Investigating the interplay of immune response and redox homeostasis in hypoxic brain injury at high altitude

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SUMMARY

Environments at high altitudes, where barometric pressure is low and oxygen levels are diminished, significantly heighten the risk of High-Altitude Cerebral Edema (HACE), a potentially life-threatening neurological disorder. HACE induces a robust inflammatory response, disrupting redox balance in the brain and causing neurocyte swelling and degeneration. This study evaluated changes in apoptotic cells, mast cells (MCs), and the expression of TNF-α, a key mediator of neuroinflammation, following acute hypobaric hypoxia. Furthermore, changes in the activities of superoxide dismutase (SOD), catalase (CAT), and lactate dehydrogenase (LDH) were examined to evaluate disruptions in the redox balance and potential disturbances in cell energy metabolism within the brain. The animals were exposed to hypobaric hypoxia for roughly 24 hours at an altitude of 7620m. The findings revealed a significant increase in MCs in the brain tissue ($p<0.001$), along with an elevated number of TNF-α and apoptotic cells, suggesting considerable neuronal degeneration ($p<0.05$). The activities of SOD and CAT were decreased ($p<0.01$), which shows weakened antioxidant defense mechanisms following the hypoxic exposure. However, the changes of LDH level remained unaltered. These results underscore the critical role of the immune reaction of the brain triggered by the hypoxic conditions that aggravate cerebral edema development. This study illustrates the interplay of inflammatory response with an antioxidant defense system in the context of blood-brain barrier (BBB) disruption, contributing to cerebral edema formation and neuronal dysfunction. This research deepens our understanding of the pathogenesis of HACE, highlighting the interactions between specific biomolecules that regulate the brain's immune reaction and redox pathways under hypoxic conditions.

Key words: High altitude cerebral edema – Apoptosis – Neuroinflammation – Mast cells – Redox balance

INTRODUCTION

Hypobaric hypoxia encountered at high altitudes (HA) is known to have deleterious effects on brain functions. People who travel to HA for various reasons including tourism and research are commonly confronted with problems such as acute mountain sickness (AMS) and high-altitude

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Submitted: October 26, 2024. **Accepted:** January 17, 2025

https://doi.org/10.52083/BKKH4373

cerebral edema (HACE) (Maiti et al., 2006; Yao et al., 2016). Altitude is characterized by low barometric pressure and, hence, reduced atmospheric oxygen partial pressure (PO_2), which decreases arterial oxygen content and leads to tissue hypoxia (Santocildes et al., 2021).

Hypoxia has long been known to diminish brain function in humans and animals. Compared to the human brain, the brain of smaller animals (such as rats) is more resistant to hypoxia because of its higher capillary density, that is, a much more severe hypoxic condition must be applied to overtly damage its brain tissues relative to humans (Qaid et al., 2017). It has been demonstrated that severe and chronic (>5500m, for 3-4 days) hypoxia causes neuronal death in the deep and peripheral brain structures like CA3, CA4, and dentate gyrus of the hippocampus, thalamus, cerebral cortex, and striatum. However, damages were visible only after 4 days (Maiti et al., 2007).

Hypoxia has been associated with increased neurodegeneration in a variety of brain nuclei, which can affect different functions (Snyder et al., 2017). The most remarkable effects of HA on human physiology are due to the low oxygen partial pressure of the inhaled air. During hypoxic stress, insufficient oxygen delivery to working muscles causes increased reactive oxygen species (ROS) production that may damage the polyunsaturated fatty acids in cell membrane structures (Esteva et al., 2010). Many events lead to hypoxia-induced oxidative stress (Bocanegra et al., 2021), with harmful effects of ROS on cellular components such as proteins, phospholipids, and DNA (Ganguly et al., 2021; Kheirbakhsh et al., 2018). Importantly, ROS is a messenger of certain signal transduction, which can activate some transcription factors and proteins, thus affecting different signal pathways and ultimately determining cell fate (He et al., 2020). The likely ROS overproduction occurring during HH can be accompanied by excess malondialdehyde (MDA) levels and oxidative stress (Ribon et al., 2016). Such oxidative damage can lead to mitochondrial dysfunction, Ca^{2+} imbalance, and apoptosis in neuronal cells. However, neurological cells are protected by antioxidants against peroxidative damage. Furthermore, detoxifying enzymes, e.g., superoxide dismutase (SOD) and catalase (CAT) may be altered in their activity or protein synthesis (Nazıroğlu, 2012). Although CAT is among the first isolated and characterized enzymes, its role *in vivo* under normal physiological conditions and in the pathogenesis of oxidant-mediated diseases remains poorly understood (Ho et al., 2004).

Brain metabolism is complex as it responds dynamically to changes in blood glucose and lactate concentrations. Lactate dehydrogenase (LDH) has an active role within the brain and lactate has also recently been implicated as a neural intracellular messenger (Valvona et al., 2015). It is suggested that the LDH-A isozyme activity and its protein expression are increased most significantly at 24 h in cells under hypoxia. However, up until now, the relationship between LDH and hypoxia has not precisely been known (Wei et al., 2016). Moreover, the mechanism of HH-induced cerebral pathology remains unclear (Koundal et al, 2014). Many central nervous system (CNS) disorders involve neuroinflammation and hypoxia-related oxidative stress (Kim et al., 2012). Hypoxia can cause brain injury, characterized by structural changes, mitochondrial dysfunction, weakened antioxidant defense system, and increased expression of apoptotic markers (Arriaza et al., 2022; Kölliker-Frersi et al., 2021).

HACE is a rare condition linked to exposure to HA, primarily driven by low blood oxygen levels, oxidative stress, and neuroinflammation (Jing et al., 2020; Hacket and Roach, 2004). HACE typically manifests in cerebral anomalies at altitudes above 3000-5000m, where oxidative damage and inflammation play key roles (Yubo et al., 2023). Specifically, elevated pro-inflammatory cytokines can worsen neuronal injury (Huaxu et al., 2021).

Mast cells (MCs), important innate immune cells, release inflammatory mediators that trigger neuroinflammation and promote immune cell infiltration into the CNS. Following hypoxic events, MC numbers increase, enhancing blood-brain barrier (BBB) permeability and contributing to brain inflammation (Germundson et al., 2022).

Our study aims to evaluate the effects of hypobaric hypoxia, which considerably causes neuroinflammatory responses and oxidative stress, contributing to the pathogenesis of HACE. Specifically, we propose that MCs, by releasing inflammatory mediators such as TNF-α, play a pivotal role in exacerbating neuroinflammation, which in turn leads to neuronal apoptosis in the brain.

Furthermore, we aim to explore how alterations in antioxidant defense mechanisms, particularly the activities of SOD and CAT, as well as LDH-related metabolic changes, contribute to the neuroinflammatory process and neuronal damage observed during HACE development. This research will elucidate the relationship between oxidative stress, immune responses, and metabolic shifts that may underlie HACE, with the broader objective of advancing understanding of the mechanisms involved in cerebral injury at high altitudes.

MATERIALS AND METHODS

Experimental design

The experiments involving 32 albino male rats weighing 200-250g were conducted in accordance with the regulations outlined in directive 2010/63/EU and were approved by the local Bioethics Committee of Armenia. To replicate cerebral edema, the HH model was utilized. The control group (n=14) was kept in normobaric conditions (6000 feet, 1829 m, FiO_2=16.6%, pO_2=609 mm Hg), while the experimental group (n=18) rats were subjected to HH in a decompression chamber for 24 hours. The specific altitude required to induce edema was chosen based on existing literature (7620 m altitude, equal to 25.000 feet, 8.1% O_2, 300 m/min velocity) with minor modifications (Sherman and Sladky, 2018; Muthuraju and Pati, 2014; Pena et al., 2022). The animals were housed in a temperature-controlled environment (22 ± 1°C) with a 12-hour light-dark cycle, and had free access to water and food. Following hypoxic exposure, animals from the experimental and control groups underwent histological and biochemical examinations.

TUNEL assay

To detect apoptotic cells, DNA fragmentation in apoptotic cell nuclei was determined using terminal deoxynucleotidyl transferase-mediated dUTP nick endlabelling (TUNEL) reaction special kit (TUNEL Assay Kit-HRP-DAB, ab206386, Cambridge, UK). After dehydration, the specimens were rinsed for 10 min in 0.1 M PBS, then treated with 20 µg/ml proteinase K for 20 min at room temperature and were treated with 3% H_2O_2 in methanol for 10min to inactivate endogenous peroxidase. After washing with PBS, specimens were incubated in the labeling reaction mixture containing terminal deoxynucleotidyl transferase and the deoxynucleotide at 4°C overnight. After incubation, all the sections were rinsed in PBS for 30 min at room temperature. Then, the sections were washed extensively with PBS for 3 min and treated with DAB solution (30 mg DAB and 200 µl H_2O_2/100 ml PBS) for 15min at room temperature in the dark. Finally, the sections were dehydrated in increasingly graded ethanol, cleared in xylene, and mounted with a cover slip. In this method, apoptotic nuclei were identified by dark brown staining. The staining was performed according to the manufacturers; instructions and literature (Kim et al., 2012). For the positive control, the incubated sections were processed with DNAse I (3000 U/ml in 50 mM Tris-HCl, pH 7.5, 1 mg/ml BSA) for 10 minutes at 15-25° C to induce DNA strand breaks, and then applied the TUNEL reaction. For the negative control, sections were incubated with only the label solution (without terminal transferase) instead of the TUNEL reaction mixture. All the sections were stained with methylene blue (MB) and observed under a light microscope (B-293, OptikamB5 Digital Camera M-114, Italy), and the images were recorded with Optika Liteview software with magnification x400. The mean number of apoptotic cells was counted in CA subfields of the hippocampus and cerebral cortex with a special focus on the prefrontal cortex via ImageJ software (n/field=10).

Examination of MCs distribution and quantitative changes

To determine the distribution and amount of MCs, toluidine blue and May-Grünwald Giemsa staining methods were applied as described in the literature (Pena et al., 2024; Rieger et al., 2013). The total amount of MCs including granulated and degranulated cells in the hippocampus and cerebral cortex (prefrontal cortex) (n=15/group) was counted in 15 observed fields under a light microscope (B-293, Italy).

Immunohistochemical study

In order to evaluate the expression of TNF-α following HH exposure, an immunohistochemical study was conducted utilizing an anti-TNF-α antibody (Abcam, 220210, Cambridge, UK). Brain slices were subjected to dewaxing and dehydration through a series of alcohol solutions of descending concentrations (95%, 70%, and 50%) for 3 minutes each. To inhibit endogenous peroxidase activity, the sections were treated with a 3% H_2O_2 solution in methanol at room temperature for 10min, followed by two washes with TBS containing 10% BSA for 1 hour. Upon removal of the blocking buffer, the primary antibody was applied, and the slides were incubated at 4°C overnight. A secondary HRP-conjugated antibody was subsequently used, and the slides were incubated for 30 minutes at 37°C. Visualization was achieved through the application of the diaminobenzidine chromogen (DAB, Abcam, UK). The specimens were mounted with DPX and viewed under a light microscope. The average number of TNF-α-positive cells in the hippocampus and prefrontal cortex was quantified utilizing the ImageJ software package.

Evaluation of antioxidant system activity

The CAT activity was assessed using the spectrophotometric method. For this purpose, the tissue samples (n=4) were washed with 0.9% NaCl solution (w/v) to eliminate blood, and 10% of brain tissue homogenate was mixed with the phosphate buffer (pH 7.4) and then centrifuged at 4000 g for 10 minutes. Afterward, 1 ml H_2O_2, 0.2 ml potassium phosphate buffer, and 1 ml ammonium molybdate were added to 0.2 ml of each sample. The optical density of the yellow complex formed by ammonium molybdate and hydrogen peroxide was measured spectrophotometrically at 374 nm at room temperature. CAT activity was expressed in the µmol/g of hydrogen peroxide (Hamza and Hadwan, 2020).

For the assessment of SOD activity, brain tissue (n=4) homogenate was homogenized with an inactivation medium containing 25M saccharose and 1 mmol/L EDTA (1:9 ratio), then was centrifuged at 6000 g for 15 minutes. Subsequently, 1.8 ml of bicarbonate buffer (pH 10.55) and 0.1 ml of adrenaline were added to 0.1 ml of the sample. The activity of SOD was determined at room temperature by measuring the absorbance at 480 nm using a spectrometer based on the auto-oxidation of adrenaline. One unit of SOD activity was defined as the amount of enzyme inhibiting the adrenaline auto-oxidation by 50% (Sirota, 2012; Jacek, 2012).

Meanwhile, the LDH activity was determined based on the standard NADH assay (Hinman and Blass, 1981). For this purpose, the brain tissue samples were homogenized (10%) through the inactivation medium following the centrifugation at 14000 g for 15 minutes, then 2 ml pyruvate resolved in the 2 ml phosphate buffer (pH 7.5) along with 0.05 ml NADH was added to each sample (0.1 ml), and a standard spectrophotometric method was used to measure the quantitative reduction of NADH at 340 nm. The mean optical density was recorded during 3-5 min with 30 sec intervals (Hinman and Bass, 1981).

Statistical analysis

The data were presented as mean±SD for all used methods. The analysis of the results was performed by Student *t*-test (unpaired, two-tailed) using the Mann-Whitney test (Fig. 2C). Taking into account the nature of the data and studied groups exposed to different conditions—normobaric and hypobaric hypoxia, the Mann-Whitney test was used to compare their results.

Meanwhile, Tukey's multiple comparisons test was employed for post-hoc analysis by one-way ANOVA to make pairwise comparisons between different regions of the brain (Figures 1J and 3I). The statistical analysis was conducted via SPSS Statistics and the significance between the control and the experimental groups was considered as $p<0.05$.

RESULTS

Following HH exposure, the number of apoptotic cells was elevated in the CA3 (Fig. 1A-C) and CA2 (Fig. 1D-F) regions of the hippocampus, and the cerebral cortex (prefrontal cortex) (Fig. 1G-I). The results indicated a significant increase (Fig. 1J) in the number of apoptotic cells in the aforementioned regions of the brain, alongside cell shrinkage and hyperchromatization. Furthermore, the number of TUNEL-positive cells was significantly higher ($p<0.001$) in the HACE group in comparison to the control group, indicating the occurrence of apoptosis in response to oxidative stress caused by acute oxygen deficiency. These changes are due to an activation of apoptotic pathways that cause DNA damage among neurons. According to the literature, HH exposure causes neurodegeneration in the CA3 region of the hippocampus, glutamate excitotoxicity, and a high influx of calcium-mediated apoptosis cascade (Kumar et al., 2018). The findings are in line with the points mentioned above and therefore suggest that exposure to HH has detrimental effects on the

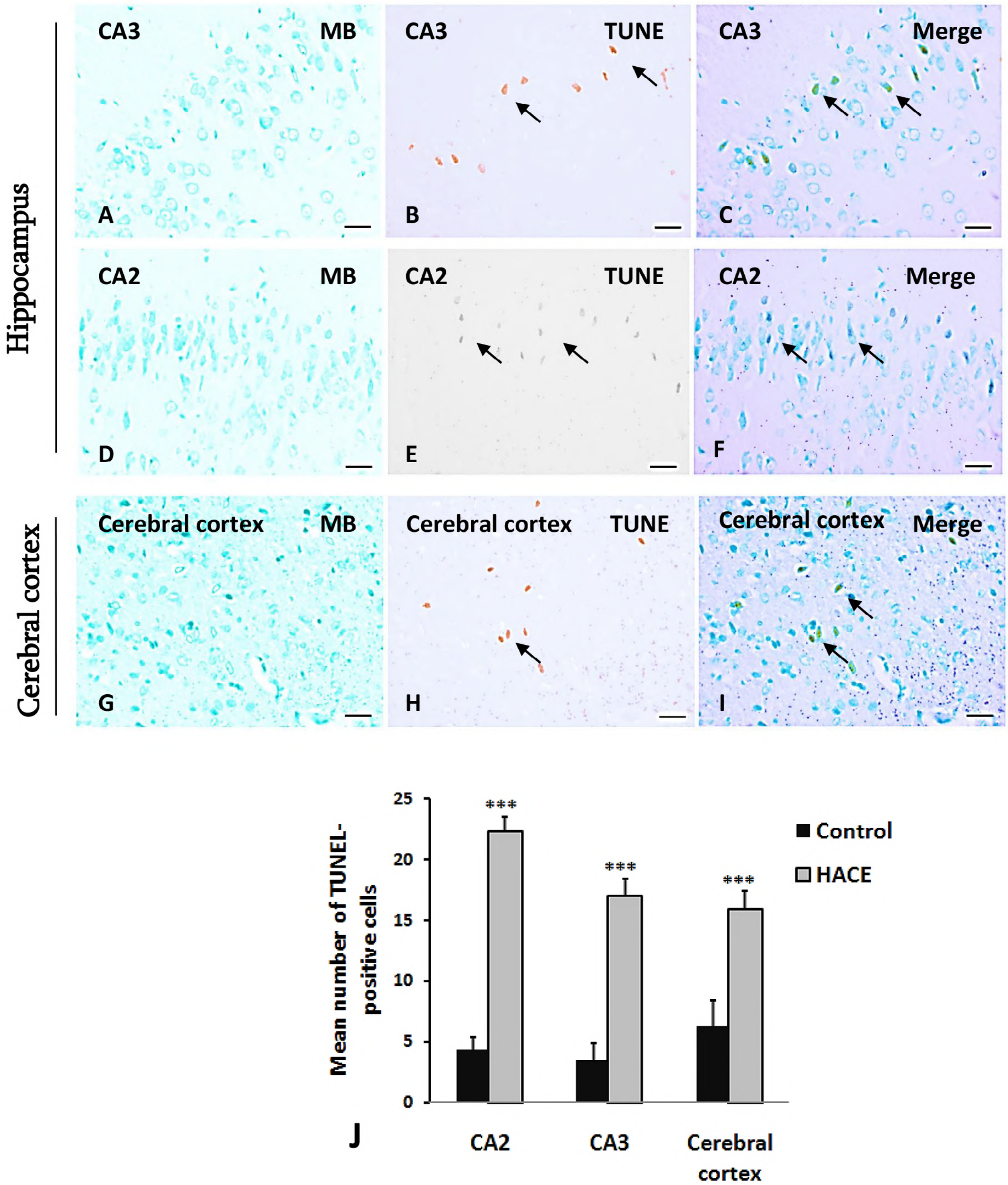

Fig. 1.- Histological sections of the CA2, and CA3 subfields of the hippocampus and cerebral cortex of negative control (**A, D, G**), positive control samples (**B, E, H**). TUNEL-positive cells (**C, F, I**) of the HACE group (arrows). Methylene blue and TUNEL staining, magnification x400, scale bar = 50 µm**.** Changes of TUNEL-positive cells in control and HACE groups (**J**). (***$p<0.001$ compared with control group according to Tukey's test, n/field=10).

brain, particularly in terms of inducing apoptosis and cellular damage.

In the brain, MCs are typically perivascular, have substantial granule content, and are frequently observed siding cortical penetrating arterioles. After hypoxia, MCs were found more abundantly in the cerebral tissue, where they release their granules, thus leading to permeability change and perivascular edema. More recently, the rapid recruitment and activation of TNF-α positive MCs within 1 h after a generalized hypoxic–ischemic challenge has been shown in the neonate rat brain, although such a rapid MC accumulation has not been documented in other ischemia models (Lindsberg et al., 2011). MCs are found adjacent to blood vessels and nerve endings; in the brain, they are located in the thalamus, hypothalamus, and median eminence. Their secretory granules store many preformed proinflammatory and neuro-sensitizing mediators, including bradykinin, histamine, TNF-α and tryptase (Theoharides et al., 2019).

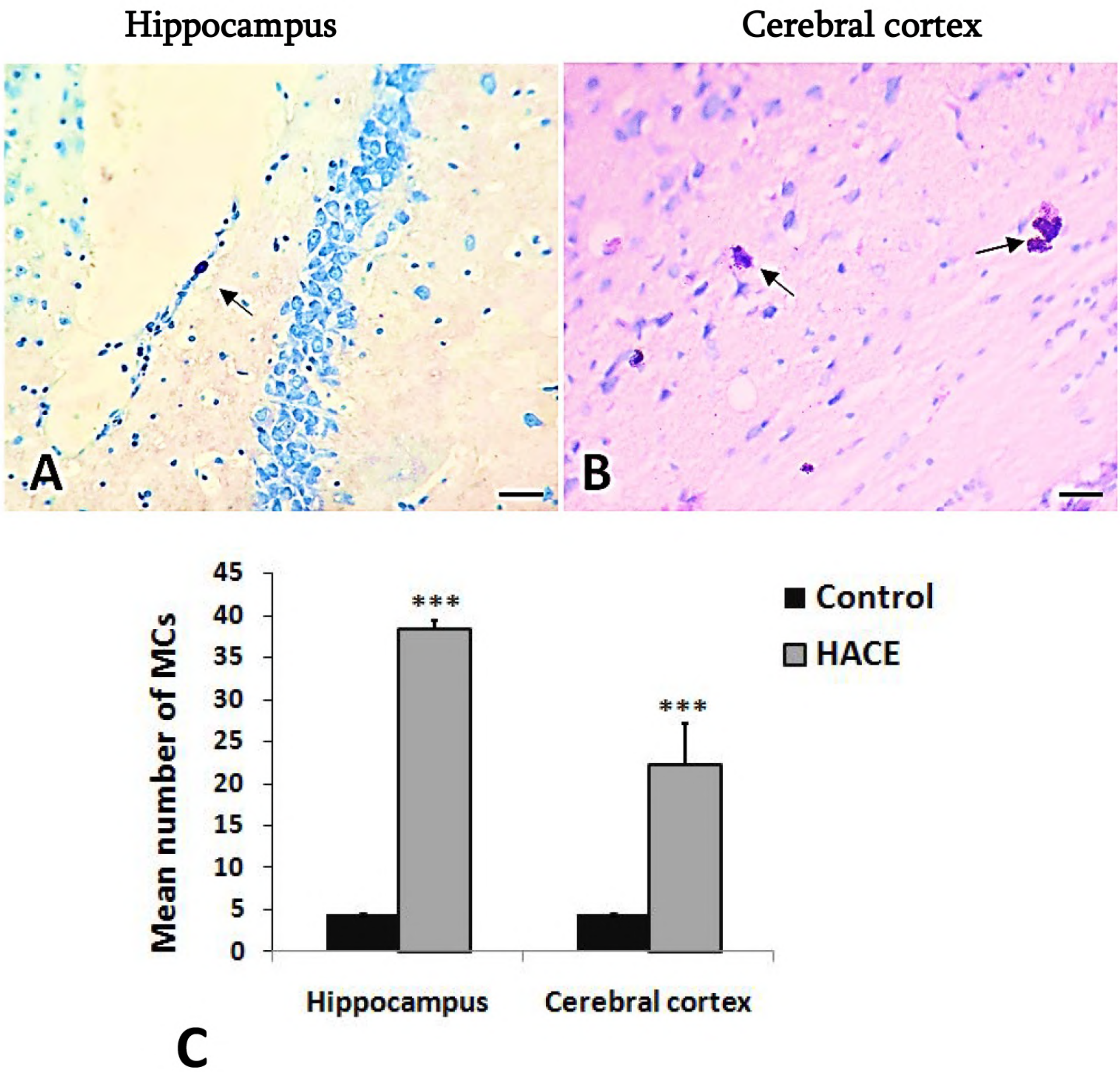

Fig. 2.- Histological sections showing MCs (arrows) in the hippocampus (**A**) (Toluidine blue staining), and cerebral cortex (**B**, arrows) of the HACE group (Giemsa staining, magnification x400, scale bar = 50 μm). Changes of MCs number between control and HACE groups (**C**). (***p<0.001 compared with control group according to Mann-Whitney test, n/field=15).

In the current study, significant changes in MC number after HH were detected. The average number of MCs is presented in Table 1. The observed MCs were mainly distributed in the hippocampus and cerebral cortex (Fig. 2A, B). Moreover, after hypoxic injury, the number of MCs in the observed brain regions has increased significantly (Fig. 2C, $p<0.001$).

In addition to the changes in MC number, the HH resulted in increased activity of TNF-α. This is linked to the brain's inflammatory response under hypoxic conditions. The TNF-α-positive cells were identified in the brain parenchyma, specifically in the hippocampus and cerebral cortex (Fig. 3 A-H). The comparison of the changes in TNF-α-positive cells in the cerebral cortex (Fig. 3D, H), CA1, CA2, and CA3 subfields of the hippocampus was presented in Fig. 3 (E-G), from which it is obvious that under hypoxic conditions the immune reactive cells were especially predominant in the hippocampus (Fig. 3I).

Table 1. The average number of mast cells (MCs) in the hippocampus and cerebral cortex between control and HACE groups.

Number of mast cells	Control Mean±SD	HACE Mean±SD
Hippocampus	3.4±0.99	30.53±7.74***
Cerebral cortex	4.4±0.97	36.5±5.23***

p<0.001 control versus HACE group

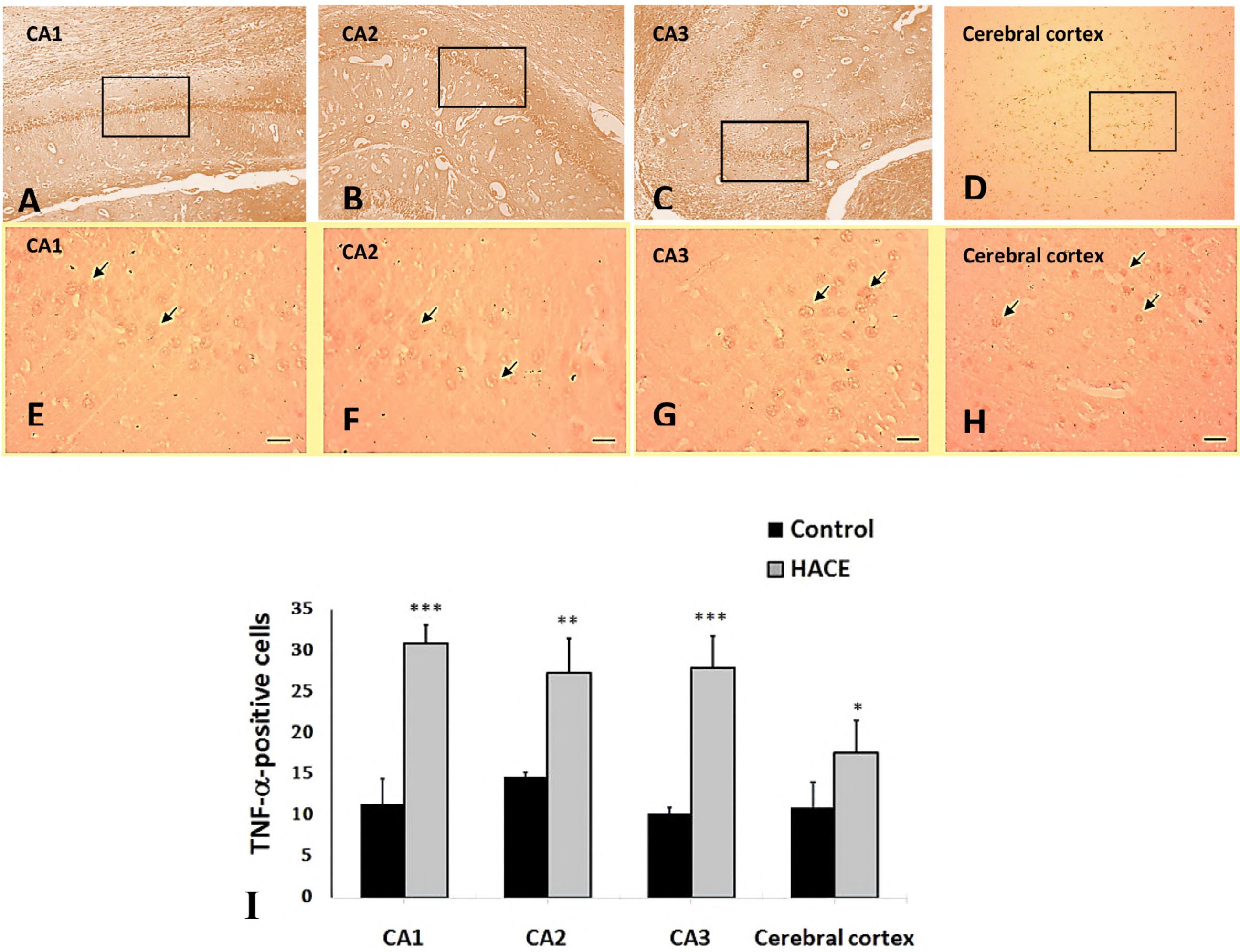

Fig. 3.- Histological sections of the TNF-α positive cells (arrows) in the CA1 (**A, E**), CA2 (**B, F**), CA3 regions (**C, G**) of the hippocampus and cerebral cortex (**D, H**) of the HACE group (magnifications x100, x400, scale bar = 50 μm). The number of TNF-α-positive cells is significantly increased between the studied groups (**E**). (***p<0.001, **p<0.01, *p<0.05 compared with the control group according to Tukey's test, n/field=10).

Table 2. The changes in CAT, SOD, and LDH activities between control and HACE groups.

Antioxidant defense system (µmol/g)	Control Mean±SD	HACE Mean±SD
CAT	2.252±0.27	1.677±0.18**
SOD	0.69±0.03	0.12±0.01***
LDH	0.386±0.02	0.396±0.03 ns

****p<0.0001, **p<0.01 significant, *ns*-non significant, control versus HACE group

In regards to the redox state, significant changes were also determined following HH exposure, specifically, the activities of SOD and CAT were undergone notable alterations. Particularly, both the SOD and CAT activities were diminished (Table 2), which was due to an excess production of ROS in the brain.

SOD is very important in the regulation of superoxide as a result of oxidative phosphorylation (Wardaya et al., 2012). It protects the cells from oxidative stress by neutralizing the superoxide radicals. SOD catalyzes the dismutation of potentially hazardous molecules of superoxide anion (O_2) into less harmful oxygen molecules (O_2) as well as H_2O_2 (Chidambaram et al., 2024), which in turn is inactivated by CAT. Furthermore, detoxifying enzymes such as those SOD and CAT may be altered in their activity in response to the brain injury (Gsell et al., 1995). Data suggest that neuronal degenerative processes could be due to the accumulation of hydrogen peroxide in some areas of the brain, as indicated by a significant decrease in CAT and SOD activities (Nistico et al., 1992).

Concomitantly, the LDH level was increased, however not significantly. This result suggests that the upregulation of LDH may enhance the brain's energy demand and facilitate glucose uptake. Consequently, the lactate metabolism within the brain after hypoxic stress remained unchanged. Furthermore, the changes in LDH activity could play a crucial role in adapting to varying energy needs during periods of oxidative stress, ultimately promoting cellular resilience and optimizing metabolic processes in the brain.

DISCUSSION

Cerebral lactate metabolism and its compartmentalization in neurons is not fully understood. It has been suggested that lactate constitutes an alternative source of energy that the brain uses under strenuous situations, thereby allowing the brain to optimize the use of energy resources and metabolism (Ross et al., 2010). When oxygen becomes scarce glycolysis becomes the main generator of ATP, which accompanies the regeneration of NAD+ from NADH. The final enzyme of this pathway, LDH, catalyzes the mutual conversion of lactate to pyruvate (Koudelova ́et al., 2006). LDH is present in all tissues and is therefore superior as a marker of cell damage (Karlsson et al., 2010). The enzyme is involved in the anaerobic metabolism of glucose when oxygen is absent or in limited supply. When cells become exposed to anaerobic or hypoxic conditions, the production of ATP by oxidative phosphorylation becomes disrupted. This process demands cells to produce energy by alternate metabolism (Farhana and Lappin, 2023).

Lactate influences NMDA receptor signaling-related plasticity via the phosphorylated extracellular signal-regulated kinase (Erk1/2) pathway. An *in vitro* study indicates that lactate inhibits Ca2+ entry in glutaminergic and GABAergic neurons of the cerebral cortex (Datta and Chakrabarti, 2018). The authors also suggested that the increase in LDH activity could be mediated by hypoxia-inducible transcription factor-1 (HIF-1). Thus, the LDH-A gene belongs to genes controlled by the HIF-1 (Koudelova ́et al., 2006). It is suggested that under hypoxic conditions, HIF1α is stabilized and forms the HIF1 transcription factor with the constitutively expressed subunit HIF1β. It promotes the transcription of target genes involved in metabolism including LDHA, angiogenesis, and apoptosis, which support cell survival in hypoxic environments (Valvona et al., 2015). LDH definitely plays a key role in this process and is considered a marker of cell damage, hence the brain metabolism responds dynamically to changes in lactate concentrations (Izuo et al., 2015).

In a recent study, LDH activity showed no significant changes following hypoxic exposure. As a result, the glucose metabolism and ATP production remained largely unaffected, ensuring that the brain continued to receive the necessary energy supply. This finding highlights the brain's remarkable ability to maintain metabolic homeostasis even under low-oxygen conditions. Moreover, the stability of LDH activity suggests that alternative metabolic pathways may be employed to support energy production during hypoxia. However, the antioxidant system enzymes seem to fail to protect the cells from the deleterious effects of hypoxia accompanied by decreased activity.

According to the literature, brain CAT activity is extremely low compared with other tissues, and during oxidative stress, certain antioxidant enzyme activities such as CAT may decrease (Nazıroğlu, 2012). We showed that after HH, the CAT and SOD activities were diminished which was due to a disrupted redox state in the brain. This is probably due to the fact that the brain antioxidant defense became weakened after acute HH causing remarkable histomorphological changes in the brain and therefore failing to entirely neutralize the harmful effects of ROS.

The up-regulation of TNF-α in MCs and other resident cells, such as macrophages, is a notable phenomenon in the brain. The activation of MCs leads to the generation of NF-κB and AP-1 and the production of many cytokines, including TNF-α, and various chemokines. The effects of TNF-α include the stimulation of histamine from MCs, which contributes to immediate inflammatory reactions. Furthermore, TNF-α may sensitize CNS functioning through the stimulation of either neurons or MCs (Ataei et al., 2018).

MCs play a significant role in the CNS, contributing a large percentage of histamine to the central pool and regulating the BBB permeability (Theoharides et al., 2019; Gu et al., 2016). Their ability to instantly degranulate and release soluble mediators from intracellular stores is crucial for optimal immune response (Hirschberg, 2013). Studies have shown that TNF-α, which is detectable as early as one hour after the onset of hypoxia, induces neuronal injury. In contrast to its negative effects, TNF-α can potentially exert beneficial or detrimental effects, depending on the pathologic context in the brain (Suzuki et al., 2009).

The major pathogenetic mechanisms of HACE include not only neuroinflammation but also oxidative stress as a promoter of brain injury. Several studies have found elevated levels of inflammation markers that activate immune cells, amplifying both inflammation and ROS generation. Hence, the neuroinflammatory response fuels oxidative stress, perpetuating inflammation. A notable consequence of neuroinflammation is the effect on the BBB, enabling the infiltration of leukocytes, which can then participate in perpetuating the inflammatory response (Kim et al., 2012).

The current study findings suggest that the increased level of MCs, along with TNF-α expression and changes in antioxidant defense system activity may be associated with BBB permeability disruption and the formation of deteriorative changes in the brain.

Summarily, these arguments further support the possibility of 'secondary' damage to neurons during HH-induced cell damage and accentuate interventional strategies aimed at preserving brain redox homeostasis in these settings (Kumar et al., 2016). Therefore, the molecular mechanisms that are responsible for adaptations against oxidative stress are still lacking and need in-depth investigation. However, this study is prone to increase our understanding of the brain immune response and its interactions with the redox landscape through its oxygen-dependent metabolic pathway in conditions caused by acute HH. By addressing these described changes, we are prone to suggest a potential use of preventive interventions for HACE utilizing the combined antioxidant and anti-inflammatory agents in order to restore the redox imbalance and mitigate the inflammation of the brain. Specifically, given the role of MCs and TNF-α in promoting neuroinflammation, targeting these mediators may help mitigate brain injury during HH exposure. The modulators of active lactate metabolism and inhibitors for pro-inflammatory pathways such as NF-κB may also be used as potential therapeutic interventions to reduce neuroinflammation and maintain the BBB integrity.

Study limitations. The timing of measurements related to antioxidant system enzyme activity and cytokine expression post-hypoxia may not capture the full temporal dynamics of these responses. Additionally, the study's limited sample size may not adequately represent the whole picture of the results. While this investigation concentrated on specific markers such as LDH, TNF-α, and MCs, it may overlook other relevant mediators and pathways involved in the hypoxic response. Hence, a more comprehensive approach could provide clearer insights into the multifaceted nature of brain injury in this context. Also, the study will benefit from larger sample sizes that could enhance the generalizability of the findings. Also, the study focuses on hypoxic exposure with a limited timeframe, however, the long-term effects of HACE should be taken into account to better understand the temporal dynamics of oxidative stress, inflammatory responses, and neuronal damage.

ACKNOWLEDGEMENTS

The work was supported by the Science Committee of MESCS RA, in the frames of the research projects № 21AA-1F041.

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