Tropical Journal of Pharmaceutical Research May 2017; 16 (5): 1051-1058
ISSN: 1596-5996 (print); 1596-9827 (electronic)

Available online at http://www.tjpr.org
http://dx.doi.org/10.4314/tjpr.v16i5.12

Original Research Article

Neuroprotective effects of α-lipoic acid against hypoxic–ischemic brain injury in neonatal rats

Xiao-han Mei and You-wen Yang
Department of Pediatrics, Xianyang City Center Hospital of Shaanxi Province, Shaanxi 712000, China

For correspondence: **Email: meixiaohanxh@hotmail.com; **Tel:** 0086-029-33222222*

Sent for review: 19 February 2017 *Revised accepted: 13 April 2017*

Abstract

Purpose: *To explore the neuroprotective efficacy of α-lipoic acid (ALA) against hypoxic-ischemic encephalopathy (HIE) in neonatal rats.*
Methods: *Forty-eight rats (P7-pups) were randomly assigned to one of four groups: group I received saline; group II (HI) underwent unilateral carotid artery ligation and hypoxia (92 % N2 and 8 % O_2) for 2.5 h; and groups III and IV (ALA 50 and 100) were treated with 50 or 100 mg ALA/kg for 7 days prior to against hypoxic-ischemic (HI) insult. Cerebral antioxidant status, edema, and the levels of inflammatory markers were determined.*
Results: *ALA administration substantially ($p < 0.01$) attenuated both cerebral infarct area and degree of edema while decreasing the levels of several inflammatory markers (TNF-α, NF-p65, IL-1β, IL-6). In addition, in the ALA groups, antioxidant enzyme (SOD, CAT, GSH) activities were significantly elevated, while the expressions of TNF-α and IL-1β protein were significantly ($p < 0.01$) down-regulated.*
Conclusion: *The neuroprotective efficacy of ALA in HIE can be attributed to its suppression of both oxidative stress and the levels of inflammatory markers.*

Keywords: *Hypoxic–ischemic brain injury, α-Lipoic acid, Cerebral infarct area, Edema, Antioxidants, Inflammatory markers*

Tropical Journal of Pharmaceutical Research is indexed by Science Citation Index (SciSearch), Scopus, International Pharmaceutical Abstract, Chemical Abstracts, Embase, Index Copernicus, EBSCO, African Index Medicus, JournalSeek, Journal Citation Reports/Science Edition, Directory of Open Access Journals (DOAJ), African Journal Online, Bioline International, Open-J-Gate and Pharmacy Abstracts

INTRODUCTION

Hypoxic-ischemic encephalopathy (HIE) is a severe complication of peripartum asphyxia that can result in severe neurological sequelae or disabilities and is often fatal [1]. The global prevalence of neonatal HIE is ~2 per 1000 term live births, of which ~55 % of the affected premature children die by the age of 2 years [2]. In neonatal HIE, the restricted blood flow ultimately causes energy failure, by triggering a cascade of immune responses that lead to increased excitotoxicity, inflammation, oxidative stress, necrosis, and apoptosis [3,4]. Thus, the underlying pathophysiology of HIE is oxidative stress and inflammation. Based on the increasing evidence of a close relationship between the degree of oxidative stress and HIE intensity [5,6], related research has focused on reining in antioxidant and inflammatory activities to reduce the severity of HIE.

Alpha-lipoic acid (ALA) is an endogenous antioxidant synthesized mainly by the mitochondria of the liver, heart, and kidney. Dihydrolipoic acid, the reduced form of ALA, has generated interest due to its antioxidant activity. ALA has two sulfhydryl (thiol) groups and a free hydroxyl group that act as electron donors and account for the free-radical scavenging activity of ALA [7]. In addition, ALA exhibits metal-chelating activity and is an essential cofactor for enzymes

involved in the Krebs cycle and the electron transport chain [8]. ALA also increases (regenerates) glutathione (GSH), thereby maintaining optimal levels of other antioxidants, such as vitamins C and E, superoxide (SOD), and catalase (CAT) [9]. Accordingly, among the many health-promoting activities of ALA are its antioxidant, anti-inflammatory, anti-apoptotic, anticancer, cardioprotective, and neuroprotective functions [10,11]. Moreover, because of its amphiphilic nature, ALA readily penetrates all cells and tissues of the body, including the blood-brain barrier (BBB). ALA also effectively increases the production of acetylcholine (neurotransmitter) and thereby maintains the integrity of the brain [12]. These findings strongly suggest a role for ALA in the treatment of various neurological disorders. However, only a few experimental studies, involving different animal and clinical models following traumatic head injury, cerebral ischemia, and seizures, have examined the neuroprotective nature of ALA [7,11,13]. The present study explored the neuroprotective efficacy of ALA against hypoxic–ischemic (HI) brain injury in neonatal rats, by evaluating cerebral infarct area, the degree of edema, and the levels of several important oxidative stress and inflammatory markers.

EXPERIMENTAL

Chemicals

Alpha-lipoic acid, dimethyl sulfoxide, and bromophenol blue were purchased from Sigma-Aldrich (St. Louis, MO, USA). Diaminobenzidine tetrahydrochloride, formaldehyde, and phosphate-buffered saline (PBS) were supplied by Beijing Zhongshan Golden Bridge Biotechnology Co. Ltd. (Beijing, China). Toluidine blue, isoflurane, pentobarbital sodium, and 2,3,5-tritriphenyl-tetrazolium chloride were purchased from Lingjin Co. Ltd. (Shanghai, China).

Experimental animals

Male Sprague-Dawley rat pups (7 days old, P7), weighing 15–20 g were obtained from the Experimental Animal Center (Shaanxi, China). The neonatal rats were maintained in a room with a temperature of 22 ± 3 °C, 55 ± 5 % humidity, and 12/12 h light/dark cycles for one week with feed and water *ad libitum*. All experimental protocols were approved by the Ethics Committee of the Center Hospital of Shaanxi Province (approval no. 162015) according to the guidelines put forth by the National Institutes of Health Guide for the Care and Use of Laboratory Animals [14].

Induction of HIE

After a week of adaptation (P7–P14), the rats were exposed to unilateral HI as described by Li *et al*, with slight modifications [15]. Unilateral common carotid artery ligation was performed with the rats initially anesthetized with 2 % isoflurane. After a 1-h recovery period, the rats were placed for 2.5 h in a hypoxia chamber (8 % O_2, 92 % N_2) maintained at a temperature of 37 °C with the assistance of a water bath. They were then moved back to their respective dams and immediately injected intraperitoneally (i.p.) with saline in the control group and ALA in the ALA groups as a one-time post-treatment. Based on a preliminary dose-dependent study in a model of HI (data not shown), the rat pups in the ALA groups were treated with 50 and 100 mg ALA/kg, administered i.p. for 7 days prior to HI induction. In the control rats, ligation was not performed and exposure was to normal atmospheric conditions rather than to hypoxia.

Experimental protocol

Of the 48 rats initially used in the current study, 5 died during HI induction (the mortality rate for HIE is 15 – 20 %). The rat pups were randomly assigned to four groups: saline (sham control, group I; n = 12), HI insult (HI group, group II; n = 10), i.p. injection of 50 mg ALA (dissolved in 2 % saline)/kg body weight for 7 consecutive days before HI insult (ALA 50+HI; group III; n=10), and i.p. injection of 100 mg ALA (dissolved in 2 % saline)/kg body weight for 7 consecutive days before HI insult (ALA 100+HI: group IV; n = 11). All four groups received a one-time post-treatment with ALA or saline after HI insult. All pups were euthanized on day 16 (P16) by i.p. sodium pentobarbital injection. Their brains were removed immediately and stored at −80 °C. One half of the brain was used for morphological analysis (infarct area) and the remaining half for biochemical and molecular analyses by homogenizing the cerebral region (pooled) in lysis buffer.

Morphological analysis

Evaluation of the infarct area

The cerebral infarct area/volume was evaluated by 2, 3, 5-triphenyl-tetrazolium chloride (TTC) staining as previously reported [15]. Four coronal sections (2 mm) were obtained from each brain. The sections were stained with 0.1 % TTC (Lingjin Co. Ltd, Shanghai, China) at 37 °C for 5 min, rinsed with PBS, and then fixed with 10 % formaldehyde. The Image J analyzing software system (ImageJ, Bethesda, MD, USA) was used

to evaluate the infarct area according to the following equation: infarct area = (total infarct area/whole brain section area) × 100%.

Evaluation of cerebral edema

The degree of the cerebral edema was determined using the gravimetric method as previously described [16]. Briefly, the wet and dry weights of the brain samples were determined and cerebral edema (CE) was then computed based on the water content according Eq 1.

CE (%) = {W - D)/W}100 …… (1)

where W and D are the wet weight and dry weight of the brain sample, respectively.

Biochemical analysis

Lipid peroxidation and antioxidant enzyme assays

The levels of the lipid peroxidation (LPO) product malondialdehyde (MDA), the enzymes SOD and CAT, and GSH were determined. MDA was measured based on the reduction of 2-thiobarbituric acid, quantified based on the absorbance at 532 nm. SOD was measured as the reduction of nitro blue tetrazolium and the absorbance at 560 nm; one unit of SOD was the amount of enzyme that inhibited the increase in absorbance at 560 nm by 50 % under the assay conditions. CAT levels were determined based on the reduction of hydrogen peroxide (H_2O_2), quantified by the absorbance at 540 nm; one unit of CAT activity was the amount that consumed 1 µmol of H_2O_2, quantified by the absorbance at 540 nm. GSH levels were measured based on the reduction of o-phthaldialdehyde, quantified by measuring the absorbance at 420 nm. All of the above assays were carried out using commercial kits according to the manufacturer's recommended procedure (Nanjing Jiancheng Bioengineering Institute, Nanjing, China). The biuret method was used to quantify the protein content using a BCA protein assay reagent kit (Biovision, California, USA).

Assay of NF-κB p65 subunit, TNF-α, IL-1β, and IL-6

The concentration of the free p65 subunit of NF-κB in the nuclear fraction (nuclear/cytosolic fractionation kit; Cell Biolabs) of the cerebral cortex was determined using an ActivELISA kit (Imgenex, San Diego, USA). The levels of the proinflammatory cytokines tumor necrosis factor (TNF)-α, interleukin (IL)-1β, and IL-6 in the cerebral homogenates were measured by ELISA kits (sandwich enzyme immunoassay) according to the manufacturer's protocol (Thermo Fisher Scientific Inc, MA, USA).

Western blot analysis

An equal amount of protein (50 µg/lane) was loaded into each well of 8 % polyacrylamide gels, subjected to SDS-PAGE, and then transferred to a polyvinylidene membrane (PVDF) using a semi-dry blotting system (Amersham, Buckinghamshire, UK). The PVDF membrane was blocked with Tween 20, Tris buffer, and 5 % skimmed milk and incubated overnight at 4 °C with polyclonal rabbit anti-TNF-α antibody (1:1000; Abcam, USA), polyclonal rabbit anti-IL-1β antibody (1:500; Abcam, USA), or rabbit anti-rat β-actin (1:500; Zhongshan Biotechnology, Beijing). The following day, the probed membrane was incubated for 1 h with horseradish-peroxidase-conjugated secondary antibody (1:1000). The membranes were developed using the enhanced chemiluminescence system and the intensity of the bands on the membrane quantified using Image J software-analyzer (ImageJ, Bethesda, USA).

Statistical analysis

All data are expressed as the mean ± standard deviation. Differences between each experimental group were measured using a one-way analysis of variance and the Statistical Package for Social Sciences software, version 21 (SPSS Inc, Chicago, USA). The least significant difference was determined using a post-hoc multiple comparison test. A p value < 0.05 was considered to indicate statistical significance.

RESULTS

Effect of ALA on cerebral infarct area and edema

The cerebral infarct area in the neonatal rats was evaluated (Figure 1A and B). In the sham control group neither cerebral infarct nor edema was detected. The infarct area, seen as white patches, was significantly larger in the brains of the HI rats (group II) than in the brains of the rats in the other groups (p < 0.01). In the two ALA treatment groups (50 and 100 mg/kg), the decreases in the infarct area compared to the HI group were significant (p < 0.01) (Figure 1C). The water content of the brain was significantly higher (p < 0.01) in the brains of pups exposed to HI insult than in brains of the control rats whereas it was significantly (p < 0.05) lower in

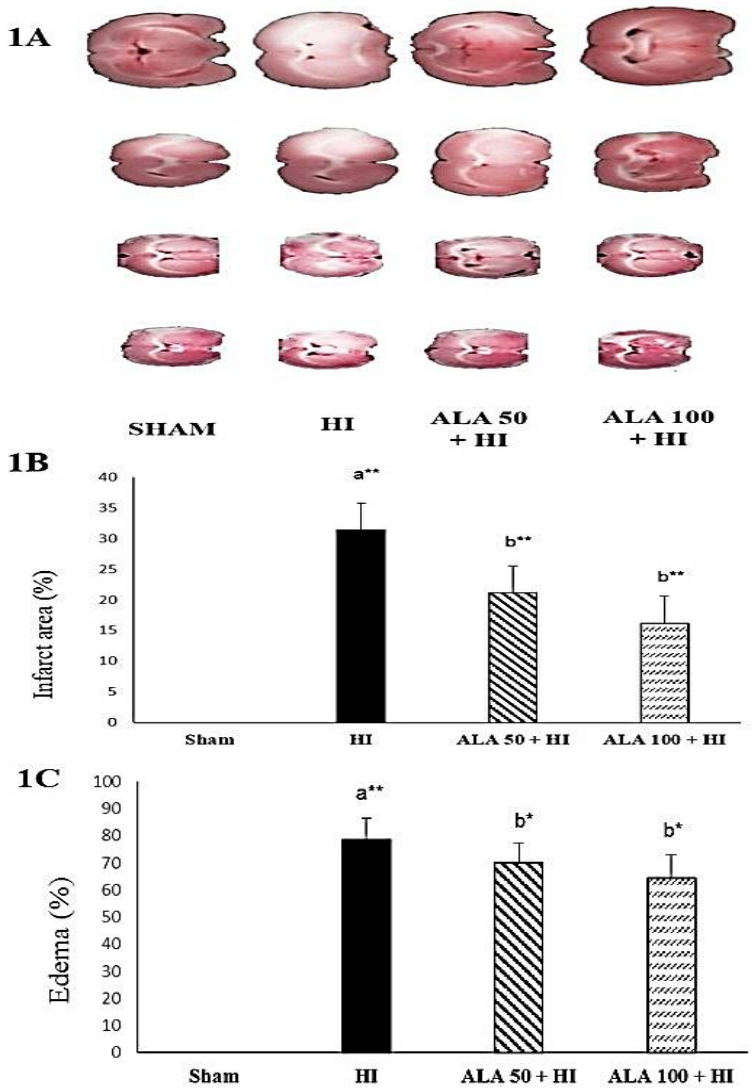

Figure 1: Effects of ALA on cerebral infarct area and edema in control and experimental neonatal rats. Coronal sections stained with TTC (A) show white patches, corresponding to ischemia whereas normal, non-ischemic tissue stains red. The area of cerebral infarct and the amount of cerebral edema (water content) were expressed as percentages (B and C, respectively); ***p < 0.01, *p* < 0.05; (a) compared with control group, (b) compared with HI group

the ALA (50 and 100 mg/kg) groups than in the control. These results demonstrate the neuroprotective potential of ALA.

Effect of ALA on LPO products and antioxidants in control and experimental rats

The effect of ALA pretreatment on the levels of LPO products (MDA) and antioxidants in control and experimental neonatal rats is shown in Table 1. The cerebral MDA level was significantly higher ($p < 0.01$) in the HI group than in the control whereas SOD and CAT activities as well as GSH levels were lower ($p < 0.01$). In the ALA pre-treated (50 and 100 mg/kg) rats, MDA levels declined significantly ($p < 0.05$ *and* $p < 0.01$, respectively) whereas SOD and CAT activities were sigificantly increased, as were GSH levels.

Effect of ALA on the activities of inflammatory markers in sham control and experimental neonatal rats

Table 2 shows the effect of ALA on the activities of key inflammatory markers (NF-κB p65 subunit, TNF-α, IL-1β, and IL-6) in control and experimental neonatal rats. Inflammatory marker levels were significantly higher in the brain tissues of HI rats ($p < 0.01$) than in those of control rats. The intraperitoneal administration of ALA (50 and 100) resulted in a substantial ($p < 0.01$) decrement in pro-inflammatory cytokine.

Table 1: Effect of ALA on lipid peroxidation (LPO) and antioxidants in control and experimental neonatal rats

Group	SOD (U/mg protein)	CAT (U/mg protein)	GSH (µg/mg protein)	LPO (nmol/mg protein)
Sham control	2.85±0.25	59.52±6.34	9.13±0.84	0.30±0.03
HI	2.21±0.22 a**	47.24±5.35 a**	6.85±0.72 a**	0.77±0.06 a**
ALA 50 + HI	2.53±0.28 b*	53.56±5.67 b*	7.58±0.53 b*	0.53±0.05 b*
ALA 100 + HI	2.72±0.34 b**	56.44±4.49 b**	7.88±0.97 b*	0.41±0.03 b**

*SOD, superoxide dismutase; CAT, catalase; GSH, glutathione; values are expressed as the mean ± SD; **p < 0.01, *p < 0.05 (a) compared with the sham control group, (b) compared with the HI-injured group*

Table 2: Effect of ALA on the activities of inflammatory markers in control and experimental neonatal rats

Group	NF-p65 (pg/mg protein)	TNF-α (ng/mg protein)	IL-1β (ng/mg protein)	IL-6 (pg/mgprotein)
Sham control	67.53±6.34	125.24±11.40	67.29±6.80	89.34±8.23
HI	195.62±18.23 a**	255.15±22.55 a**	169.53±16.61 a**	242.34±21.42 a**
ALA 50 + HI	125.57±11.03 b**	186.45±18.16 b**	120.24±11.12 b*	168.24±16.07 b*
ALA 100 + HI	84.21±9.64 b**	152.83±14.74 b**	88.34±10.20 b**	112.64±10.09 b**

*TNF-α, tumor necrosis factor-α; IL, interleukin. Values are the mean ± SD: **p < 0.01, *p < 0.05 (a) compared with the sham control group, (b) compared with the HI injured group*

concentrations, consistent with its anti-inflammatory potential

Effect of ALA on TNF-α and IL-1β protein expression in sham control and experimental neonatal rats

The effect of ALA on the levels of TNF-α and IL-1β in control and experimental rats is shown in Figure 2. Both cytokines were significantly up-regulated ($p < 0.05$) in the HI group vs. the control group. ALA treatment of HI-induced rats resulted in a significant down-regulation in the levels of these two markers compared to the HI group. The agreement between the results of the molecular analysis (protein) and the biochemical assay provided clear evidence of the anti-inflammatory effect of ALA. Both doses of ALA improved neurological status, although the neuroprotective activity of 100 mg of ALA (group IV) was superior.

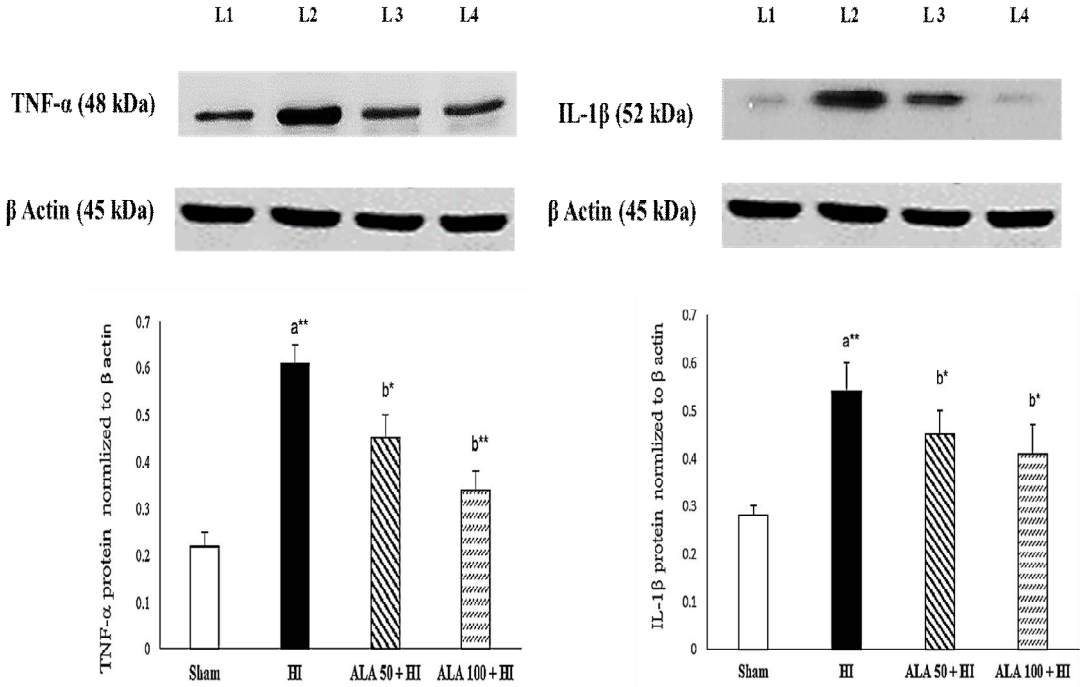

Figure 2: Effect of ALA on TNF-α (A) and IL-1β (B) protein expression in the cerebral region of control and experimental neonatal rats.Values are expressed as the mean ± SD; Lane 1: sham control group; lane 2; HI-induced group; lane 3: ALA 50 group: lane 4 ALA 100 group; **$p < 0.01$, *$p < 0.05$ (a) compared with the control group, (b) compared with the HI group.

DISCUSSION

Hypoxic–ischemic brain injury in neonates can lead to severe complications, including cerebral palsy, epilepsy, motor impairment, and delayed neural development, and in many cases, is fatal [17]. Among the factors implicated in HIE are the over-production of free radicals and the resulting inflammatory response [18,19]. Based on the well-known antioxidant and anti-inflammatory activity of ALA, it was tested in this study as a neuroprotective agent against HI injury in neonatal rats. Whereas large infarct areas, seen as white patches because of impeded blood flow (hypoxic condition), were seen in rats in the HI group, ALA pre-treatment resulted in a substantially smaller infarct area. Connell and Saleh similarly demonstrated that the administration of lipoic acid together with apocynin significantly reduced the infarct area in a rat model of ischemia/reperfusion [20], in addition to markedly increasing cerebral edema levels due to the lack of BBB integrity. In that study, treatment with ALA (50 and 100) substantially reduced cerebral edema. Our data also agree with the findings of Erşahin *et al* [21], who showed that treatment with lipoic acid reduced cerebral edema levels by restoring the integrity of the BBB and by inhibiting neutrophil activation, thereby conferring neuronal protection.

The neonatal brain, with its high oxygen utilization rate, low levels of antioxidants, high content of polyunsaturated fatty acids (PUFA), high water content, low degree of myelinization, and low availability of redox-active iron, is highly susceptible to oxidative stress (damage) [17], which is a primary contributor to HIE [22,23]. An imbalance between antioxidants and oxidants may lead to oxidative stress and thus to damage in the form of LPO, especially for neurons in the compensatory mode [24].

LPO is a potent oxidative marker used to quantify oxidative stress [25]. The cerebral LPO (MDA) level is increased during hypoxia because the respective neurons are rich in PUFA and have a low antioxidant capacity. ALA administration, especially 100 vs. 50 mg, significantly reduced LPO levels to near normal. Emmez and coworkers [12] showed that ALA, with its free-radical scavenging activity, significantly suppressed LPO.

SOD and CAT are important enzymatic antioxidants that, together with the antioxidant GSH, control the cellular levels of free radicals. The activities of SOD, CAT, and GSH were notably suppressed in the HI group, which may have been due to free-radical generation. Both ALA doses (50 and 100 mg/kg) substantially improved the activities of SOD, CAT, and GSH. The cytoprotective effect of ALA is derived from its ability to limit the generation of reactive oxygen species, by inhibiting NADPH and xanthine oxidase and by effectively activating the PI3k/Akt pathway and related proteins [26]. In addition, Skibska and Goraca [8], reported that thioredoxin (Trx), a redox signaling molecule similar to HO-1 and Nrf2, which are highly regulated during oxidative stress (hypoxia), is up-regulated in ALA-treated rats and indirectly activates antioxidant expression. Our results are also in agreement with those of Turamanlar and colleagues [27], who reported that in rats with sciatic nerve ischemic injury the elevated MDA, SOD, GSH peroxidase, and GSH levels revert to normal in response to ALA.

Oxidative stress and inflammation are interlinked responses. The levels of nuclear factor NF-p65 and pro-inflammatory cytokines such as TNF-α, IL-1β, and IL-6 were greatly augmented in the HI group. During oxidative stress, the release of various pro-inflammatory cytokines is stimulated by microglia/astrocytes, which account for the increased levels of these inflammatory markers during ischemia. Rats treated with two different doses of ALA (50 and 100 mg) had significantly lower levels of these markers, with much greater anti-inflammatory activity achieved with the higher dose. Our results are well supported by those of Toklu *et al* [10], who demonstrated that the anti-inflammatory effect of lipoic acid can be attributed to diminished levels of inflammatory cytokines and to the suppression of neutrophil infiltration. In addition, ALA abolishes microglial/astrocyte activation by inhibiting the translocation of activated NF-κB p65 subunits from the cytosol to the nucleus, which hampers the expression of downstream pro-inflammatory cytokines [11,28].

TNF-α and IL-1β play crucial roles in the early phase of the inflammatory response during ischemia. As such, they serve as markers of the severity of HIE [1,3]. In this study, TNF-α and IL-1β expression in the cerebral cortex was markedly up-regulated in the HI group vs. the control group but significantly down-regulated in the ALA-treated groups, subsequent to the suppression in the latter of both neutrophil infiltration and microglial/astrocytes activation. Chen and Kunsch [29] proposed that compounds activating the Nrf2 pathway significantly lower the inflammatory response while acting as effective antioxidants. Pre-treatment of the rat pups in this study with 50 or 100 mg ALA effectively improved their neurological status, although the higher dose showed superior neuroprotective

activity based on the larger reductions in oxidative stress and inflammatory markers. Thus, by enhancing the antioxidant status and suppressing various inflammatory markers ALA could attenuate HIE in neonatal rats.

There were two major limitations to this study. First, ALA was administered for 7 days and the rats were euthanized on day 16 after HIE induction; thus, the long-term neuroprotective effect of ALA is unclear. Also, we did not examine specific neuronal responses, such as apoptosis, glutamate excitotoxicity, and mitochondrial dysfunction. These will be investigated in future studies.

CONCLUSION

The findings of this study demonstrate the antioxidant and anti-inflammatory activities of ALA. In a neonatal rat model of HIE, ALA pretreatment decreases cerebral infarct volume, the degree of edema, and the levels of several key oxidative stress and inflammatory markers. Thus, ALA merits further investigation its mechanisms of action, any related complications during treatment.

DECLARATIONS

Acknowledgement

Financial support was provided by Xianyang City Center Hospital of Shaanxi Province, Shaanxi 712000, China.

Conflict of Interest

No conflict of interest associated with this work.

Contribution of Authors

The authors declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by them.

REFERENCES

1. *Liu F, McCullough LD. Inflammatory responses in hypoxic ischemic encephalopathy. Acta Pharmacol Sin 2013; 34(9): 1121-1130.*
2. *Pierrat V, Haouari N, Liska A, Thomas D, Subtil D, Truffert P; Groupe d'Etudes en Epidémiologie Périnatale. Prevalence, causes, and outcome at 2 years of age of newborn encephalopathy: population based study. Arch Dis Child Fetal Neonatal Ed 2005; 90(3): F257-F261.*
3. *Seema S, Kumar GA, Mamta P, Sumitra B. Correlation of oxidative stress biomarker and serum marker of brain injury in hypoxic ischemic encephalopathy. Int J Med Appl Sci 2014; 3(1): 106-115.*
4. *Brekke EM, Morken TS, Widerøe M, Håberg AK, Brubakk AM, Sonnewald U. The pentose phosphate pathway and pyruvate carboxylation after neonatal hypoxic-ischemic brain injury. J Cereb Blood Flow Metab 2014; 34(4): 724-734.*
5. *Perrone S, Tataranno ML, Negro S, Longini M, Marzocchi B, Proietti F, Lacoponi F, Capitani S, Buonocore G. "Early identification of the risk for free radical-related diseases in preterm newborns," Early Hum Dev 2010; 86(4): 241-244.*
6. *Schulz JB, Lindenau J, Seyfried J, Dichgans J. Glutathione, oxidative stress and neurodegeneration. Eur J Biochem 2000; 267(16): 4904-4911.*
7. *Jia Z, Zhu H, Vitto MJ, Misra BR, Li Y, Misra HP. Alpha-lipoic acid potently inhibits peroxynitrite-mediated DNA strand breakage and hydroxyl radical formation: implications for the neuroprotective effects of alpha-lipoic acid. Mol Cell Biochem 2009; 323: 131-138.*
8. *Skibska B, Goraca A. The Protective Effect of Lipoic Acid on Selected Cardiovascular Diseases Caused by Age-Related Oxidative Stress. Oxid Med cell longev 2015; 2015: 313021.*
9. *El-Beshbishy HA, Bahashwan SA, Aly HA, Fakher HA. Abrogation of cisplatin-induced nephrotoxicity in mice by alpha lipoic acid through ameliorating oxidative stress and enhancing gene expression of antioxidant enzymes. Eur J Pharmacol 2011; 668(1): 278-284.*
10. *Toklu HZ, Hakan T, Bi˙ ber N, Solakoğlu S, Öğünç AzV, Şener G: The protective effect of alpha lipoic acid against traumatic brain injury in rats. Free Rad Res 2009; 43(7): 658-667.*
11. *Maczurek A, Hager K, Kenklies M, Sharman M, Martins R, Engel J, et al. Lipoic acid as an anti-inflammatory and neuroprotective treatment for Alzheimer's disease. Adv Drug Deliv Rev 2008; 60: 1463-1470.*
12. *Emmez H, Yildirim Z, Kale A, Tönge M, Durdağ, E, Börcek AÖ, Uçankuş LN, Baykaner MK. Anti-apoptotic and neuroprotective effects of alpha-lipoic acid on spinal cord ischemia–reperfusion injury in rabbits. Acta Neurochirurgica 2010; 152(9): 1591-1601.*
13. *Richard MJ, Connell BJ, Khan BV, Saleh TM. Cellular mechanisms by which lipoic acid confers protection*

during the early stages of cerebral ischemia: a possible role for calcium. Neurosci Res 2011; 69(4): 299-307.

14. Clark JD, Gebhart GF, Gonder JC, Keeling ME, Kohn DF. The 1996 guide for the care and use of laboratory animals. ILAR J 1997; 38(1): 41-48.
15. Li Y, Xiao D, Dasgupta C, Xiong F, Tong W, Yang S. Zhang L. Perinatal Nicotine Exposure Increases Vulnerability of Hypoxic–Ischemic Brain Injury in Neonatal Rats Role of Angiotensin II Receptors. Stroke 2012; 43(9): 2483-2490.
16. Mdzinarishvili A, Kiewert C, Kumar V, Hillert M, Klein J. Bilobalide prevents ischemia-induced edema formation in vitro and in vivo. Neurosci 2007; 144(1): 217-222.
17. Tataranno ML, Perrone S, Longini M, Buonocore G. New Antioxidant Drugs for Neonatal Brain Injury. Oxid Med cell longev 2015; 2015: 384372.
18. Garcia-Bonilla L, Benakis C, Moore J, Iadecola C, Anrather J. Immune mechanisms in cerebral ischemic tolerance. Front Neurosci 2014; 8: 44.
19. Lu Q, Wainwright MS, Harris VA. Increased NADPH oxidase derived superoxide is involved in the neuronal cell death induced by hypoxia ischemia in neonatal hippocampal slice cultures. Free Radic Biol Med 2012; 53: 1139-1151.
20. Connell BJ, Saleh TM. Co-administration of apocynin with lipoic acid enhances neuroprotection in a rat model of ischemia/reperfusion. Neurosci Lett 2012; 507(1): 43-46.
21. Erşahin M, Toklu HZ, Çetinel Ş, Yüksel M, Erzik C, Berkman MZ, Yeğen BÇ, Şener G. Alpha lipoic acid alleviates oxidative stress and preserves blood brain permeability in rats with subarachnoid hemorrhage. Neurochem Res 2010; 35(3): 418-428.
22. Sheldon RA, Christen S, Ferriero DM. Genetic and pharmacologic manipulation of oxidative stress after neonatal hypoxia-ischemia. Int J Dev Neurosci 2008; 26(1): 87-92.
23. Buonocore G, Groenendaal F. Anti-oxidant strategies. Semin Fetal Neonatal Med 2007; 12: 287-295.
24. Dalazen GR, Terra M, Jacques CED, Coelho JG, Freitas R, Mazzola PN, Dutra-Filho CS. Pipecolic acid induces oxidative stress in vitro in cerebral cortex of young rats and the protective role of lipoic acid. Metab Brain Dis 2014; 29(1): 175-183.
25. Fathi F, Oryan S, Rafieian-Kopaei M, Eidi A. Neuroprotective effect of pretreatment with Mentha longifolia L. extract on brain ischemia in the rat stroke model. Arch Biol Sci 2015; 67(4): 1511-1163.
26. Deng C, Sun Z, Tong G, Yi W, Ma L, Zhao B, Cheng L, Zhang J, Cao F, Yi D. α-Lipoic acid reduces infarct size and preserves cardiac function in rat myocardial ischemia/reperfusion injury through activation of PI3K/Akt/Nrf2 pathway. PLoS One 2013; 8(3): e58371.
27. Turamanlar O, Özen OA, Songur A, Yağmurca M, Akçer S, Mollaoğlu H, Aktaş C. Protective Effect of Alpha Lipoic Acid on Rat Sciatic Nerve Ischemia Reperfusion Damage. Balkan J Med 2015; 32(2): 196-202.
28. Rocamonde B, Paradells S, Barcia J, Barcia C, Verdugo JG, Miranda M, Gómez FR, Soria J. Neuroprotection of lipoic acid treatment promotes angiogenesis and reduces the glial scar formation after brain injury. Neurosci 2012; 224: 102-115.
29. Chen, C, Kunsch. Induction of cytoprotective genes through Nrf2/antioxidant response element pathway: a new therapeutic approach for the treatment of inflammatory diseases, Curr Pharm Des 2004; 10: 879-891.