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THE EVALUATION OF MAST CELLS, TNF-α EXPRESSION AND TUNEL-POSITIVE NEURONS IN THE BRAIN DURING INFLAMMATORY RESPONSE INDUCED BY ACUTE HYPOBARIC HYPOXIA

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Elevated altitudes accompanied by low barometric pressure and acute oxygen deficiency, pose a significant risk for the development of High-Altitude Cerebral Edema in the brain. The resultant HACE is a potentially fatal neurological disorder that triggers a neuroinflammatory response and leads to significant changes in the redox homeostasis of the brain, ultimately resulting in neurodegeneration. In the current study, the level of apoptotic cells and mast cells, as well as the expression of TNF-α as an essential modulator of neuroinflammation was investigated following acute hypobaric hypoxic exposure. The animals were exposed to acute hypobaric hypoxia for approximately 24 *h* at an altitude of 7620 *m*. The study findings indicate an increased level of mast cells in the brain parenchyma ($p < 0.0001$), accompanied by elevated TNF-α expression and TUNEL-positive cells ($p < 0.0001$), suggestive of neuronal degeneration following acute hypobaric hypoxia. The current study reveals the critical role of mast cells in edema formation during the neuroinflammatory response of the brain under hypoxic conditions. Additionally, it underscores the involvement of pro-inflammatory cytokines, such as TNF-α, in the process of blood brain barrier disruption, and the subsequent development of cerebral edema. This study provides a more in-depth understanding of the pathogenesis of HACE, where the studied biomolecules contribute as essential neuroinflammatory modulators.

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***Keywords*:** HACE, apoptosis, mast cells, neuroinflammation, cerebral edema, TUNEL.

Introduction. Numerous central nervous system (CNS) disorders are postulated to encompass a neuroinflammatory component, coupled with oxidative stress consequential to hypoxia-ischemia [1]. Hypoxia-induced brain injury exhibits morphological changes, mitochondrial dysfunction, and upregulation of proteins and genes associated with apoptosis [2].

High altitude cerebral edema (HACE) is an infrequent and enigmatic ailment caused by exposure to the rarefied air of high altitude. The primary pathogenic mechanisms of this malady encompass not only oxidative stress, but also neuroinflammation as a promoter of brain injury [3]. Although the fundamental

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cause of HACE is low blood oxygen saturation caused by high altitude hypobaric hypoxia (HH), the pathophysiological mechanism of HACE is exceedingly complex and not comprehensively understood [4]. HACE usually denotes cerebral anomalies of high-altitude (above 3000–5000 *m*) sickness [5] that is mediated by oxidative stress damage caused by an imbalance of redox homeostasis, and inflammation mediating the onset of HACE [6]. Furthermore, heightened pro-inflammatory cytokines may exacerbate neuron injury [7].

Mast cells (MCs) that are innate immune cells can secrete factors that serve as inflammatory mediators triggering neuroinflammation, and immune cell infiltration into the CNS. The number of MCs rises after hypoxic-ischemic events, increasing blood–brain barrier (BBB) permeability to facilitate an inflammatory state in the brain. These findings suggest that MCs accumulate in the CNS as one of the initial responders to neuroinflammatory disorders [8]. In this study, the potential role of mast cells and TNF-α as neuroinflammatory mediators during HACE formation is considered along with the apoptosis that usually co-exists with the inflammatory response of the brain following hypoxic exposure.

Materials and Methods.

***Experimental Design*.** All experiments with animals (24 albino male rats, weighing 200–250 *g*) have been carried out by the rules of the National Center for Bioethics of Armenia. In order to mimic the cerebral edema, the HH model was used. The control group animals ($n = 10$) were kept in normobaric conditions, while the rats from the experimental group ($n = 14$) underwent HH in the decompression chamber. The experimental animals were kept under acute hypoxic conditions for 24 *h*. The desired altitude to induce edema was selected according to the literature (7620 *m* altitude, equal to 25 000 feet, 8.1% O_2, 300 *m*/*min* velocity) with some modifications [9–11]. The animals were kept in an air-conditioned room with a controlled temperature of 22±1°C, under relative humidity in the controlled 12-hour light-dark cycled cages with free access to water and food. After the hypoxic exposure, animals from the experimental and the control group underwent histological examination.

***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). First, tissue sections were deparaffinized with xylene, rehydrated through descending concentrations of ethanol, rinsed for 10 *min* in 0.1 *M* PBS, and then treated with 20 $\mu g/mL$ proteinase K for 20 *min* at room temperature. The specimens were treated with 3% H_2O_2 in methanol for 10 *min* 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 15 *min* 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 [1]. 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 *min* 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 ×400.

Mast Cell Staining. To determine the distribution and amount of MCs, Toluidine blue and May-Grünwald Giemsa staining methods were applied as described in the literature [12–14]. The total amount of MCs including granulated (metachromatically intact cells) and degranulated cells (n = 10/group), and counted in 10 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 *min* each. To inhibit endogenous peroxidase activity, the sections were treated with a 3% H_2O_2 solution in methanol at room temperature for 10 *min*, followed by two washes with TBS containing 10% BSA for 1 *h*. 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 *min* 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.

Statistical Analysis. The data were presented as mean ± SD for all methods used. Student *t*-test, using GraphPad software, was used to compare the studied groups. A significance level of $p < 0.05$ was considered.

Results and Discussion. Following hypobaric hypoxic exposure, the number of apoptotic cells was increased in CA3 (Figs. 1, A–C) and CA2 (Figs. 1, D–F) regions of the hippocampus, and in the cerebral cortex (Figs. 1, G–I). The results indicated a significant increase 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 ($p < 0.0001$) in the HACE group than in the control group, indicating the occurrence of apoptosis in response to oxidative stress caused by acute oxygen deficiency. The findings suggest that exposure to HH has detrimental effects on the brain, particularly in terms of inducing apoptosis and cellular damage.

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 high influx of calcium-mediated apoptosis cascade [5].

In the brain, MCs are typically perivascular, have substantial granule content, and are frequently observed siding cortical penetrating arterioles. After ischemia, 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 cells within 1 *h* after a

generalized hypoxic-ischemic (HI) challenge has been shown in the neonate rat brain, although such a rapid MCs accumulation has not been documented in other ischemia models [8].

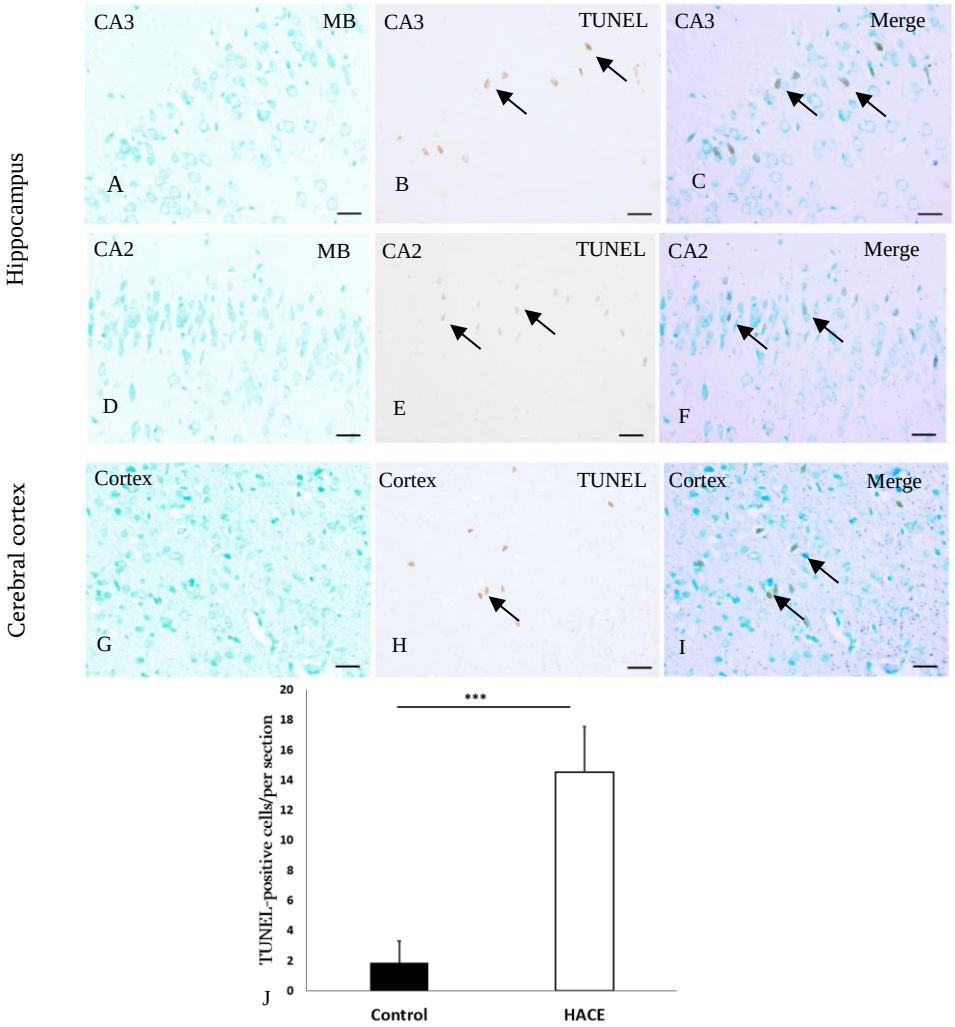

Fig. 1. Histological photomicrographs of the CA2, and CA3 subfields of the hippocampus and cerebral cortex of negative control (A, D, G), positive control (B, E, H), and TUNEL-positive (C, F, I) of the HACE group (arrows), MB and TUNEL staining, magnification ×400, scale bar = 50 μm. Changes of TUNEL-positive cells in control and HACE groups (J). Data presented as mean ± SD, *** $p < 0.0001$ compared with control according to Student *t*-test.

*The average number of MCs in the brain parenchyma in control and HACE groups (*** $p < 0.0001$ significance control vs HACE group based on Student t-test)*

Number of mast cells	Control mean ± SD	HACE mean ± SD
	4.2 ± 1.74	40.5 ± 5.92***

In the current study, a significant increase in MCs after HH was detected. The average number of MCs is presented in Table.

MCs are found adjacent to blood vessels and nerve endings; in the brain, they are located in the thalamus, hypothalamus, and median eminence. MC secretory granules store many preformed proinflammatory and neuro-sensitizing mediators including bradykinin, histamine, TNF-α, and tryptase [15]. The observed MCs were mainly distributed in the hippocampus and cerebral cortex (Fig. 2, A–C).

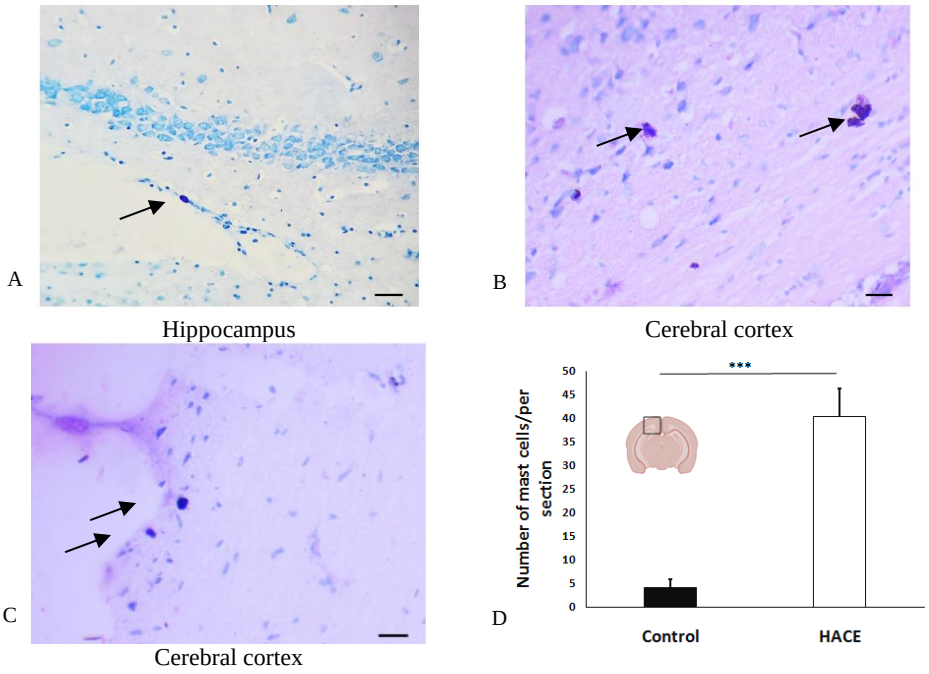

Fig. 2. Histological photomicrographs showing MCs (arrows) in the hippocampus (A) (Toluidine blue staining), and cerebral cortex (B, C, arrows) of the HACE group (Giemsa staining, magnification ×400, scale bar = 50 μm). Changes of MCs number in control and HACE groups (D). Data presented as mean ± SD, *** $p < 0.0001$ compared with control according to Student *t*-test.

In addition to the changes in MCs 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 (Figs. 3, A–D).

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 [16].

Mast cells play a significant role in the CNS, contributing a large percentage of histamine to the central pool and regulating the blood-brain barrier permeability [15, 17]. Their ability to instantly degranulate and release soluble mediators from intracellular stores is crucial for optimal immune response [18].

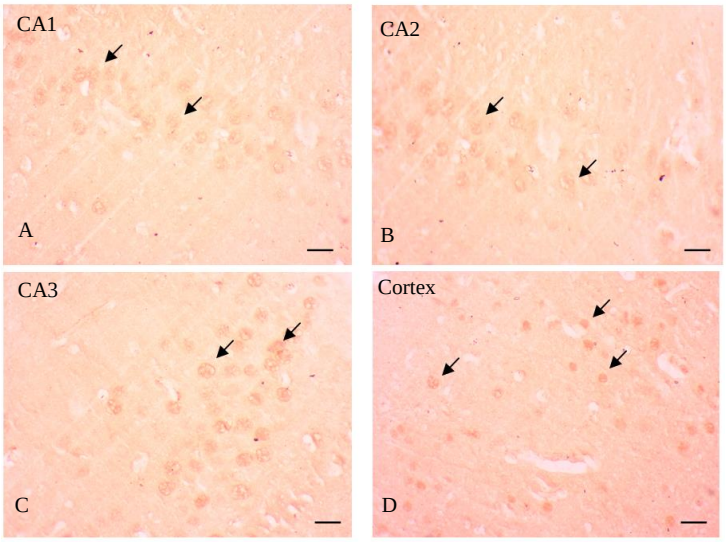

Fig. 3. Histological microphotographs of the TNF-α positive cells (arrows) in the hippocampus (A, B, C) and cerebral cortex of (D) of the HACE group **(**magnification ×400, scale bar = 50 μm).

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 [19].

The major pathogenetic mechanisms of HACE include neuroinflammation and oxidative stress as promoters of brain injury. Several studies have found elevated levels of inflammation markers that activate immune cells, amplifying both inflamemation and reactive oxygen specie 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 [20].

Conclusion. The current study findings suggest that the increased level of MCs, along with TNF-α expression, may be associated with BBB permeability disruption and the formation of deteriorative changes in the brain. Evaluating the role of these inflammatory mediators in the pathological mechanisms underlying cerebral edema induced by hypoxic exposure is a significant challenge due to the complexity of the underlying processes of inflammation. However, this study could shed light on the particular biomolecule and cells, mainly the MCs and TNF-α that play a considerable role in HACE pathogenesis and contribute to cell apoptosis and disruption of BBB integrity.

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Ռ. Ա. ՇՈՒՇԱՆՅԱՆ

ՊԱՐԱՐՏ ԲՋԻՋՆԵՐԻ, ՈՒՆԳ-α ԷՔՍՊՐԵՍԻԱՅԻ ԵՎ TUNEL-ԴՐԱԿԱՆ ՆԵՅՐՈՆՆԵՐԻ ԳՆԱՀԱՏՈՒՄԸ ՍՈՒՐ ՀԻՊՈԲԱՐԻԿ ՀԻՊՕՔՍԻԱՅԻ ՀԵՏԵՎԱՆՔՈՎ ԱՌԱՋԱՑԱԾ ԲՈՐԲՈՔԱՅԻՆ ՊԱՏԱՍԽԱՆԻ ԺԱՄԱՆԱԿ

Լեռնային մեծ բարձրությունները, որոնք բնութագրվում են ցածր բարոմետրիկ ճնշմամբ և թթվածնի սուր անբավարարությամբ, մեծ վտանգ են ներկայացնում բարձրլեռնային ցերեբրալ այտուցի (ԲՑԷ) զարգացման համար: ԲՑԷ-ն պոտենցիալ մահացու նյարդաբանական խանգարում է, որը հրահրում է նեյրոբորբոքային ռեակցիա և հանգեցնում գլխուղեղում ռեդոքս հոմեոստազի զգալի փոփոխությունների, ինչն ի վերջո, հանգեցնում է նեյրոնների դեգեներացիայի: Սույն հետազոտության շրջանակներում ուսումնասիրվել է ապոպտոտիկ և պարարտ բջիջների քանակության փոփոխությունները, ինչպես նաև TNF-α-ի էքսպրեսիան որպես նեյրոբորբոքման կարևոր մոդուլյատոր՝ սուր հիպոբարիկ հիպօքսիայի ազդեցությունից հետո: Կենդանիները ենթարկվել են սուր հիպոբարիկ հիպօքսիայի մոտ 24 *ժ* տևողությամբ 7620 *մ* բարձրության վրա: Հետազոտության արդյունքները ցույց են տվել, որ գլխուղեղի պարենխիմայում պարարտ բջիջների քանակը մեծացել է ($p<0.0001$), որն ուղեկցվել է TNF-α-ի և TUNEL-դրական բջիջների ավելացմամբ ($p<0.0001$), ինչը վկայում է նեյրոդեգեներացիայի մասին: Սույն ուսումնասիրությունը ցույց է տալիս պարարտ բջիջների կարևոր դերը հիպօքսիկ պայմաններում դրսևորված նեյրոբորբոքային ռեակցիայի ժամանակ, որն ընկած է ցերեբրալ այտուցի զարգացման հիմքում: Ավելին, հետազոտությամբ ցույց է տրվում պրոբորբոքային ցիտոկինների, մասնավորապես TNF-α-ի ներգրավվածությունը արյունաուղեղային պատնեշի խանգարման և ցերեբրալ այտուցի հետագա զարգացման գործընթացում: Այս ուսումնասիրությունը թույլ է տալիս է առավել համապարփակ պատկերացում կազմել բարձրլեռնային ցերեբրալ այտուցի պաթոգենեզի մասին, որում ուսումնասիրված կենսամոլեկուլները կարևոր դեր են խաղում որպես նեյրոբորբոքային մոդուլյատորներ:

Р. А. ШУШАНЯН

ОЦЕНКА ТУЧНЫХ КЛЕТОК, ЭКСПРЕССИИ TNF-α И TUNEL-ПОЗИТИВНЫХ НЕЙРОНОВ ГОЛОВНОГО МОЗГА ВО ВРЕМЯ ВОСПАЛИТЕЛЬНОЙ РЕАКЦИИ, ВЫЗВАННОЙ ОСТРОЙ ГИПОБАРИЧЕСКОЙ ГИПОКСИЕЙ

Повышенные высоты, сопровождающиеся низким барометрическим давлением и острым дефицитом кислорода, представляют значительный риск развития высотного отека головного мозга (ВОГМ). Возникающий ВОГМ представляет собой потенциально смертельное неврологическое расстройство, которое запускает нейровоспалительную реакцию и приводит к значительным изменениям в окислительно-восстановительном гомеостазе головного мозга, что в конечном итоге приводит к нейродегенерации. В настоящей работе исследован уровень апоптотических и тучных клеток, а также экспрессия TNF-α как важного модулятора нейровоспаления после острого гипобарического гипоксического воздействия. Животные подвергались острой гипобарической гипоксии (ГГ) в течение примерно 24 *ч* на высоте 7620 *м*. Результаты исследования указывают на повышенный уровень тучных клеток в паренхиме головного мозга ($p < 0,0001$), сопровождающийся повышенной экспрессией TNF-α и TUNEL-позитивными клетками ($p < 0,0001$), что указывает на дегенерацию нейронов после острой ГГ. Настоящее исследование раскрывает решающую роль тучных клеток в формировании отека при нейровоспалительной реакции головного мозга в условиях ГГ. Кроме того, это подчеркивает участие провоспалительных цитокинов, таких как TNF-α, в процессе разрушения гематоэнцефалического барьера и последующего развития отека мозга. Данное исследование дает более полное представление о патогенезе ВОГМ, в котором изученные биомолекулы играют важную роль в качестве нейровоспалительных модуляторов.