

Original Article

The effect and mechanism of UCH-L1 inhibitor LDN-57444 on hypoxic/ischemic injury in neonatal rats

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Abstract: To investigate how the UCH-L1 inhibitor LDN-57444 regulate hypoxic/ischemic injury in neonatal rats and its mechanism. Methods: 60 SD rats were randomly divided into three groups, the sham group, HIBD model group and intervention group, each group contained 20 cases. The three groups were further randomly divided into five sub-groups: 0 h group, 24 h group, 48 h group, 96 h group and 7 d groups (each group n=4). HIBD rat model was prepared by modified Rice method. HE (hematoxylin-eosin) staining was used to observe the pathological changes, and western-blot observe the expression of UCH-L1 and NF- κ B. With the expression of UCH-L1 was blocked by LDN-57444, the pathological changes and the expression of UCH-L1 and NF- κ B in the rat brain tissue were observed further. Results: Pathological brain structure shown that the sham group had normal brain tissue, there was no edema, necrosis and liquefaction; HIBD group and intervention group showed varying degrees of edema, necrosis and liquefaction at different times after modeling, with the extension of modeling time, edema, liquefaction and necrosis of brain tissue gradually increased. At the same time, the expression of protein UCH-L1 and NF- κ B gradually increased in rat brain tissue ($P<0.01$). With the effect of UCH-L1 was blocked of intervention group, the degree of edema, liquefaction and necrosis of brain tissue in rats significantly reduced, further observed NF- κ B protein expression levels in rat brain tissue, it also decreased significantly ($P<0.01$). Conclusion: High expression of UCH-L1 is closely related to hypoxic-ischemic brain damage in rats, the progress of hypoxic-ischemic brain damage of rats can be slowed down when the UCH-L1 was blocked, and UCH-L1 regulated this progress may be by regulating the expression level of NF- κ B in the brain tissue.

Keywords: UCH-L1, inhibitor, neonatal, hypoxia, ischemia

Introduction

Hypoxic-ischemic encephalopathy (HIE) is encephalic lesions caused by cerebral ischemia and hypoxia, which can be induced by a variety of reasons. The most commonly form of HIE is the neonatal hypoxic ischemic encephalopathy, There would be 1-3 newborns appear neonatal hypoxic ischemic encephalopathy in per 1000 full-term newborns [1]. About 15-20% of the fetus with HIE will die after birth, and about 25% of the rest surviving children will suffer serious permanent neurological sequelae [2]. According to the estimation of World Health Organization (WHO), 98% of neonatal deaths occur in developing countries, and the neonatal deaths caused by perinatal asphyxia and birth injury accounts for about 29% [3].

Neonatal hypoxic ischemic encephalopathy is encephalic lesions caused by lacking of oxygen

in the perinatal stage of neonatal, and its common pathogenesis is intrauterine fetal distress, which can be caused by various of causes such as umbilical cord around the neck, abnormality in amniotic fluid and so on. It is also often found in asphyxia in the process of delivery and post birth, as well as (though rare) in brain damage caused by other conditions [4-8]. Moreover, recent studies have shown that, the abnormal expressions of many proteins and signal transduction molecules are closely related to the occurrence and development of neonatal hypoxic ischemic encephalopathy, such as S-100 protein, UCH-L1 (ubiquitin C-terminal hydrolase L1), JNK signal pathway and NF- κ B signal pathway, etc. Among them, S-100 protein belongs to Ca²⁺-binding protein family, including two family members of S-100 and S-100 β . According to the research of Shi et al. [9], the expression level of S-100 β in serum is closely related to the clinical manifestations and severity of neonates with

hypoxic ischemic encephalopathy; the research of Li, et al. [10] showed that JNK /FOXO3a/Bim signal pathway is closely related to brain cell apoptosis, and that the activation of JNK signal pathway is closely related to brain damage induced by hypoxia and ischemia; and the research of Kielland showed that NF- κ B signal pathway is also involved in the process [11].

Ubiquitin C-terminal hydrolase L1 (UCH-L1) is a multi-functional protein highly expressed in neurons throughout the brain [12]. It is one of the most abundant proteins in the brain, accounting for about 2% of total brain proteins. The main function of UCH-L1 is to stabilize the ubiquitin monomer [13] and inhibit its degradation [14]. It is highly specifically distributed in the neurons which results in its specific expressional mode [14], and makes it an important marker of the nervous system. Studies have showed that UCH-L1 can closely interact with the cytoskeletal proteins, and play important roles in the axonal transportation and the maintenance of axonal integrity [16, 17]. The study of Zaigham et al. [18] showed that the concentration of UCH-L1 in umbilical cord blood is closely related to the development of neonatal hypoxic ischemic encephalopathy. The study of Douglas-Escobar et al. [19] showed that the serumal concentration of UCH-L1 is closely related to the severity of neonatal hypoxic ischemic encephalopathy. At present, the mechanism of neonatal hypoxic ischemic encephalopathy led by UCH-L1 has not been reported in the literature. In this study, the model of rats with hypoxic-ischemic brain damage (HIBD) was constructed, the role of UCH-L1 in HIBD was observed, and the related mechanism was discussed. Results are reported as follows.

Materials and methods

Reagents

Hematoxylin-eosin (HE) staining kit was purchased from Beijing Biyuntian Biological Reagent Co., Ltd.; UCH-L1 inhibitor LDN-57444 was purchased from MCE company in the United States; Goat anti mouse antibody against UCH-L1 and Goat anti mouse antibody against NF- κ B were purchased from Cell Signaling Technology company in the United States; Antibody against actin as internal control reference was purchased from Santa Cruz company; Optimal cutting temperature com-

pound (OCT) for brain tissue embedded was purchased from American Sakura company; Mounting reagents were purchased from Zhongshanjinjiao Biotechnology Co., Ltd.; Protein concentration detection kit and CBA kit were purchased from American Thermo company.

Rats with hypoxic-ischemic brain damage (HIBD) model construction method

7-day old SD rats were purchased from Beijing Weitong Lihua Experimental Animal Technology Co. Ltd. Each rat was 18-20 g weight, with no limitation of sex, and the total number of rats was sixty. Sixty SD rats were randomly divided into 3 groups (20 rats in each group), respectively for HIBD model group, sham operation group and intervention group. The sham operation group and HIBD group were randomly divided into five subgroups (4 rats in each subgroup): 0 h, 24 h, 48 h, 96 h group and 7 d group, respectively. HIBD rat model was constructed according to the modified Rice method reported in the literature [20], the specific steps were: firstly, SD rats were placed in a jar with ether, the SD rats were taken out after completely anaesthetized without limbs muscle tension; the rat was supine placed on the operating table, and its limbs and head were fixed; the neck skin of SD rat was routinely iodophor sterilized, then the iodine was removed with 75% alcohol; an incision with length about 0.5 cm was made in the middle part of the neck of SD rats; the sternocleidomastoid muscle on the left side of the neck was found, the muscle was separated down along the sternocleidomastoid muscle, until the carotid triangle (be composed of common carotid artery and its veins and vagus nerve) was found; the left common carotid artery was separated with ophthalmic scissors; double ligaments were performed on the left common carotid artery with NO. 5-0 surgical silk thread, then, the left common carotid artery was cut off with ophthalmic scissors between the two sides of the ligaments; the SD rat skin of the neck was sutured, and iodophor sterilized again; SD rats were returned to the maternal side after operation and resumed for 2 h; the rats were hypoxic treated for 2 h subsequently (rats were placed in a sealed organic glass hypoxic cabin equipped with a water bath incubator at the constant temperature of 37°C, 92% N₂ and 8% O₂ mixed humidified gases were supplied into the sealed cabin at

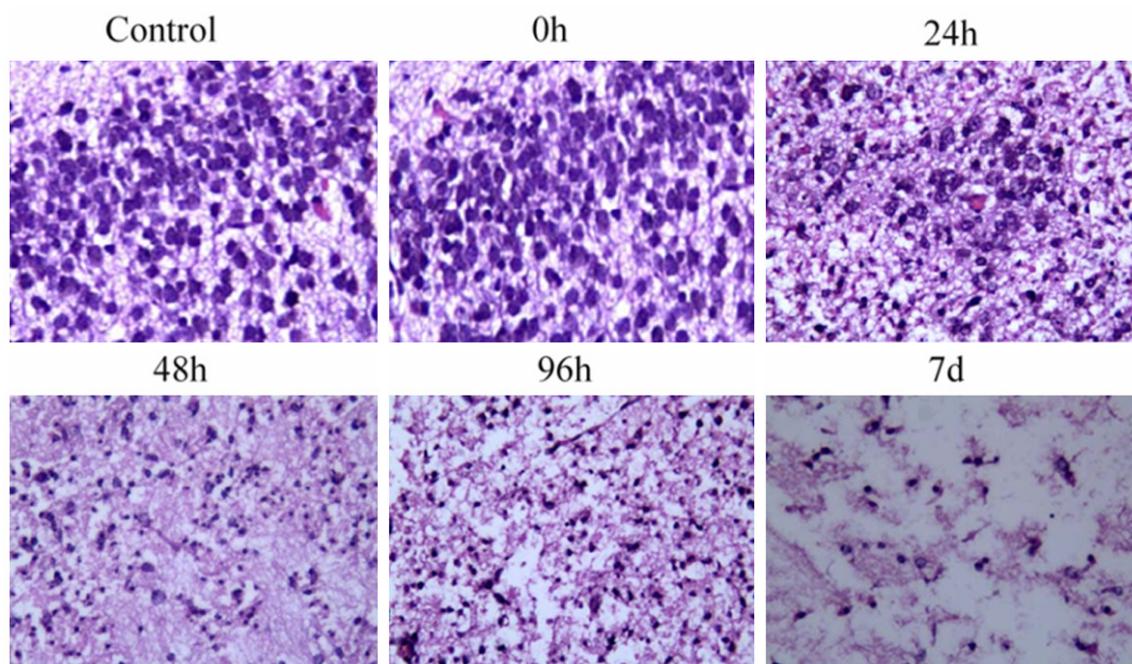


Figure 1. Pathological conditions in rats' brain tissue at different time points (10×40).

the speed of 2 L/min, observed the situation of SD rats at any time). Operated ibid for the sham operation group, the left common carotid artery were isolated but not ligated and cut off, no hypoxic treatment was performed after the rats were sutured and resumed neither. In the intervention group, rats should be hypoxic ischemic treated immediately after the effect of UCH-L1 was blocked with intravenous administration of 0.03 mg/kg of LDN-57444 inhibitor, the dose of LDN-57444 was reported in the literature [21].

HE staining of rats' brain tissue

The rats brain tissue was obtained by OCT and prepared into frozen section. The frozen sections were placed in distilled water for 2 min; stained with hematoxylin staining solution for 5-10 min (adjust the staining time according to the staining results and requirements); the sections were rinsed with tap water for about 10 min to remove the excess staining fluid, then washed again by distilled water (only for a few seconds). The sections were placed in 95% ethanol for 5 S; stained with eosin staining solution for 30 s-2 min (adjust the time according to the staining results and requirements). Washed the sections with 70% ethanol for 2 times if observe directly. Operated as the follow-up

steps if mounting was required post dehydration and transparency. The sections were dehydrated with 95% ethanol for 2 min; re-dehydrated with fresh 95% ethanol for 2 min; The sections were made transparent with xylene for 5 min; re-transparent with fresh xylene for 5 min. The sections were mounted with neutral resin. The nucleus was blue and the cytoplasm was pink or red under the view of microscope.

Western-blot method to detect the expression of UCH-L1 and NF-κB in rats' brain tissue

Method to obtain the brain protein: CaSki cells were washed with ice-PBS twice, centrifuged at 400× g for 5 min; the supernatants were discharged, 100× proteinase inhibitors and 100 μl CytoBuster protein extraction reagent were added into the precipitation of the CaSki cells, the mixture was placed at room temperature for 15 min after mixing evenly; centrifuged at 12000× g for 15 min at 4°C; the supernatant fractions were the total protein, parts of the samples were taken for the detection of the protein concentration, the remained parts were packaged for 15 ml/tube, and stored at -80°C. The concentration of the brain tissue proteins were determined by CBA method. The specific steps were: prepare the standard and working solution according the instruction of the kit.

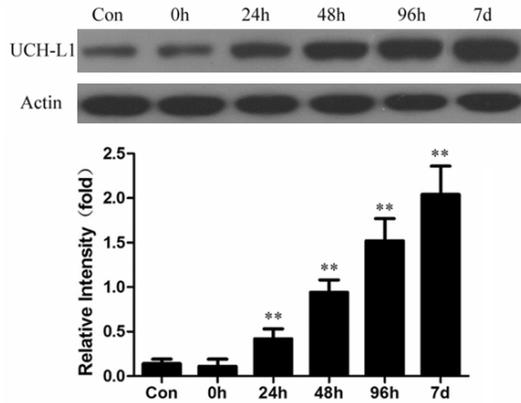


Figure 2. The expression of UCH-L1 in rats' brain tissue at different time points.

200 uL/well of working solution was added into each well of the 96-well plate, and then added 25 uL of standard solution or sample solution to each well, set 3 duplicated wells for each sample. The OD value at 562 nm wavelength was measured after incubation for 30 min at 37°C. Standard curves were drawn according the optical density value and the concentration gradient of the standard product, the protein concentration of the sample was calculated according to the standard curve and the OD value of the samples. The equivalent amount of extracted brain tissue proteins were separated by 8% SDS-PAGE separation gel and 5% concentrated gel, and then the product was transferred into the semi dry PVDF film, blocked the film with TBST containing 5% BSA at room temperature for 1 h, then, added the first antibody into the solution and incubated at 4°C overnight. Washed the film with 0.1% TBST for 3 times on the next day, each time for 5 min, added the goat anti mouse second antibody labeled with HRP to the film, and incubated at room temperature for 1 h. Washed the film with 0.1% TBST, then, the bands were developed with chemiluminescence substrate which was sensitive to Supersignal West Femto HRP. Actin was used as an internal control. All experiments were repeated at least 3 times.

Statistical methods

SPSS15.0 software was applied in the statistical analysis, *t* test method was used for the data comparison between two groups, ANOVA method was used in the statistical analysis for data comparison in multi groups. $P < 0.05$ repre-

sented the difference has statistical significance.

Results

Pathological conditions of the rats' brain tissue at different time points

No changes in the structures of rat brain tissues were found in the sham operation group at 0 h, 24 h, 48 h, 96 h and 7 d (**Figure 1**, the control group): the structures of the brain tissues were clear, cells were aligned, and no brain cells degeneration were found. Meanwhile, as shown in **Figure 1** of the model group, the structures of the brain tissues were clear, cells were aligned, no brain cells degeneration were found at 0 h; the brain cells showed degeneration and necrosis, the cellular gap widened at 24 h; the brain cell degeneration and necrosis were further aggravated, a large number of nuclear pyknosis and nuclear fragments were found under the view of microscope at 48 h; large areas of cellular liquefaction necrosis in the brain tissue of rats were found at 96 h, accompanied with a large number of cytolysis, the nuclear pyknosis and nuclear fragments were further increased; the cellular liquefaction necrosis in the brain tissue of rats were more obvious at 7 d, the number of cells were significantly reduced, the brain cells had became unobservable in some parts of the brain, and the cavity structures were found.

The expression of UCH-L1 in rats' brain tissue at different time points

As shown in **Figure 2**, the expression level of UCH-L1 protein in the rat brain tissue was gradually increased ($P < 0.01$) with the time duration of the model construction.

Pathological conditions in rats' brain tissue after UCH-L1 was blocked

As shown in **Figure 3**, compared with the pathological conditions of rat brain tissue in the model group at 7 days, the number of the necrotic brain cells significantly reduced when the UCH-L1 was blocked, and only a few brain cells appeared degeneration and necrosis. Slight nuclear pyknosis appeared in the brain cell nuclei, but the brain nuclei were still visible, and the intercellular gaps in the brain tissue

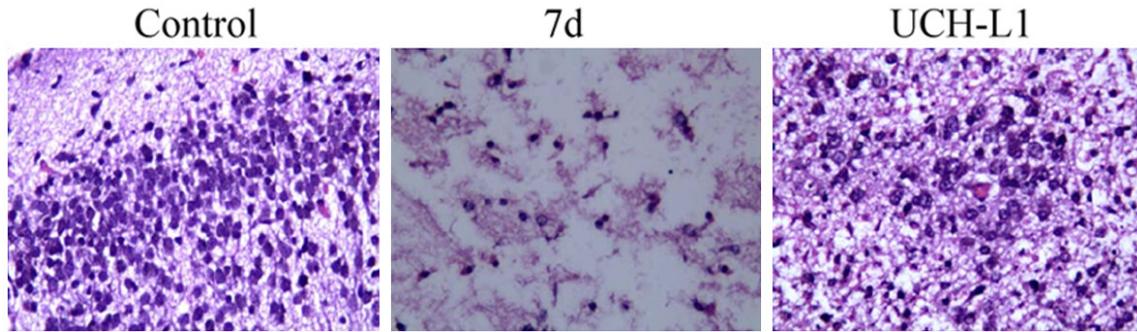


Figure 3. Pathological conditions in rats' brain tissue after UCH-L1 was blocked.

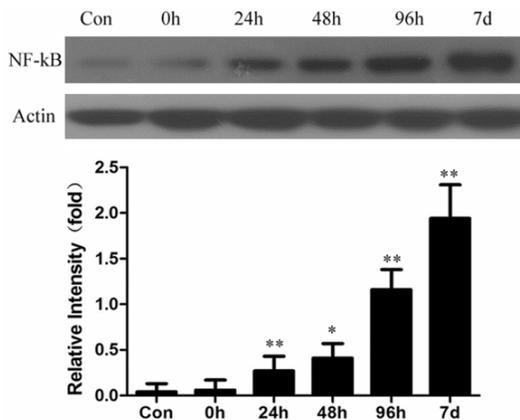


Figure 4. The expression of NF-κB in rats' brain tissue at different time points.

were significantly reduced compared with those in the model group at 7 days.

The expression of NF-κB in rats' brain tissue at different time points

As shown in **Figure 4**, the expression level of NF-κB protein in rat brain tissue was gradually increased ($P < 0.01$) with the time duration of the model.

The expression of NF-κB in rats' brain tissue after UCH-L1 was blocked

As shown in **Figure 5**, compared with that of the model group at 7 days, the expression level of NF-κB in rat brain tissue was significantly decreased ($P < 0.01$) when the UCH-L1 was blocked.

Discussions

HIE is a fetal or neonatal brain injury, which is caused by partial or complete hypoxia, cerebral

blood flow reduction or suspension resulted from a variety of perinatal asphyxia. The incidence of HIE in premature infants is significantly higher than in full term infants. Previous studies have indicated that hypoxia is the core of HIE, wherein perinatal asphyxia is the main pathogeny. At present, the mechanism of the pathogenesis of neonatal hypoxic ischemic encephalopathy is not clear. As previously mentioned, the S-100 protein, UCH-L1, JNK/FOXO3a/Bim signal pathway and NF-κB signal pathway are closely associated with the brain injury induced by hypoxia and ischemia. But the relations between the UCH-L1 and the occurrence and development of the neonatal hypoxic ischemic encephalopathy are less reported in the literature, and the research on the related mechanisms is especially limited. Therefore, the rat HIBD model was constructed to observe the effect of UCH-L1 in HIBD and discuss the related mechanism, and the following results were obtained.

In this study, the HIBD model was prepared by the modified Rice method. The pathological changes of rat brain tissue were observed by HE staining. The expression of UCH-L1 in rat brain was observed by Western-blot method, and the role of UCH-L1 in the development of HIBD was clearly defined. The cerebral histopathological observations showed that in the sham operation group, the brain structure was normal, and there was no edema, liquefaction and necrosis; different degrees of edema, liquefaction and necrosis appeared in the HIBD group at different time of modeling, suggesting that the HIBD model was constructed successfully. The pathological structures of the brain tissue in HIBD model group at different time showed that, the degree of edema, liquefaction and necrosis of brain tissue in SD rats aggra-

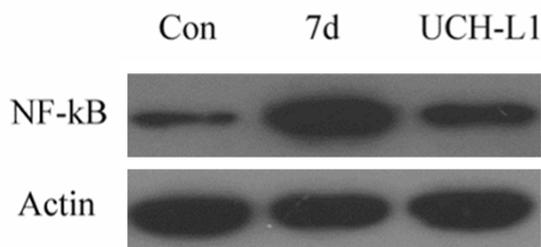
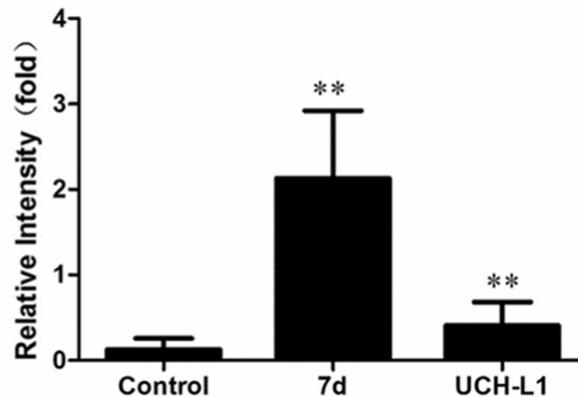


Figure 5. The expression of NF- κ B in rats' brain tissue after UCH-L1 was blocked.



vated gradually with the time duration of modeling. The expression of UCH-L1 protein in the brain tissue of rats was further observed by Western-blot method in this study, and the results showed that with the time extension of the model establishment, the expression of UCH-L1 protein in brain tissue of rats increased gradually ($P < 0.01$). When the effect of UCH-L1 was blocked by LDN-57444, the inhibitor of UCH-L1, the pathological changes of rat brain showed that the degrees of edema, liquefaction and necrosis of rat brain were significantly reduced. These results suggested that UCH-L1 was closely related to the occurrence and development of HIBD in rats.

NF- κ B signal pathway plays important roles in the complex cytokine network of inflammatory response, thus NF- κ B signal pathway is considered to be one of the most important regulators in the inflammatory cascade reactions after cerebral ischemia [22-24]. In order to further study the mechanism of UCH-L1 in the occurrence and development of HIBD in rats, whether the occurrence and development of HIBD in rats could be promoted by the expression of NF- κ B signal pathway regulated by UCH-L1 was investigated in this study. The results showed that the expression level of NF- κ B protein increased gradually ($P < 0.01$) with the time of model establishment, which was consistent with the expression of UCH-L1 protein. These results suggested that the NF- κ B signal pathway was closely related to the occurrence and development of HIBD in SD rats. The expression of NF- κ B protein in rat brain tissue was further observed by blocking the role of UCH-L1 with UCH-L1 inhibitor LDN-57444, and the results showed that the expression level of NF- κ B decreased significantly ($P < 0.01$) in rat brain tissue after the blockade of UCH-L1 effect.

These results suggested that UCH-L1 may promote the occurrence and development of HIBD in rats by regulating the expression of NF- κ B signal pathway.

In summary, high expression of UCH-L1 is closely related to hypoxic-ischemic brain damage in rats, the progress of hypoxic-ischemic brain damage of rats can be slow down with the effect of UCH-L1 was blocked, and UCH-L1 regulates this progress by regulating the expression level of NF- κ B in the brain tissue probably.

Disclosure of conflict of interest

None.

Authors' contribution

All authors contributed the same. All authors read and approved the manuscript.

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References

- [1] Graham EM, Ruis KA, Hartman AL, Northington FJ, Fox HE. A systematic review of the role of intrapartum hypoxia-ischemia in the causation of neonatal encephalopathy. *Am J Obstet Gynecol* 2008; 199: 587-595.
- [2] van Handel M, Swaab H, de Vries LS, Jongmans MJ. Long-term cognitive and behavioral consequences of neonatal encephalopathy following perinatal asphyxia: a review. *Eur J Pediatr* 2007; 166: 645-654.
- [3] Lawn J, Shibuya K, Stein C. No cry at birth: global estimates of intrapartum stillbirths and intrapartum-related neonatal deaths. *Bull World Health Organ* 2005; 83: 409-417.

- [4] Arriaga Redondo M, Rodriguez Sánchez de la Blanca A, Lowy Benoliel A, Navarro Patiño N, Villar Castro S, Blanco Bravo D, Sánchez-Luna M. Stridor in neonates with hypoxic-ischaemic encephalopathy subject to selective cerebral or whole body hypothermia. *An Pediatr (Barc)* 2015; [Epub ahead of print].
- [5] Rocha-Ferreira E, Phillips E, Francesch-Domenech E, Thei L, Peebles DM, Raivich G, Hristova M. The role of different strain backgrounds in bacterial endotoxin-mediated sensitization to neonatal hypoxic-ischemic brain damage. *Neuroscience* 2015; 311: 292-307.
- [6] El-Mazary AA, Abdel-Aziz RA, Mahmoud RA, El-Said MA, Mohammed NR. Correlations between maternal and neonatal serum selenium levels in full term neonates with hypoxic ischemic encephalopathy. *Ital J Pediatr* 2015; 41: 83.
- [7] Burton VJ, Gerner G, Cristofalo E, Chung SE, Jennings JM, Parkinson C, Koehler RC, Chavez-Valdez R, Johnston MV, Northington FJ, Lee JK. A pilot cohort study of cerebral autoregulation and 2-year neurodevelopmental outcomes in neonates with hypoxic-ischemic encephalopathy who received therapeutic hypothermia. *BMC Neurol* 2015; 15: 209.
- [8] Davidson JO, Wassink G, Heuij LG, Bennet L, Gunn AJ. Therapeutic Hypothermia for Neonatal Hypoxic-Ischemic Encephalopathy-Where to from Here?. *Front Neurol* 2015; 6: 198.
- [9] Shi LH, Zhou Y, Guo MF, Liu JS, Li CX, Wang GF, Liu W, Tian L. Serum levels of S-100 β correlate with the clinical status and severity of hypoxic-ischemic encephalopathy in neonates. *Genet Mol Res* 2015; 14: 14760-14771.
- [10] Li D, Li X, Wu J, Li J, Zhang L, Xiong T, Tang J, Qu Y, Mu D. Involvement of the JNK/FOXO3a/Bim Pathway in Neuronal Apoptosis after Hypoxic-Ischemic Brain Damage in Neonatal Rats. *PLoS One* 2015; 10: e0132998.
- [11] Kielland A, Camassa LM, Døhlen G, Munthe LA, Blomhoff R, Amiry-Moghaddam M, Carlsen H. NF- κ B activity in perinatal brain during infectious and hypoxic-ischemic insults revealed by a reporter mouse. *Brain Pathol* 2012; 22: 499-510.
- [12] Larsen CN, Price JS, Wilkinson KD. Substrate binding and catalysis by ubiquitin C-terminal hydrolases: identification of two active site residues. *Biochemistry* 1996; 35: 6735-6744.
- [13] Lucas K Campbell, Roby J Thomas, Laura W Lamps, Bruce R Smoller, Andrew L Folpe. Protein gene product 9.5 (PGP 9.5) is not a specific marker of neural and nerve sheath tumors: an immunohistochemical study of 95 mesenchymal neoplasms. *Mod Pathol* 2003; 16: 963-969.
- [14] Meray RK, Lansbury PT. Reversible monoubiquitination regulates the Parkinson's disease-associated ubiquitin hydrolase UCH-L1. *J Biol Chem* 2007; 282: 10567-10575.
- [15] Haglund K, Dikic I. Ubiquitylation and cell signaling. *EMBO J* 2005; 24: 3353-3359.
- [16] Liu Y, Fallon L, Lashuel HA, Liu Z, Lansbury PT. The UCH-L1 gene encodes two opposing enzymatic activities that affect alpha-synuclein degradation and Parkinson's disease susceptibility. *Cell* 2002; 111: 209-218.
- [17] Sakurai M, Sekiguchi M, Zushida K, Yamada K, Nagamine S, Kabuta T, Wada K. Reduction in memory in passive avoidance learning, exploratory behaviour and synaptic plasticity in mice with a spontaneous deletion in the ubiquitin C-terminal hydrolase L1 gene. *Eur J Neurosci* 2008; 27: 691-701.
- [18] Zaigham M, Lundberg F, Hayes R, Undén J, Olofsson P. Umbilical cord blood concentrations of ubiquitin carboxy-terminal hydrolase L1 (UCH-L1) and glial fibrillary acidic protein (GFAP) in neonates developing hypoxic-ischemic encephalopathy. *J Matern Fetal Neonatal Med* 2015; 10: 1-7.
- [19] Douglas-Escobar MV, Heaton SC, Bennett J, Young LJ, Glushakova O, Xu X, Barbeau DY, Rossignol C, Miller C, Old Crow AM, Hayes RL, Weiss MD. UCH-L1 and GFAP Serum Levels in Neonates with Hypoxic-Ischemic Encephalopathy: A Single Center Pilot Study. *Front Neurol* 2014; 5: 273.
- [20] Tang Q, Liu FR, Luo YL, Hei MY. Effect of IGF-1 on long-term anxiety-like behavior in rats after hypoxic-ischemic brain damage. *Zhongguo Dang Dai Er Ke Za Zhi* 2014; 16: 295-300.
- [21] Xie M, Wang SH, Lu ZM, Pan Y, Chen QC, Liao XM. UCH-L1 inhibition involved in CREB dephosphorylation in hippocampal slices. *J Mol Neurosci* 2014; 53: 59-68.
- [22] Yang D, Sun YY, Lin X, Baumann JM, Dunn RS, Lindquist DM, Kuan CY. Intranasal delivery of cell-penetrating anti-NF- κ B peptides (Tat-NBD) alleviates infection-sensitized hypoxic-ischemic brain injury. *Exp Neurol* 2013; 247: 447-455.
- [23] van der Kooij MA, Nijboer CH, Ohl F, Groenendaal F, Heijnen CJ, van Bel F, Kavelaars A. NF-kappaB inhibition after neonatal cerebral hypoxia-ischemia improves long-term motor and cognitive outcome in rats. *Neurobiol Dis* 2010; 38: 266-272.
- [24] Ke RH, Xiong J, Liu Y, Ye ZR. Adenosine A2a receptor induced gliosis via Akt/NF-kappaB pathway in vitro. *Neurosci Res* 2009; 65: 280-285.