, USA).Two-group comparisons were made using the Mann–Whitney U-test, while multiple groups were compared with repeated measures analysis of variance (ANOVA) followed by Bonferroni post hoc analysis for pairwise comparisons between groups. All data are represented as mean ± standard error of the mean (SEM), and $p < 0.05$ was considered to indicate a statistically significant difference.

## III. RESULTS

1. Confirming the neuroprotective effect of dexamethasone by assessment of neuropathology

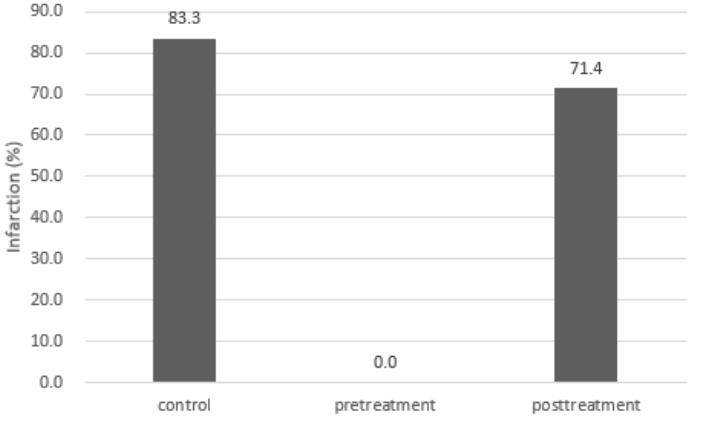



**Figure 1.** Incidence of gross cerebral infarction

Firstly, We examined the potential neuroprotective effects of dexamethasone pretreatment by animal model by assessing the neuropathology of HI brain injury. At the time of sacrifice, we inspected the brain to check presence of and degree of HI brain damage. Incidence of gross cerebral infarction was markedly

decreased in pretreatment group (Figure.1).

We conducted a morphometric analysis to confirm the effect of dexamethasone treatment in HI brain injury by measuring the diameter of cerebral hemisphere and cortical thickness by microscope. We measured the diameter of the cerebral hemisphere at the level of the dorsal hippocampus. (Table.2)

(a)

| Group | Hemisphere diameter (mm) | | p value |
|---|---|---|---|
| | Right | Left | |
| Control | 5.7 ± 0.2 | 7.9 ± 0.2 | 0.000 |
| Pretreatment | 8.0 ± 0.2 | 8.3 ± 0.5 | 0.012 |
| posttreatment | 5.4 ± 0.4 | 8.8 ± 0.2 | 0.000 |

(b)

| Group | Hemisphere diameter (mm) | | p value |
|---|---|---|---|
| | Right | Left | |
| Control | 4.2±-0.1 | 5.8±0.1 | 0.000 |
| Pretreatment | 5.3±0.2 | 5.9±0.1 | 0.022 |
| posttreatment | 3.7±0.3 | 4.7±0.2 | 0.002 |

(c)

| Group | Cortical thickness (mm) | | p value |
|---|---|---|---|
| | Right | Left | |
| Control | 0.72±-0.6 | 1.21±-0.6 | 0.000 |
| Pretreatment | 0.98±-0.1 | 1.22±0.09 | 0.088 |
| posttreatment | 0.63±0.08 | 0.94±0.1 | 0.031 |

**Table 2.** Morphologic analysis of hypoxic-ischemic injured brain

(a)Comparison of gross inspection (b) Comparison of diameter of cerebral

hemisphere under a microscope (c) Comparison of cortical thickness under a microscope. The black box represents the ipsilateral

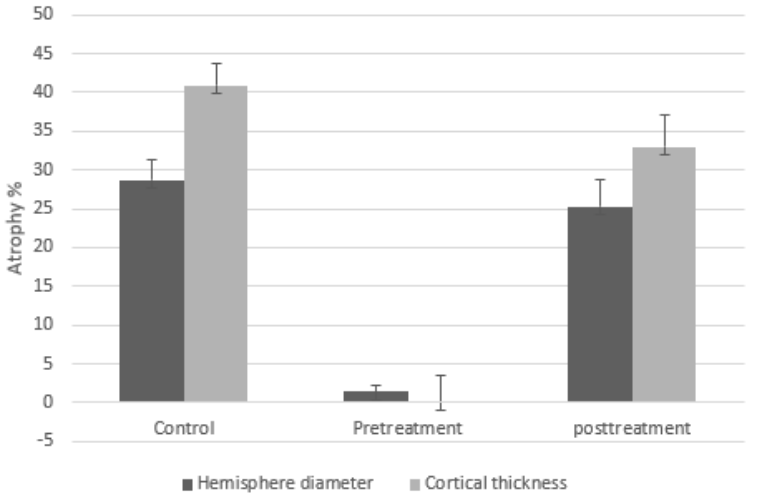



**Figure 2**. Comparison of the extent of cerebral and cortical atrophy between control group and treatment groups. Extent of atrophy was expressed % atrophy from the change in right vs left hemisphere diameter and cortical thickness measured under microscope.

Control group and post treatment group showed that hypoxic injured right hemisphere diameter and cortical thickness decreased compared to left hemisphere. Based on the data, we calculated degree of atrophy by both hemisphere diameter and cortical thickness. Pretreatment group showed significant decrease of degree of atrophy which is consistent that dexamethasone has a neuroprotective effect.

2. Identification of DEGs

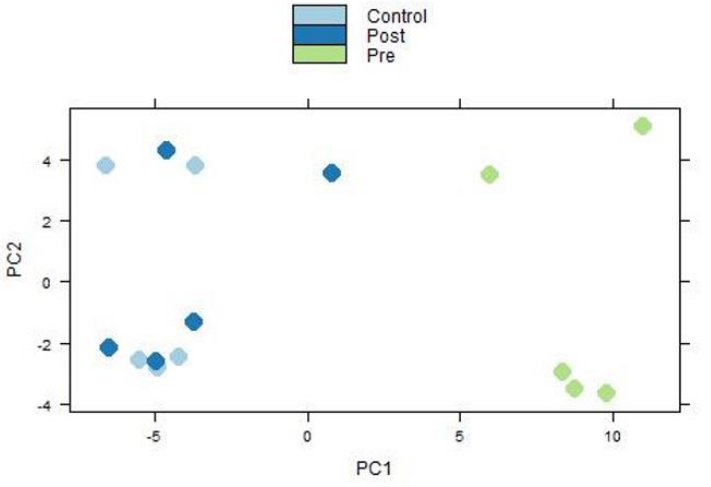



**Figure 3.** Principal component analysis (PCA) plot of dexamethasone pre, post treatment groups.
Pretreatment group show distinctive pattern in PCA plot compared to other groups

Transcriptional profiling of control, pre/post treatment group are done. First, we confirmed neuroprotective effect of dexamethasone pretreatment by principal component analysis (PCA plot). PCA analysis showed the obviously distinguished pattern in pretreatment group compared to other groups. Respectively, control group and post treatment group showed similar pattern. (Fig.2) After preprocessing of the raw data, a total of 962 DEGs (P value 0.01) were identified between pretreatment group and control group. Among these DEGs, we found 407 upregulated DEGS with p value 0.01 and 555 downregulated DEGs. In contrast, in comparing post treatment group and control group, only 28 DEG (p value 0.01) were found, which indicates that post dexamethasone group is very similar to control group. Also, from the comparison between pretreatment group and post treatment group, 282 DEGS were upregulated and 463 DEGS were down regulated.

We assumed that excluding 16 genes which was upregulated in post treatment group from upregulated DEGs in pretreatment group (A-C) can be a key DEG in neuroprotective effect. DEGs in the intersection set among E group and (A-C) seem to increase sensitivity. In the same way, we identified the intersection set among (B-D) and F for down regulated DEGs.

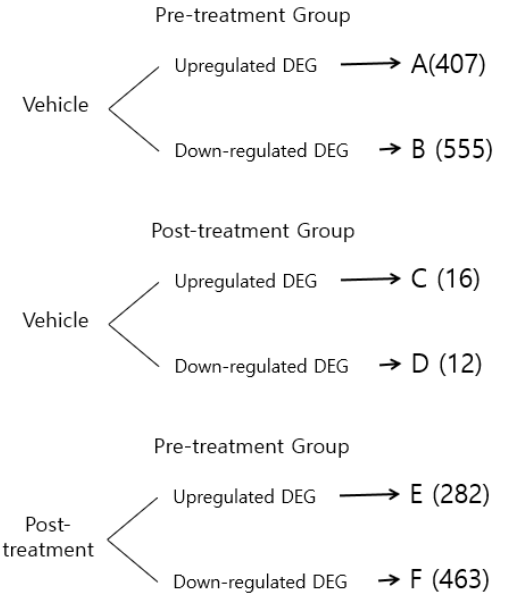



**Figure 4.** Identification and grouping of differently expressed genes (DEGs)

### 3. Functional and pathway enrichment analysis and network analysis

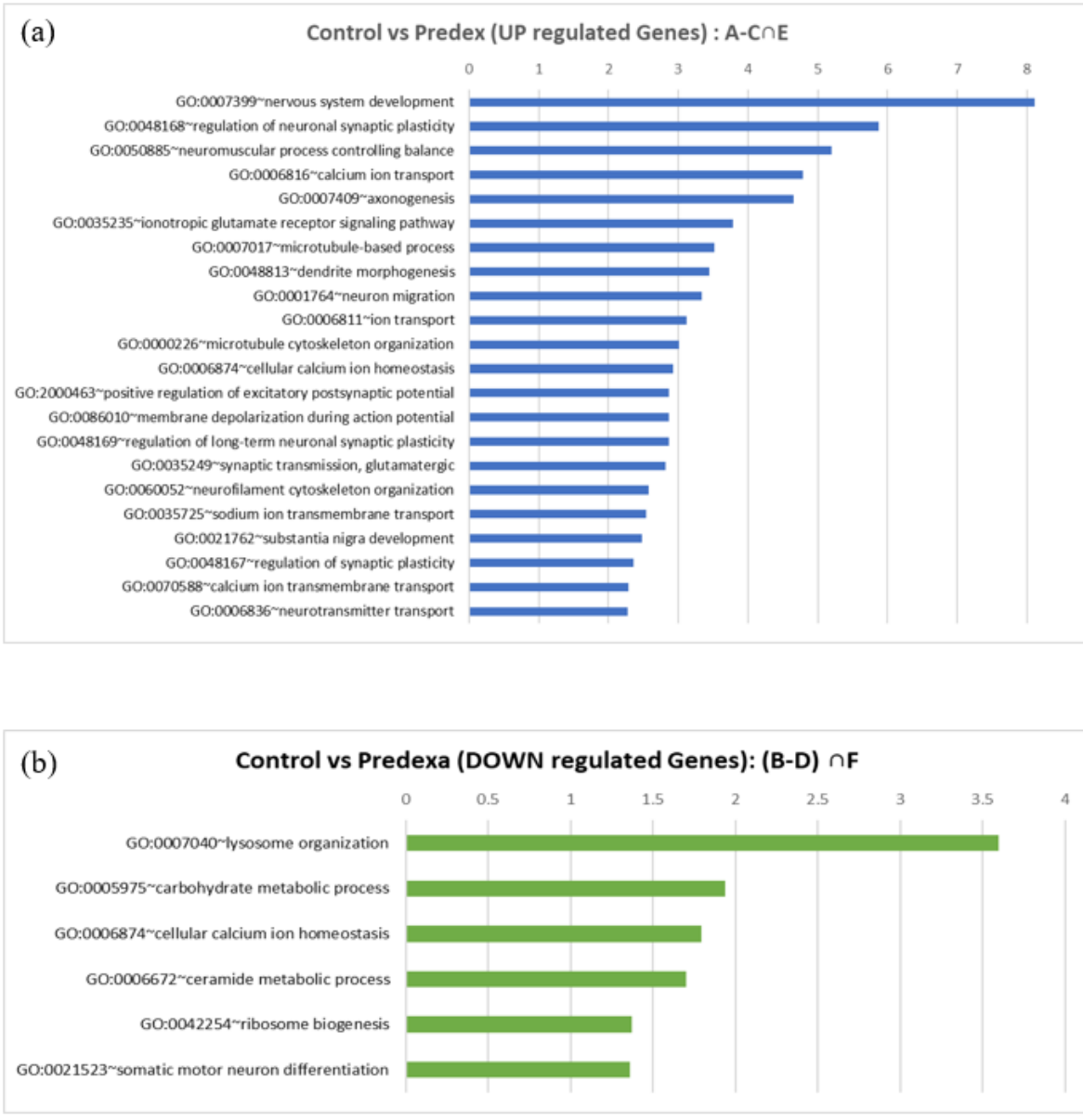



**Figure 5.** Top gene ontology(GO) terms from the fold enrichment analysis of significant differently expressed genes (DEGs) (a) shows the top enriched pathways from upregulated DEGs (b) shows top enriched pathways from down regulated DEGs.

To correlate biological information for describing the regulatory mechanism and molecular networks related in neuroprotective effect of dexamethasone, we identified the enriched pathways associated with DEGs. DEGs were assessed based on DAVID database. DEGs were conducted with functional and pathway enrichment analyses by GO terms. Up regulated DEGs in pre-treatment of dexamethasone were related to ionic homeostasis such as ion transport including calcium, inotropic glutamate receptor signaling, membrane depolarization during AP and synapse function such as neuronal synaptic plasticity, neuro migration, axon genesis, dendrite morphogenesis, microtubule skeleton organization, and glutamatergic synaptic transmission.

Down regulated DEGs were related to cell metabolism such as lysosome organization, carbohydrate metabolic process, ribosome biogenesis, phosphatidylinositol dephosphorylation, phospholipid biosynthesis process and ceramide metabolic process. We identified the core genes in enriched pathway. DLG4, Grin1, Grik5, CAMK2A, Calm1, Shank3, Nefl and other genes in figure were upregulated in enriched pathway. HEXA, HEXB, IDUA were the main down regulated genes.

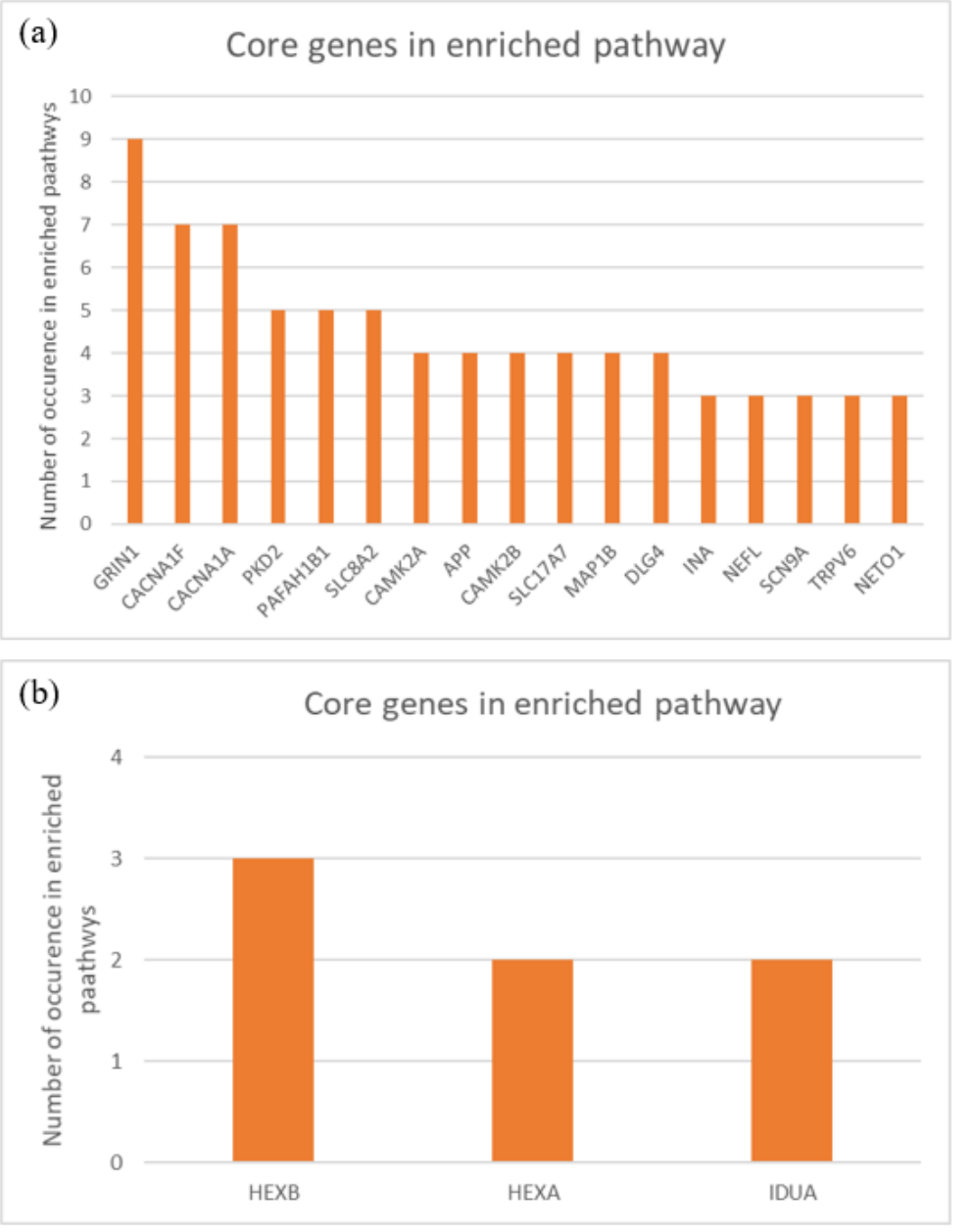
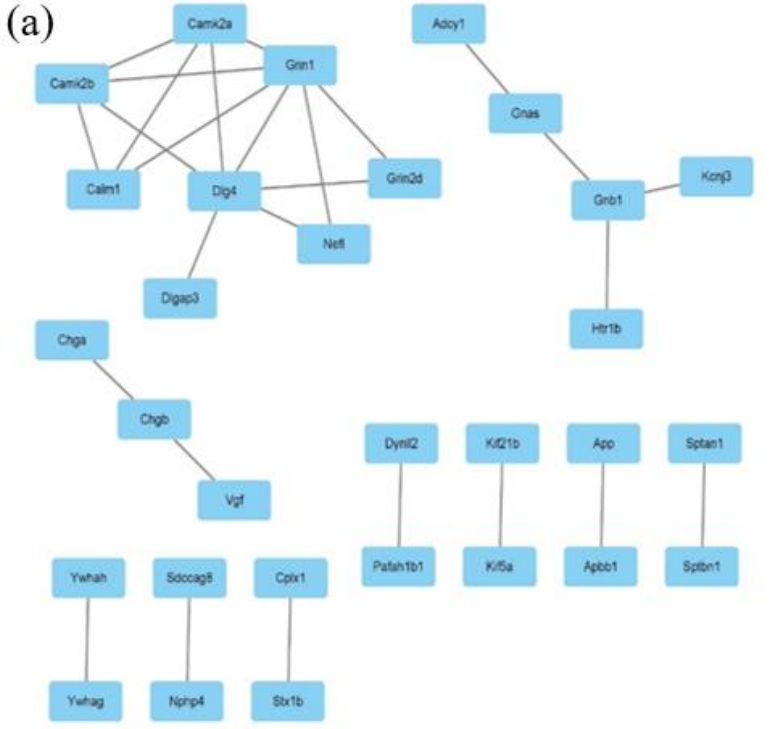



**Figure 6.** The high occurrence differently expressed genes (DEGs) from enriched pathways (a) shows the top nodes in upregulated DEGS (b) shows top nodes in down regulated DEGs.

4. PPI Network analysis

PPI network was constructed to identify topological and functional characteristics involving HI brain injury. In this study, the 962 DEGs were analyzed to construct PPI network, combined with previous reported GO classification and enrichment, for the purpose of comprehensively unraveling the molecular regulatory mechanisms



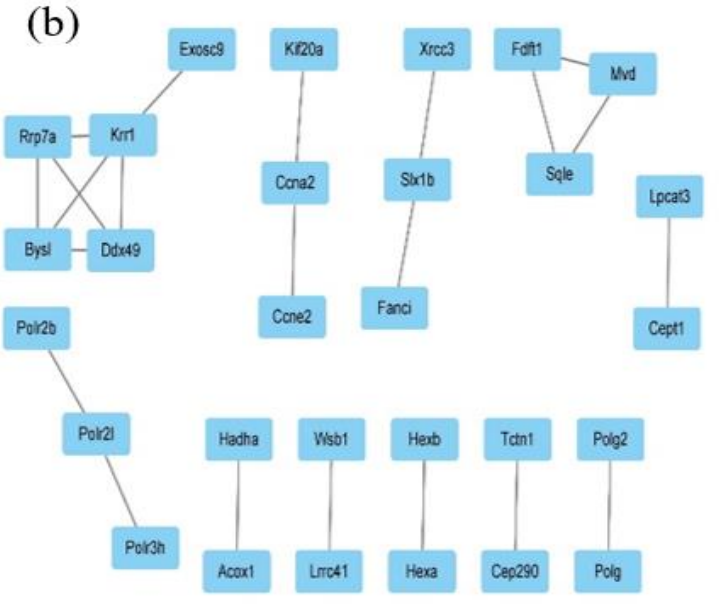



**Figure 7.** Protein-protein interaction network among differently expressed genes(DEGs)

(a) Interactions between up-regulated nodes in pretreatment group; (b) interactions between down-regulated nodes in pretreatment group. The Sub-Network Corresponds to the Significant Cluster.

Among various networks of upregulated DEGs, we visualized and sorted based on degree value and the top 0.1% of the network were selected as hub nodes and visualized as Fig4. The top hub nodes in the entire PPI network with their degrees are shown. The nodes implicated in the significantly enriched pathways that are relevant to neuroprotective effect of dexamethasone were displayed with degrees. A group including 11 nodes are seen. A cluster with DLG4, CALM1, GRIN1 are characterized by highest betweenness values in compare to the other nodes. Among 384 genes of down regulated DEGs, DDx49, Krr1, RRP7a, BYsl showed a cluster. It can be assumed that these cluster are a

suitable candidate for the genes that affect the neuroprotective effect of dexamethasone.

5. qRT- PCR

Real-time reverse transcription PCR (RT-PCR) is now widely used for quantifying levels of expressed gene transcripts. RT-PCR was performed on all samples with each primer (DLG4, Grin1, Calm1) set to determine expression of control and pre/post treatment groups. We chosee to investigate genes with highest interaction among upregulated DEGs. Results are expressed as Mean ± S.E.M. Consistent with the microarray data the PCR data showed increased tendency of expression of DLG4,GRIN1, CALM1 in pretreatment group. Especially, GRIN(p=0.001) and CALM1(p=0.000) were significantly increased in pretreatment group.

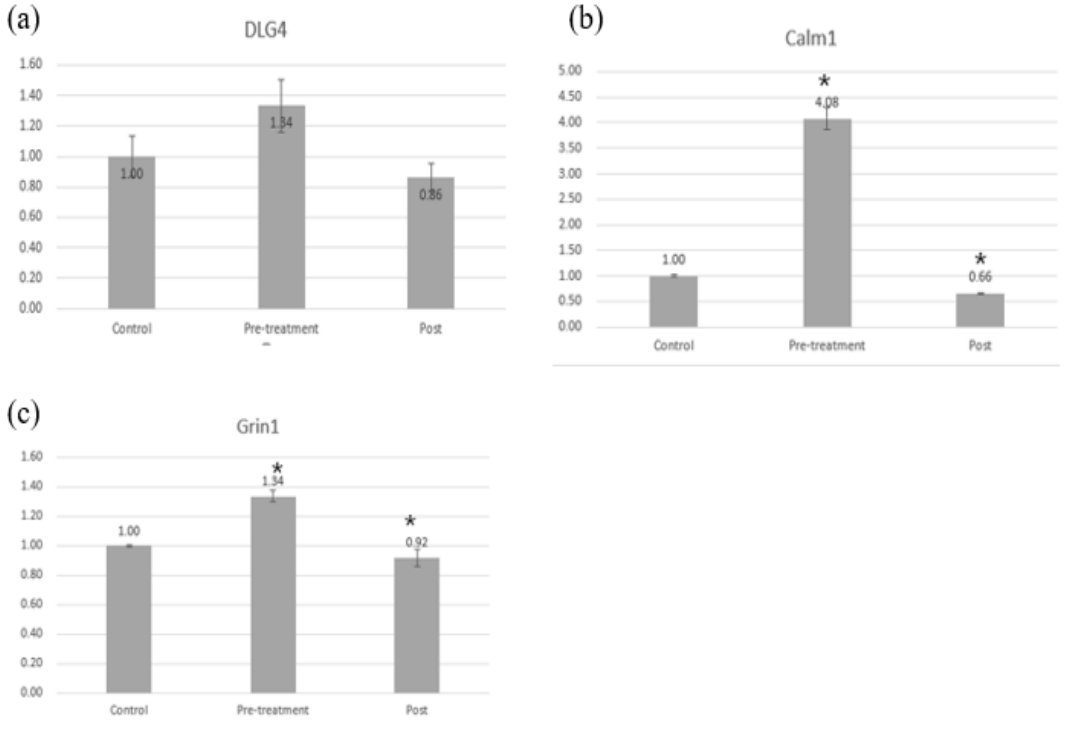


**Figure 8.** RT- PCR results for validating differently expressed genes (DEGs) identified by next generation sequencing (NGS). (a) DLG4 (b) Calm1 (c)Grin1. Data are mean fold changes in expression (± S.E.M.) Each bar represents the Mean ± S.E.M. for each of the conditions.
*Represents statistical significance ($p < 0.001$)

## IV. DISCUSSION

Our present study presents novel findings of revealing in-depth mechanism of neuroprotective effect of dexamethasone pretreatment in HI brain injury which can lead to develop new treatment options for HI brain injury. We confirmed the neuroprotective effect of dexamethasone by assessing neuropathology. RNA sequencing followed by functional enrichment analysis and PPI network analysis of DEG was performed identify the relationship and cascade of the genes involving to the neuroprotective effect. Functional enrichment analysis showed that up regulated genes play a role in ionic homeostasis and synaptic transmission regulation. Down regulated genes involved in cell metabolism.
The dense part of a network including some nodes that are closely connected together is called cluster, and main cluster of the network can provide novel information in PPI network.[35] Analysis of this sub-network leads to introduce some important proteins. Based on degree value of nodes of cluster network, it is possible that the genes to be categorized in several groups. In our analysis, we focused on several cluster, especially 3 hub genes which were upregulated and showed closest interaction by PPI analysis. It can be inferred from the PPI network that the essential biological processes are functionally connected. By module exploring, the up-regulated nodes, including DLG4, Grin1, Calm1 implicated in the pathways of cellular response to ionic homeostasis and synaptic transmission regulation. These genes were identified by qRT-PCR to

confirm their neuroprotective effect.

DLG4 shows high degree of interaction among other genes, and showed substantial increase in pretreatment group but there was no significant difference between control and pre, post treatment group. DLG4 is post-synaptic-associated gene which acts as neurodevelopmental role in synaptic plasticity in neurons, especially in the microglial inflammatory response.[36] Genetic variation in DLG4 is associated with structural differences of brain development and impacts inter-individual susceptibility to injury.[37] DLG4 interact with both NMDA receptors and form and maintain synaptic junctions.[38] Alteration of DLG4 expression by HI brain injury may cause excessive release of glutamate under pathological conditions leads to the over-activation of NMDA receptors, resulting in excessive $Ca^{2+}$ influx that activates downstream death signaling pathways and finally leads to cell necrosis or apoptosis, which is known as excitotoxicity.[39] As neonatal brains are more susceptible to excitotoxic damage[40], DLG4 may play an important role for treating neonatal HI brain injury to prevent long term outcomes related to synaptic plasticity inducing impairment of cognition, learning, and memory formation.

GRIN1 and Calm1 were significantly expressed in pretreatment group. GRIN1 is known to encode critical subunit of N-methyl-D-aspartate receptors. These subunits play a key role in the plasticity of synapses, which is believed to involve memory and learning. GRIN1 acts as hetero-tetrameric, ligand-gated ion channels with high calcium permeability and voltage-dependent sensitivity to magnesium. Channel activation requires binding of the neurotransmitter glutamate to the epsilon subunit, glycine binding to the zeta subunit, plus membrane depolarization to eliminate channel inhibition by Mg(2+).[41] Dexamethasone inducing upregulation of both DLG4, GRIN can be part of neuroprotective mechanism by modulating NMDA receptor.

Calm1 is a calcium ion modulator, which involves in cell cycle and growth. It is also known to be essential for the migration of pre-cerebellar neurons.[42] Calcium/calmodulin-dependent protein kinase II (CaMK2) is a multifunctional protein kinase that is highly expressed in the central nervous system and is activated by the binding of $Ca^{2+}$/calmodulin. It plays a crucial role in memory processing, learning and neuroplasticity in the central neurologic system.[43] Calcium channel involves in the early stage of cellular cascade of HI brain injury by influx of $Ca^{2+}$ leading to disruption of ionic homeostasis. [44] One study revealed that Calm1 showed significant fold change in human astrocyotes under hypoxia with or without hypothermic intervention. [45] Minimizing the first steps of cascade by modulating Calm1 pathway combined with hypothermia can lead to profound effect on the neurologic outcome in HI brain injury.

Other than these genes with highest interaction, upregulated genes are mainly related to synaptic regulation. Hypoxia can trigger a cascade of neuronal pathophysiologic changes such as dysregulation of synapse are one of the followed by hypoxia. Grik5 encodes a kinate-type glutamate receptor subunit.[46]Abundant GRIK5 mRNA levels have been found in the basal ganglia, especially in the striatum, substantia nigra pars compacta, and subthalamic nucleus in rat brain, which regions that are critically involved in movement control.[47] NEFL gene encodes the light chain neurofilament protein, one of the subunits that compose neurofilaments. Neurofilaments are essential for the radial growth of axons during development, the preservation of axon diameter, and the transmission of electrical impulses. Dysfunction of the NFL protein could give rise to pathology in either of the two parts of the nervous system.[48] SHANK3 plays critical roles in spine development and excitatory synaptic function. Alteration of SHANK3 is known to induce hypoactivity in pyramidal neurons.[49,50]

Among significantly down regulated genes, HEXA, HEXB and IDUA which

are related to cell metabolism are most significantly down regulated. Dexamethasone seems to have a neuroprotective effect by altering metabolism. It is according to the mechanism that therapeutic hypothermia leads to a general slowing down effect that includes reducing metabolic rate by altering enzymatic cascade and interruption of cell metabolism by various pathways.[51,52] HEXB gene produces a subunit of two related enzymes that include beta-hexosaminidase A and beta-hexosaminidase B which are mainly found in lysosomes. They break down sphingolipids, oligosaccharides and glycoproteins, and are critical in the central nervous system as they have very essential roles in breaking down the GM2 ganglioside in this system, preventing the accumulation of this product and its damage to the nervous system.[53,54] IDUA gene codes an enzyme that is required for the lysosomal degradation. Mutations in this gene are known to result mucopolysaccharidosis type I .[55] RT-PCR confirmed the mechanistic consequences of gene expression.

Limitation of this study is that our results are obtained from bioinformatics analysis and in vitro experiment, so more in-depth experiments involving human and animals should be conducted to further confirm these findings.
Brain samples in this study were collected from various dissections of mice brain without considering cell diversity depending on anatomical region. Further study obtaining transcriptional profiling from different anatomical region can identify more functional information related to neuroprotective effect of dexamethasone.

In conclusion. a total of 962 DEGs were identified. DEGs involved to ionic homeostasis, synaptic regulation were up regulated by dexamethasone pretreatment. In contrast, DEGs involved to cell metabolism are down regulated by pretreatment. More in-depth experiments should be conducted for detailed functional studies. We highlight that mediating DLG4, Grin1, Calm1 seems to enhance neuroprotective effect of dexamethasone. We expect that these genes

can be the promising therapeutic targets for preventing and treating HI brain injury.

## V. CONCLUSION

Our present study reveals a novel and detailed molecular mechanism underlying the dexamethasone mediated neuroprotective effects in HI brain injury animal model. Genes mediating ion homeostasis, synapse regulation, and cell metabolism play a key role in protection against HI injury. Although recent advance in neonatal treatment, therapeutic hypothermia is the only effective treatment in HI brain injury. We expect that these genes can be the future therapeutic options for preventing and treating HI brain injury which can improve the neurologic outcome of HI brain injury.

Our data, for the first time analyzed and validated DEGs of dexamethasone's neuroprotective mechanism. This study can lead to new insights on the pathogenesis of HI brain injury and offer promising interventional molecular targets leading to well-being of HI brain injured newborns.

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ABSTRACT(IN KOREAN)

## 신생아 저산소성-허혈성 뇌손상 동물모델에서 덱사메타손의 신경보호효과와 NGS를 통한 신호전달 경로의 분석

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임주희

신생아 저산소성-허혈성 뇌 손상 (Neonatal hypoxic-ischemic brain injury) 은 신생아에서 정신지체, 뇌성마비, 난치성 간질, 청력 및 시력장애 등의 중증 신경학적 후유증을 유발하는 중추신경계질환으로 유병률은 1000명 신생아 중 1-8명 정도에 이른다. 지난 수십 년 동안 고위험 신생아에 대한 주산기 치료가 획기적으로 발전하였고 새로운 신경 보호제 개발을 위한 많은 연구가 진행되었음에도 불구하고, 최근 치료적 저체온증 적용 시 중등도 이상의 저산소성 허혈성 뇌 손상에서 제한된 신경보호 효과를 보인다는 결과 외에, 신생아 저산소성 허혈성 뇌 손상에 대한 특별한 치료법이 없으며, 주로 일반적 대증요법이 진행되고 있는 실정이다.

본 연구에서는 저산소성 뇌손상 동물모델에서 덱사메타손 전처치군에서 신경병리학적으로 신경보호 효과를 확인하였으며 RNA를 표적으로 하는 NGS (transcriptome sequencing)의 시행을 통해 덱사메타손의 신경 보호 효과에 관여하는 중추적 신호전달 경로를 규명하고자 한다.

덱사메타손 전처치군에서 차별화되어 발현되는 유전자를 규명한 뒤 차별화 발현된 유전자들을 중합효소 연쇄반응을 통해 검증하였다. 전치군에서 962개의 차별화된 유전자가 발현되었다. 407개가 상향조절, 555 개는 하향 조절됨을 확인하였다. 상향조절된 차별화 유전자는 대부분 신경계의 발달, 이온과 시냅스 조절에 관여하였고 하향 조절된 차별화된 유전자는 대부분 라이소좀 구성과 탄수화물, 리보솜, 지질 등의 대사와 관련된 기능에 연관됨을 확인하였다.

증강된 신호전달 경로에서 발견된 핵심유전자들의 상호작용과 분포를 보기위해 protein protein interaction network를 시행하였으며, DLG, Grin1, Calm1을 중심으로 한 개의 군집을 이루며 상호작용이 증가함을 확인하였다. 하향 조절된 유전자에서는 유의미한 군집이 발견되지 않았다.

PCR을 통해 DLG, Grin1, Calm1의 발현이 덱사메타손 전처치군에서 증가함을 확인하였으며, 이들이 덱사메타손의 신경보호효과에 연관 될것으로 생각된다.
덱사메타손 전 처치와 치료적 저체온 요법을 병행하는 것은 저산소성 허혈성 뇌 손상의 신경학적 발달을 향상시키는데 긍정적으로 기여할 것으로 생각된다.

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핵심되는 말: 저산소성허혈성 뇌손상, 신생아, 덱사메타손, NGS