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ORIGINAL ARTICLE

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Effect of humic acid on oxidative stress and neuroprotection in hypoxic-ischemic brain injury: part 1

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ABSTRACT

Background: Aimed to investigate in an animal model the efficacy of humic acid by showing its antioxidant and anti-apoptotic effect comparing with the histopathological and neurological outcomes for the hypoxic-ischemic brain injury.

Methods: 28 Wistar-Albino rats who were on the 7th postnatal day and weighting between 9 and 19 g randomly divided into four groups with developed HIE model under the gas anesthesia. 20 mg/kg and 10 mg/kg intraperitoneal HA were given to Group I and II respectively. Saline was given to Group III and the sham group was Group IV. The brain tissues were stained with cresyl-violet histochemistry for grading neuronal cell injury and caspase immunohistochemistry.

Results: The neuronal cell injury was statistically lower in all neuroanatomical lands in HA treatment groups. The degree of ischemia was significantly smaller in HA groups. Caspase-3 immunoreactivity was decreased in the HA groups compared with the saline group. When the groups were compared, there were no serious neuronal injury in Group I.

Conclusions: This is the first study which investigates the role of HA in HIE model. HA reduces apoptosis and neuronal injury in cerebral tissue of the rats. This findings suggest that HA may be viable protective agent against HIE.

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Hypoxic-Ischemic; neonate; apoptosis; Humic acid

Introduction

Hypoxic-ischemic encephalopathy (HIE) is one of the major leading causes of mortality and morbidity in children. World wide frequency of HIE may be seen approximately with a frequency of 1–8 per 1000 neonates in high-income countries and is as high as 26 per 1000 births in underdeveloped countries [1]. Hypoxia and ischemia cause primary neuronal damage by neuronal necrosis and apoptosis during perinatal asphyxia [2]. It has been recognized that HIE can induce a cascade of intracellular events which causes to cell apoptosis by activation of proapoptotic genes and caspase-3 [3].

There is still no consensus about medical treatment options. To date, therapeutic hypothermia is the only treatment method following perinatal asphyxia [4]. Neuroprotective mechanisms involved are inhibition of acute metabolic disturbance and inhibition of apoptotic cell death [5]. Difficulty in the treatment of the aspyxtic newborn has led to the intensive investigation of many mediators such as glutamate receptor antagonists, cyclooxygenase inhibitors, melatonin and erythropoietin. These agents are expected to be supported by further studies [6].

Humic acid (HA) is a high polyphenolic substance which is used effectively in veterinary and agriculture. It contains multiple carboxylic acid side chains and complex structure of polyaromatic and heterocyclic chemicals [7]. It is an active substance which are used in prophylaxis and as therapeutical drug in veterinary in Europe [8]. HA is administered as anti-inflammatory, anti-bacterial, anti-ulserogenic and anti-allergic features [9]. HA has antioxidant features through phenol, carboxyl acid and quinone in its structure. HA reduces in the activity of antioxidant systems in human cells [10].

In asphyxia, if the energy need is not met, there is a shift from aerobic metabolism to anaerobic metabolism, leading to an accerelation of glycolysis and an

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increase in lactate production. These changes cause a decrease in ATP concentration, which leads to loss of cell membrane potential and leads to cell death. Another important mechanism in asphyxia is excitoxicity in neuronal damage. The increase in extracellular glutamate level leads to cell death by stimulating glutamate receptors [11].

To our knowledge, the efficacy of HA in HIE has not been evaluated in the literature. Therefore in this study, we aimed to investigate in an animal model the efficacy of HA by showing its antioxidant and anti-apoptotic effect comparing with the histopathological and neurological outcomes.

Materials and methods

Experimental procedure

This present study was approved by the Aegean University Animal Experiments Local Ethics Committee in Izmir (approval number 2019-011). There were 28 Wistar-Albino rats in total amount of male and female between each group. Study group consist of the rats who were on the 7th postnatal day and weighting between 9 and 19 g. Due to the presence of Wistar Albino strain in Aegean University Faculty of Medicine Experimental Animals Laboratory and compatibility with the literature, this type was preferred as a subject. All animals were kept at controlled temperature (21 °C−23 °C) and 12 h light 12 h dark condition.

For the anesthesia application, the subjects were placed in the glass lantern with sections where the anesthetic gas could enter and exit, and anesthesia would be performed by giving the system a 2.5% concentration of halo in 100% O_2. The gas mixture from the halothane vaporizer was kept constant by monitoring it on the anesthetic gas monitor (Anesthesia Gas Monitoring 1304, Denmark). For the surgical procedure, when the experimental animal was removed from the glass lantern, the maintenance of anesthesia was achieved with a mask and connector adapted to the mouth and nose of the subject. Oxygen and halothane levels were kept constant by monitoring them on the anesthetic gas monitor. For the experimental model of HIE, the skin of the head of the rat was shaved and then prepared with poviodone-iodine. In the supine position with a midline neck incision, as Kocher's thyroidectomy incision, the left common carotid arteries were presented. The left carotid artery was identified after dissection and was ligated with a 6/0 silk suture under the microscope. After confirming that there was no pulsation in the carotid artery under the microscope, the incision site was sutured. Anesthetic gas intake was stopped and the animal was awakened. The experimental animal was left with his mother for 2 h for compilation and feeding.

After a 1–2 h recovery period animals were placed in a trasparent fiberglas chamber and exposed to a continuous flow of 8% oxygen and 92% nitrogen for 2.5 h to induce systemic hypoxia.

28 Wistar-albino rats were randomly divided into four groups as follows:

- Group 1 ($n = 7$, HA 20 mg/kg): The group with developed HIE model and given intraperitoneal HA 20 mg/kg
- Group 2 ($n = 7$, HA 10 mg/kg): The group with developed HIE model and given intraperitoneal HA 10 mg/kg
- Group 3 ($n = 7$, Control group): The group that developed the HIE model and will be given 0.3 ml intraperitoneal saline
- Group 4 ($n = 7$, Sham group): The group that no intervention was done

Humic acid injection was done immediately after HIE procedure in HA groups (group 1 and 2). Following a reoxygenation period in the room air pups were sacrificated. For the sacrification procedure, under gase anesthesia all rats were decapitated. The brain tissues were obtained for the pathological evaluation and brain weights were noted.

Histopathological evaluation

Pathological analysis held at Dokuz Eylül University Schoool of Medicine Department of Histology and Pathology. Pathological specimens were extracted after the sacrification of the rats. Brain samples extracted from the trauma applied region and put in a formalin solution of 10%. Histological assessments were conducted by investigators blinded to the groups/treatments

The brains were removed whole and stored in 4% formaldehyde for one week post-fixation. The cerebral hemispheres were cut into coronal (5 mm) blocks through defined neuroanatomical landmarks and underwent tissue processing [12]. The slides for microscopy were prepared from each block using 6 μm-thick sections, and stained with cresyl violet histochemistry for grading of neuronal cell injury and caspase immunohistochemistry.

Histopathological grading of hypoxic ischemic injury

The histopathological grading was used to assess the severity of neuronal cell injury in the parietal cortex, hippocampus (in the pyramidal layer of sector CA1 and extending into the stratum oriens and stratum radiatum), and prefrontal cortex semi-quantitatively. The viable and injured cells were counted in an ocular field. The grading system of Hoque et al. [13] was modified as follows: a Grade of 0 described no detectable hypoxic ischemic damage; Grade $1 \leq 10\%$ neurons injured; Grade 2, 20–30%; Grade 3, 40–60% and Grade $4 > 75\%$. In each specimen, the neurons were counted in 8 neighboring, non-overlapping ocular fields of 400× magnification.

Viable neurons were typically large cells with large, round and pale nuclei. The nucleus contained one or more, well defined nucleoli and was surrounded by a rim of slightly darker cytoplasm. In contrast, the cells with ischemic injury had nuclear hyperchromasia and pyknosis, loss of nucleoli as well as cytoplasmic shrinkage and microvacuolation. The neurons were distinguished from neighboring cells by strict morphological criteria.

Caspase immunohistochemistry and assessment of positivity

For immunohistochemical analyses 5 μ thick sections were used. In brief, the deparaffinization procedure was accomplished in xylene for 1 h. Rehydration was done in 100%, 95%, 80% and 70% alcohol series for 2 min each. After leaving in distilled water for 5 min, the tissues samples were washed in phosphate buffer solution (PBS) for 10 min and than left in trypsin for 15 min. Then the primary antibody (Invitrogen, Thermo Fisher Scientific, USA) diluted as 1:100 was applied in an incubator and washed with PBS. Afterwards the biotinylated secondary antibody was applied, washed with PBS before incubating with 3.3-diaminobenzidine tetrahydrochloride (DAB). The whole procedure was finished after staining the sections with hematoxylin. Caspase-3 expression was calculated by counting the positively-stained cells per 10 HPF in the parietal cortex, hippocampus and prefrontal cortex.

Statistical analysis

SPSS 25.0 (IBM Corporation, Armonk, New York, United States) program was used to analyze the variables. The conformity of the univariate data to normal distribution was evaluated with the Shapiro-Wilk and Shapino-Francia test and variance homogeneity with the Levene's test. One-Way Anova (Robust Test: Brown-Forsythe) test and Kruskal-Wallis H Tests were used to compare more than two groups according to quantitative data, while Dunn's Test and Tukey HSD test were used for Post Hoc analysis. In comparison of categorical variables, Fisher-Freeman-Holton test was tested by Monte Carlo Simulation technique and column ratios were compared and expressed according to Bonferroni corrected *p*-value results. Quantitative variables mean ± SD in the tables. (standard deviation) and median (minimum/maximum), while categorical variables were shown as *n* (%). Variables were examined at a 95% confidence level, and *p* value was considered as less than 0.05.

Results

The study population consisted of pups 13.7 ± 2.93 (mean ± SD) grams (range 9.7–19.4). During the study period 28 pups included into the trial but 22 puppies with sufficient sections of tissues were included in the analysis. No significant differences were observed between the groups in terms of weight and gender. The mean body weight of groups was respectively follows Group 1 was 16.46 ± 3.4 g, Group 2 was 14.8 ± 2.7 g, Group 3 was 12.2 ± 1.4 g and Group 4 was 11.6 ± 1.33 g.

The defined neuroanatomical landmarks were shown in Table 1. The count of neuronal damaged cells was statistically demonstrated in Table 2. The neuronal cell injury was statistically lower in all neuroanatomical lands in HA treatment groups. Grade 3 neuronal damage was not seen in any areas in high dose HA treatment group. Also in hippocampal region there were no grade 3 damage in HA groups. Grade 2 and grade 3 damage was statistically higher in no HA group (Figure 1). Caspase-3 immunoreactivity was decreased in the HA groups compared with the saline group (Figure 2). When the groups were compared, there were no serious neuronal injury in HA with 20 mg/kg/dose group. Grade 3 neuronal injury was statistically higher in parietofrontal lobe (PFL) and parietal lobe (PL) areas without HA groups.

Caspase-3 expression was calculated by counting the positively-stained cells in Table 3 and Figure 2. Neuronal apoptotic cells were higher in no treatment group and in all neuroanatomical lands ($p < 0.001$). When the groups were compared, there were statistically different between the groups. Group 3 had higher apoptotic cells than the other groups. However apoptotic index levels were similar between Group 1 and

Table 1. Neuroanatomical landmarks.

		Hippocampus (HC) Median (Min. / Max.)	Parietal lobe (PL) Median (Min. / Max.)	Parietofrontal lobe (PFL) Median (Min. / Max.)
High dose HA, ($n = 4$)	I	1.5 (2/1)	1 (2/1)	1 (1/1)
Low dose HA, ($n = 6$)	II	2.5 (3/1)	2 (3/2)	1 (2/1)
Saline group, ($n = 6$)	III	3 (3/2)	2.5 (3/2)	1.5 (2/1)
Sham, ($n = 6$)	IV	0 (0/0)	0 (0 / 0)	0 (0 / 0)
P		**<0.001**	**<0.001**	**<0.001**
Multiple Comparisons	I→II	0.239	0.117	0.489
	I→III	0.103	0.066	0.299
	I→IV	0.094	0.144	**0.027**
	II→III	0.611	0.763	0.699
	II→IV	**0.001**	**0.001**	**0.001**
	III→IV	**<0.001**	**<0.001**	**<0.001**

The bold values are statistically significant.

Table 2. Statistical analysis of groups according to cresyl violet histochemistry for grading of neural cell injury.

	High dose HA *n* (%)	Low dose HA *n* (%)	Saline group *n* (%)	Sham *n* (%)	*p*
Parietofrontal lobe (PFL)					
Grade 0	0 (0)	0 (0)	0 (0)	6 (100)^{ABC}	**<0.001**
Grade I	2 (50)	1 (16.7)	0 (0)	0 (0)	
Grade II	2 (50)	2 (33.3)	2 (33.3)	0 (0)	
Grade III	0 (0)	3 (50)^{AD}	4 (66.7)^{AD}	0 (0)	
Parietal lobe (PL)					
Grade 0	0 (0)	0 (0)	0 (0)	6 (100)^{ABC}	**<0.001**
Grade I	3 (75)^{BCD}	0 (0)	0 (0)	0 (0)	
Grade II	1 (25)	4 (66.7)^{D}	3 (50)^{D}	0 (0)	
Grade III	0 (0)	2 (33.3)	3 (50)^{D}	0 (0)	
Hippocampus (HC)					
Grade 0	0 (0)	0 (0)	0 (0)	6 (100)^{ABC}	**<0.001**
Grade I	4 (100)^{D}	4 (66.7)^{F}	3 (50)^{D}	0 (0)	
Grade II	0 (0)	2 (33.3)	3 (50)^{D}	0 (0)	
Grade III	0 (0)	0 (0)	0 (0)	0 (0)	

Fisher Freeman Halton Test(Monte Carlo); Post Hoc Test; Bonferroni Correction, ^{A}Significant compared to group 1, ^{B}Significant compared to group 2, ^{C}Significant compared to the untreated group, ^{D}Significant compared to sham group; The bold values are statistically significant.

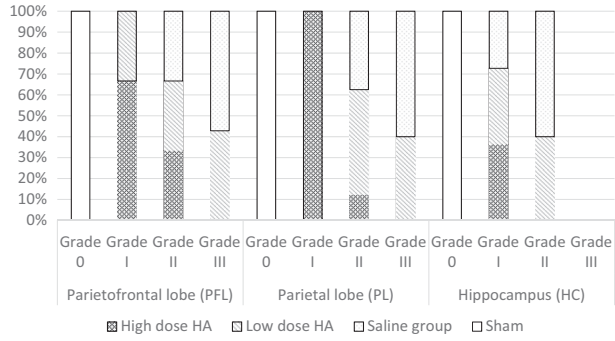


Figure 1. Statistical analysis of groups according to cresyl violet histochemistry for grading of neural cell injury.

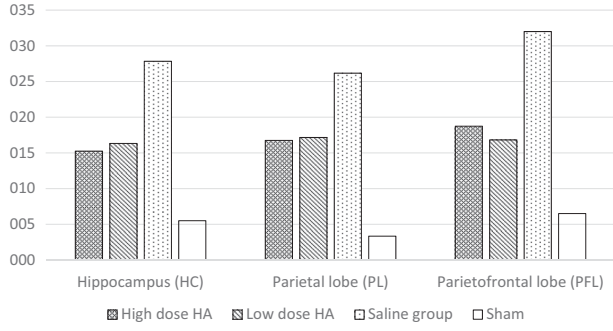


Figure 2. Analysis of groups according to Caspase assay in neuroanatomical lands.

Group 2. The degree of ischemia was significantly smaller in HA groups than the control group.

Caspase-3 immunoreactivity was decreased in the HA groups compared with the control group (Figure 2). When the groups were compared, there were no serious neuronal injury in HA with 20 mg/kg/dose group. Grade 3 neuronal injury was statistically higher in PFL and PL areas with no treatment group.

The photos of injured and noninjured areas were given in Photos 1–5. Our results demonstrated that HA reduces apoptosis and neuronal injury in cerebral tissue of the rats.

Discussion

HA is a polyphenolic antioxidant and a strong free radical scavenger. We aimed to investigate in an

animal model the efficacy of humic acid by showing its antioxidant and anti-apoptotic effect comparing with the histopathological and neurological outcomes for the hypoxic-ischemic brain injury. neuronal injury and apoptosis in an experimental model of HIE and to evaluate the effect of humic acid on the neuronal injury. We showed that HA reduces apoptosis and neuronal injury in cerebral tissue of the rats.

Table 3. Statistical comparison of groups according to Caspase assay.

		Hippocampus (HC) Mean ± SD.	Parietal lobe (PL) Mean ± SD.	Parietofrontal lobe (PFL) Mean ± SD.
High dose HA, ($n = 4$)	I	15.25 ± 1.26	16.75 ± 1.50	18.75 ± 3.30
Low dose HA, ($n = 6$)	II	16.33 ± 1.51	17.17 ± 2.32	16.83 ± 2.04
Saline group, ($n = 6$)	III	27.83 ± 1.60	26.17 ± 1.83	32.00 ± 2.37
Sham, ($n = 6$)	IV	5.50 ± 1.76	3.33 ± 1.03	6.50 ± 1.52
p		**<0.001**	**<0.001**	**<0.001**
Multiple Comparisons	I→II	0.712	0.983	0.571
	I→III	**<0.001**	**<0.001**	**<0.001**
	I→IV	**<0.001**	**<0.001**	**<0.001**
	II→III	**<0.001**	**<0.001**	**<0.001**
	II→IV	**<0.001**	**<0.001**	**<0.001**
	III→IV	**<0.001**	**<0.001**	**<0.001**

Oneway Analysis of Variance (ANOVA) (Robuts Statistic:Brown-Forsythe); Post Hoc Test: Tukey HSD, SD.: Standard deviation; The bold values are statistically significant.

HIE is characterized by condition occurring in babies born over 35 weeks gestational age in which there is disturbed neurological function. It is known that HIE is not a single event, it is an evolving process that can continue for days and weeks [14]. Cerebral blood flow delivers oxygen and glucose to the fetal brain and maintains homeostasis and meet cellular energy demands. A variety of situations may reduce plasental perfusion and the hypoxia leads to a decrease in fetal cardiac output. Depending on the timing of injury, a partial recovery occurs during the 30–60 min after the acute injury [15]. The latent phase is characterized by oxidative stress, intensive inflammation and activation of apoptosis [16]. This phase is characterized by initial transient recovery of cerebral oxidative matabolism. Glutamate excitotoxicity and mitochondrial dysfunction occur within secondary damage mechanisms and leads to oxidative stress. The increase of free oxygen radicals and the intracellular Ca^{+2}, and activation of apoptotic pathways are the major events occuring in the second phase of the neuronal damage. Oxidative stress causes an incease in oxygen radicals and deficiency of antioxidant defense mechanisms are shown as a cause of neuronal injury in HIE [17]. These compounds bind to the cell

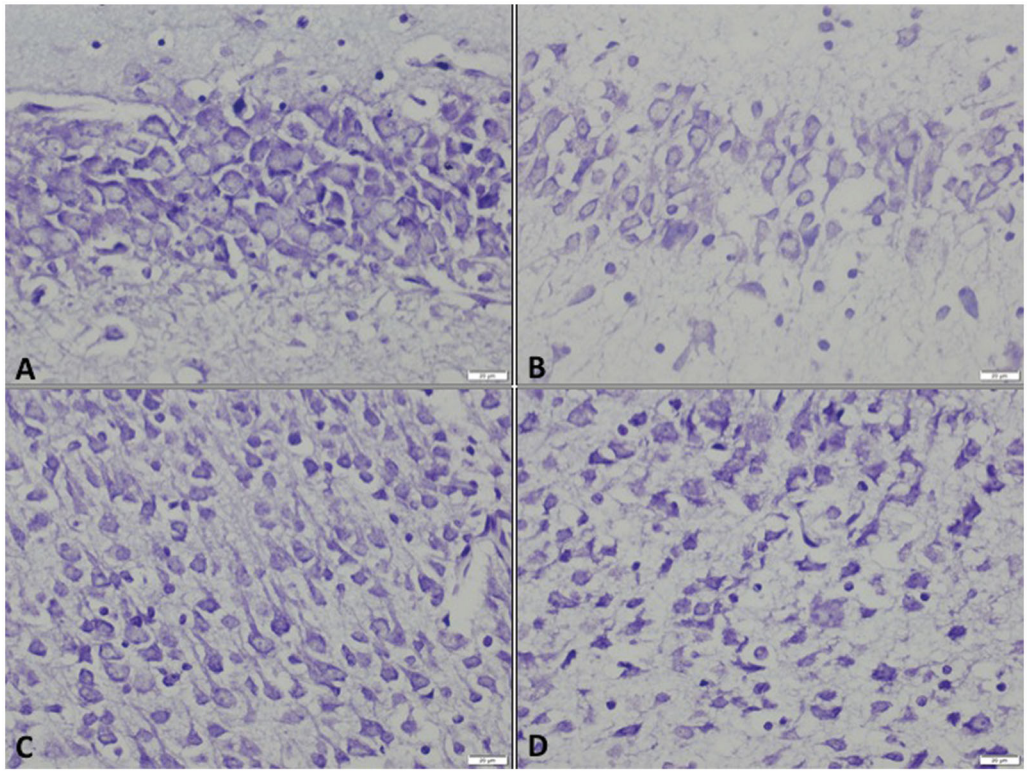


Photo 1. Representative photomicrographs of the hippocampus CA1 and prefrontal cortex stained with cresyl violet. (A) Hippocampus CA1 region 400× magnification showing normal appearance and healthy neurons. (B) Severe ischemic changes in hippocampus showing neuronal loss, cytoplasmic shrinkage, nuclear condensation and disruption of tissue architecture. (C) Prefrontal cortex 400× magnification with healthy neuronal cells. (D) Ischemic cells in the prefrontal cortex.

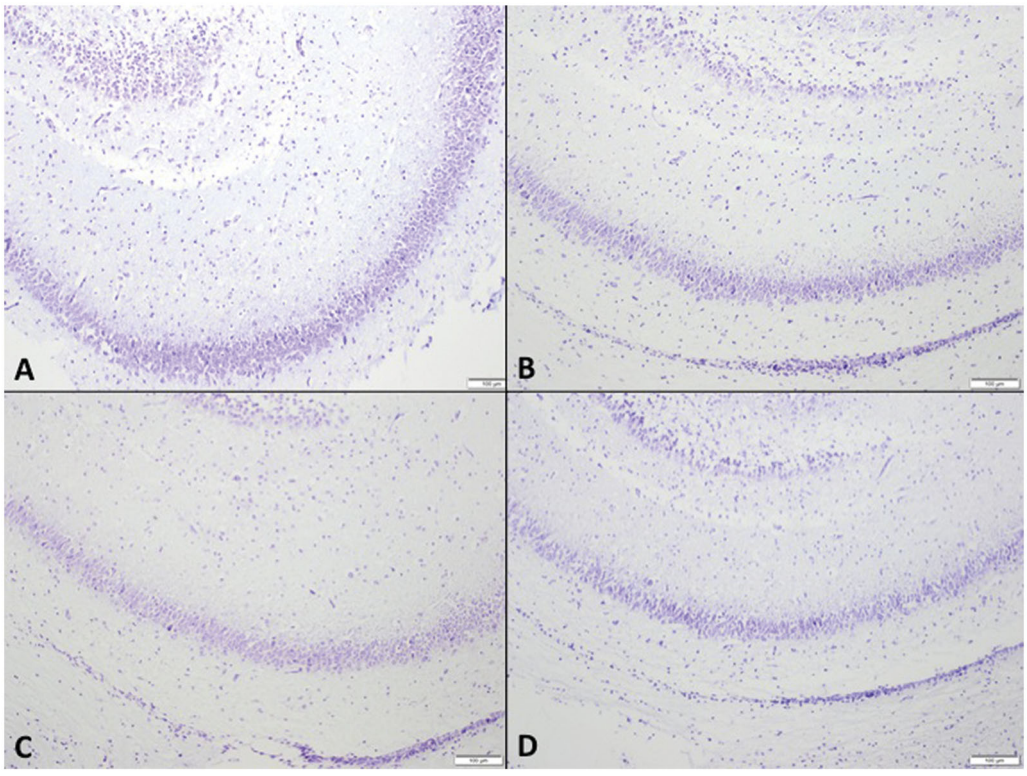


Photo 2. Coronal section of hippocampus. A: sham group; B: no treatment group; C: low-dose treatment group; D: high-dose treatment group. Cresyl violet staining ×100.

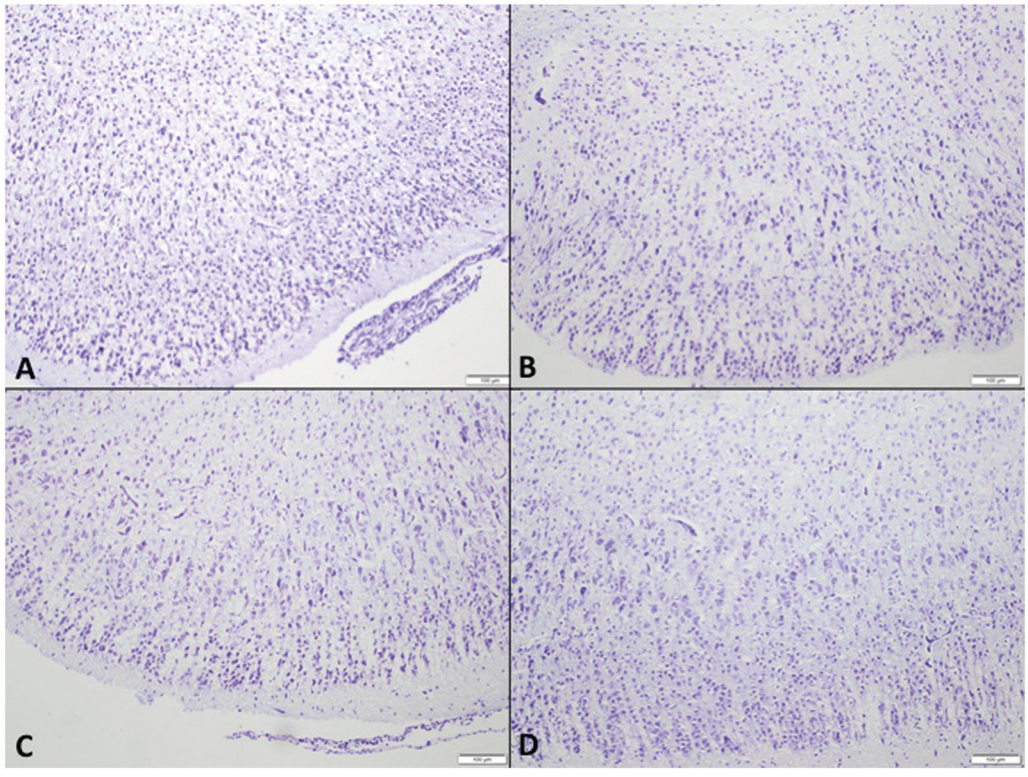


Photo 3. Coronal section of parietal cortex. A: sham group; B: no treatment group; C: low-dose treatment group; D: high-dose treatment group. Cresyl violet staining ×100.

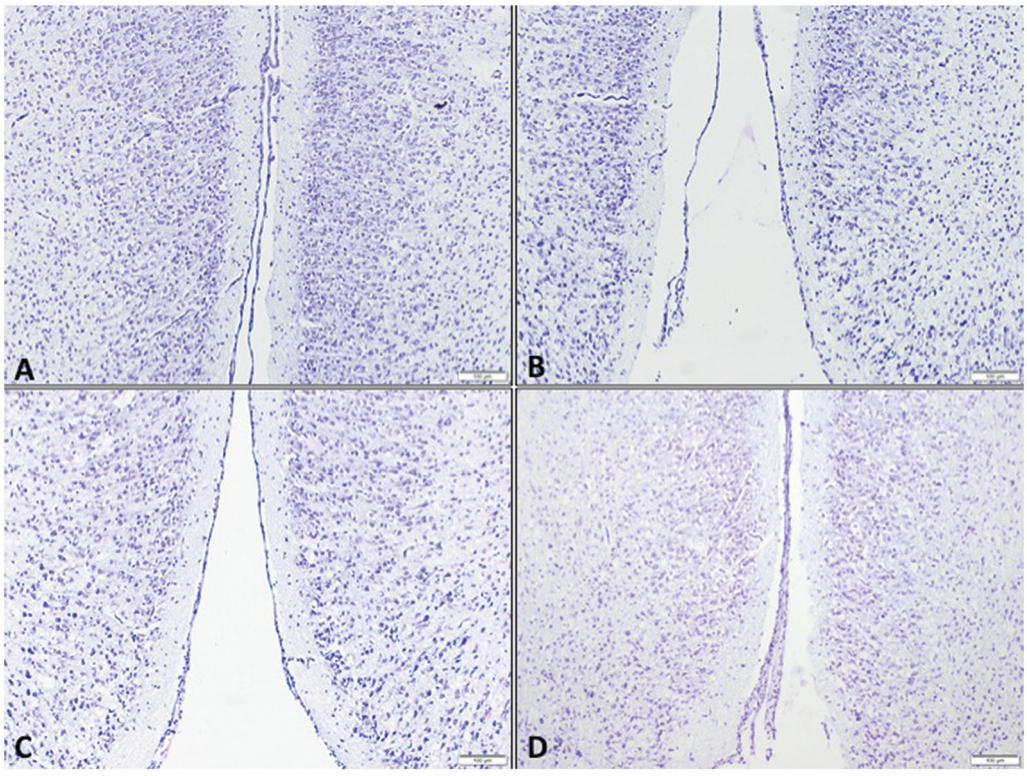


Photo 4. Coronal section of prefrontal cortex. A: sham group; B: no treatment group; C: low-dose treatment group; D: high-dose treatment group. Cresyl violet staining ×100.

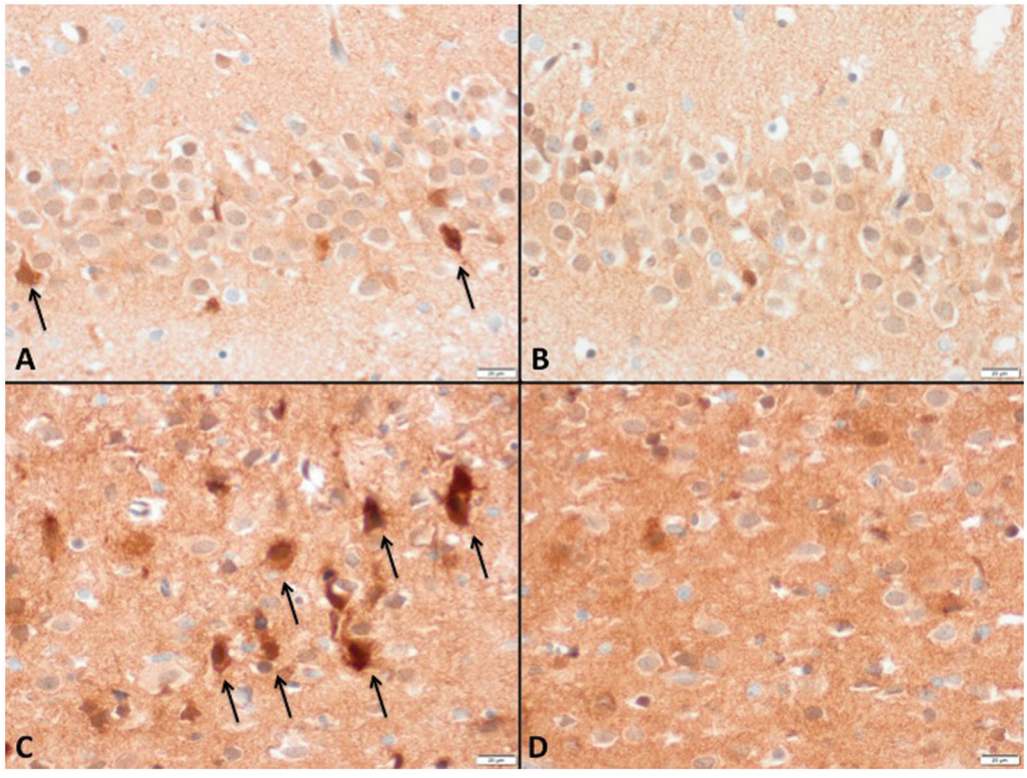


Photo 5. Photomicrograph of the brain tissue immunostained for caspase-3. A (hippocampus): non-treatment group, B (hippocampus): high dose treatment.

membranes, enter reactions with lipids, proteins and cause lipid peroxidation leading to the brain damage. Antioxidant materials clear free oxygen radicals endogenously.

HA is commonly used in agriculture industry and trials to improve ecologic production. It consists of a group of natural high molecular weight macromolecules composed of aromatic rings which has a complex structure in the presence of phenolic, hydroxyl, phenolic hydroxyl, ketonyl, quinone, carboxyl, carbonyl and alkoxyl groups [18]. It has been used for growth stimulation in plants and as therapeutics in animals for many years [19]. It has local analgesic properties, immunostimulatory, antioxidant, antimicrobial, antiinflammatory and antiviral effects [18]. It has been used as a pharmacologic agents in clinical trials.

There are many papers on HIE and the other effects of HA, but there is no data found in the literature. In our study, we found that HA had a serious effect to reduce the degree of damage to HIE. Additionally, histopathological changes linked to HIE remained at minimal levels in the group given high dose of HA.

Ischemia and reperfusion injury leads to cellular necrosis and apoptosis. Apoptosis starts later in HIE than other mechanisms and continues for a longer time. Therefore, administration of HA during the therapeutic window period may be effective in reducing the injury. The other important mechanism is inflammation triggered by ischemia and reperfusion is usually associated with the formation of free oxygen radicals. We speculate that HA may not remove all free oxygen radials in damaged cells, but reperfusion injury can be prevented by reducing oxidative stress. In Akbas trial [20] they investigated the role of HA in renal ischemia and they found that total antioxidant status were increased by giving HA. Also in histopathological evaluation they showed that the apoptosis were deteriorated in no HA group [20]. The renal ischemia was attenuated by HA administration. In adult stroke studies there are many molecules with antioxidant and antiinflammatory effects in order to reduce injury. But there is only one paper on the efficacy of HA on adult stroke model [21]. Ozkan et al. [21], studied in focal cerebral ischemia model. They showed that cerebral edema, vacuolization, degeneration and destruction of neural elements were reduced by giving HA. According to the their trial, caspase-3 immunoreactivity was decreased in the HA group. They also found that that cerebral ischemia was attenuated and they speculated that HA may have potential therapeutic agent in stroke cases. Similarly in our study we showed the decrease of injured neuron levels by HA administration. Also we compared with two different dose levels but dose levels did not make any sense.

In another experimental model Lasukova et al. [22]. investigated the cardiovascular effects of the HA on the model of global ischemia. They showed that the administration of the substances of humic origin before ischemia reduced reperfusion contracture and necrotic death of cardiomyocytes. They were hypothesized that HA are mediated *via* a signaling mechanism associated with activation of NO synthase. Similarly Zykova et al. [23] found that the active substances in HA produced a vasodilating effect and this effect may be related with the regulation of NO synthesis in cardiomyocytes. In HIE mechanism, release of NO is important step of the mitochondrial dysfunction. Mitochondria also play a role in energy metabolism of the brain injury. In literature mitochondrial biogenesis contributes to improved post-ischemic recovery of brain functions and it releases nuclear transcription factor-1 which has an important effect of antioxidant and antiinflammatory effects [24]. However the mechanism of HA is still not well-known. Some researchs showed that HA may induce oxidative DNA damage and may increase NO synthesis and apoptosis [25,26]. Cheng et al. [27] reported that HA generate superoxide anion and reduce glutathione and several antioxidant systems and could induce DNA damage and apoptosis. In contrast to these findings Kodama et al. [28] determined the anticancer effect of HA extract on L1210 leukemia mice model [28]. In another trial, they showed the antiinflammatory effects of HA aganist to Trypanosoma brucei infection [29].

Also the anti-inflammatory effects of HA has been used and tested in literature [30]. The antiinflammatory effects of humic substances may contribute to the inhibition of inflammation-related molecules and other components [30]. It had been evaluated in previous studies either by blocking adhesion molecules or inhibiting the phagocytic stimulants [31]. It is showed that humic substances causes anti-inflammation by inhibiting the degranulation and adherence of neutrophils [31]. Goel et al. [32]., showed that there was a strong antiinflammatory effect by reducing paw edema . They found a relation between HA and blood flow stimulating effects, that may be related to anti-inflammatory features [32]. In another trial, the efficacy of HA substances significantly reduced the paw volume of carrageenan-induced edema in rats [33]. In a study conducted by European Agency for Evaluation of Medicinal Products (EMEA), protective effects on the intestinal mucosa, antitoxic and antimicrobial effects

were demonstrated [34]. Also there are a few researchs on the antiviral effect of HA in invitro trials [35].

Here, we showed the effect of HA on HIE model. This is the first study which investigates the role of HA in HIE model. Apoptotic cells were significantly decreased in the HIE model. Also normal neuronal cell levels were more common in HA administation group. This may be related with the reduction of apoptosis and oxidative stress. This findings suggest that HA may be a viable protective agent against HIE. HA may be a new agent in the treatment of HIE, being also inexpensive and easily available.

In the light of the results of this study, authors are working on the second phase of the study which investigates the effect of humic acid on neurodevelopment.

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Disclosure statement

All authors declare that there is no conflict of interest.

Author contributions

Senem Alkan Ozdemir and Nail Ozdemir designed the study and wrote the manuscript.

Nail Ozdemir, Burak Kınalı and Ozgur Aksan designed the experimental model.

Erdener Ozer, Güven Erbil and Gökçen Bilici Güler evaluated the histopathological analysis.

Esra Ozer revised the manuscript.

References

[1] Escobar MD, Weiss MD. Hypoxic-ischemic ensephalopathy a review for the clinician. JAMA. 2015;169(4):397–403.

[2] Volpe JJ. Perinatal brain injury: from pathogenesis to neuroprotection. Ment Retard Dev Disabil Res Rev. 2001;7(1):56–64.

[3] Plessis AJ, Volpe J. Perinatal brain injury in the preterm and term newborn. Curr Op Neurol. 2002;15:151–157.

[4] Wyckoff MH, Aziz K, Escobedo MB, et al. American Heart Association guidelines update for cardiopulmonary resuscitation and emergency cardiovascular care (reprint). Pediatrics. 2015;136(Supplement):S196–S218.

[5] Wassink G, Lear CA, Gunn KC, et al. Analgesics, sedatives, anticonvulsant drugs, and the cooled brain. Semin Fetal Neonatal Med. 2015;20(2):109–114.

[6] Davidson OJ, Dean JM, Fraser M, et al. Perinatal brain injury: mechanisms and therapeutic approaches. Front Biosci. 2018;23:2204–2226.

[7] Yoruk MA, Gul M, Hayirli A, et al. The effects of supplementation of humate and probiotic on egg production and quality parameters during the late laying period in hens. Poult Sci. 2004;83(1):84–88.

[8] EMEA. Humic acids and their sodium salts, summary report. Committee for Veterinary Medical Products. European Agency for the Evaluation of Medicinal Products. 1999. Available from: http://www.ema.europa.eu/docs/en_GB/document_library/Maximum_Residue_Limits-Reports/2009/11/WC500014416.pdf.

[9] Erel O. A novel automated direct measurement method for total antioxidant capacity using a new generation, more stable ABTS radical cation. Clin Biochem. 2004;37(4):277–285.

[10] Hseu YC, Huang HW, Wang SY, et al. Humic acid induces apoptosis in human endothelial cells. Toxicol Appl Pharmacol. 2002;1182(1):34–43.

[11] Akisu M, Kumral A, Canpolat FE. Turkish Neonatal Society Guideline on neonatal encephalopathy. Turk Pediatri Ars. 2018;53(Suppl 1):S32–S44.

[12] Defazio R, Criado A, Zantedeschi V, et al. Neuroanatomy-based matrix-guided trimming protocol for the rat brain. Toxicol Pathol. 2015;43(2):249–256.

[13] Hoque N, Sabir H, Maes E, et al. Validation of a neuropathology score using quantitative methods to evaluate brain injury in a pig model of hypoxia ischaemia. J Neurosci Methods. 2014;230:30–36.

[14] Cosar H, Ozer E, Topel H, et al. Neuronal apoptosis in the neonates born to preeclamptic mothers. J Matern Fetal Neonatal Med. 2013;26(11):1143–1146.

[15] Bennet L, Roelfsema V, Pathipati P, et al. Relationship between evolving epileptiform activity and delayed loss of mitochondrial activity after asphyxia measured by near-infrared spectroscopy in preterm fetal sheep. J Physiol. 2006;572(1):141–154.

[16] Wassink G, Gunn ER, Drury PP, et al. The mechanisms and treatment of asphyxial encephalopathy. Front Neurosci. 2014;8:40.

[17] Arguelles S, Machado MJ, Ayala A, et al. Correlation between circulating biomarkers of oxidative stress of maternal and umblical cord blood at birth. Free Radic Res. 2006;40:565–570.

[18] Chen Y, Katan J, Gamliel T, et al. Involvement of soluble organic matter in increased plant growth in solarized soils. Biol Fertil Soils. 2000;32(1):28–34.

[19] Hartenstein R. Sludge decomposition and stabilization. Science. 1981;212(4496):743–749.

[20] Akbas A, Silan C, Gulpinar MT, et al. Renoprotective effect of humic acid on renal ischemia-reperfusion injury: an experimental study in rats. Inflammation. 2015;38(6):2042–2048.

[21] Ozkan A, Sen HM, Sehitoglu I, et al. Neuroprotective effect of humic acid on focal cerebral ischemia injury: an experimental study in rats. Inflammation. 2015;38(1):32–39.

[22] Lasukova TV, Zykova MV, Belousov MV, et al. The role of NO Synthase in the cardioprotective effect of substances of humic origin on the model of ischemia and reperfusion of isolated rat heart. Bull Exp Biol Med. 2019;166(5):598–601.

[23] Zykova MV, Belousov MV, Lasukova TV, et al. Cardiovascular effects of high-molecular-weight compounds of humic nature. Bull Exp Biol Med. 2017; 163(2):206–209.
[24] Zhang Q, Wu Y, Zhang P, et al. Exercise induces mitochondrial biogenesis after brain ischemia in rats. Neuroscience. 2012;205(205):10–17.
[25] Hu CW, Yen CC, Huang YL, et al. Oxidatively damaged DNA induced by humic acid and arsenic in maternal and neonatal mice. Chemosphere. 2010;79(1):93–99.
[26] Gau RJ, Yang HL, Suen JL, et al. Induction of oxidative stress by humic acid through increasing intracellular iron: a possible mechanism leading to atherothrombotic vascular disorder in blackfoot disease. Biochem Biophys Res Commun. 2001;283(4):743–749.
[27] Cheng ML, Ho HY, Huang YW, et al. Humic acid induces oxidative DNA damage, growth retardation, and apoptosis in human primary fibroblasts. Exp Biol Med (Maywood). 2003;228(4):413–423.
[28] Kodama H, DENSOAntitumor effect of humus extract on murine transplantable L1210 leukemia. J Vet Med Sci. 2007;69(10):1069–1071.
[29] Kodama H, Okazaki FD, Ishida S. Protective effect of humus extract against *Trypanosoma brucei* infection in mice. J Veterinary Med Sci. 2008;70(11):1185–1190.
[30] van Rensburg CE. The antiinflammatory properties of humic substances: a mini review. Phytother Res. 2015; 29(6):791–795.
[31] Joone GK, van Rensburg CE. An in vitro investigation of the anti-inflammatory properties of potassium humate. Inflammation. 2004;28(3):169–174.
[32] Naude PJW, Cromarty AD, Jansen AE. Potassium humate inhibits carrageenan-induced paw oedema and a graft-versus-host reaction in rats. Inflammopharmacology. 2010;18(1):33–39.
[33] Goel RK, Banerjee RS, Acharya SB. Antiulcerogenic and antiinflammatory studies with shilajit. J Ethnopharmacol. 1990;29(1):95–103.
[34] Keuth U, Leinenbach A, Beck HP, et al. Separation and characterization of humic acids and metal humates by electrophoretic methods. Electrophoresis. 1998; 19(7):1091–1096.
[35] Joone GK, Dekker J, van Rensburg CE. Investigation of the immunostimulatory properties of oxihumate. Z Naturforsch C J Biosci. 2003;58(3–4):263–267.