Calcium-binding protein, S100b, in the blood as a biochemical marker of the neurological state of men in warzones

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Acute and chronic stress are two of the most challenging issues faced by men situated in warzones. According to the main concept of G. Selye, stress response is the most important link during the adaptation of an organism to environmental factors. However, excessively intense or prolonged exposure to stress creates the threat of sustained homeostasis disorder. At present, the primary indicators in clinical studies are the general biochemical parameters of blood – alanine aminotransferase, aspartate aminotransferase, bilirubin, creatine, urea, total protein, and glucose levels. But these indicators are not classical markers for determining the state of the nervous system itself and cannot reflect a patient's mental state. In this regard, we tested for a different biomarker; we made an attempt to determine the neurospecific S100b protein in the blood serum by analyzing the correlation between classical and specific methods for diagnosing the state of the nervous system. For this study, blood was collected from 20 patients aged 25 to 45 years old. Participants were divided into two groups: one group of provisionally healthy men and another group of patients who were admitted to the Department of Neurology of the I. Mechnikov Regional Clinical Hospital after military operations (from the territory of the ATU). The standard test kits of PrAT Reagent Company (Dnipro) were used to assess biochemical indicators in accordance with the given instruction in the supplier's protocol. The activity of each of alanine aminotransferase, aspartate aminotransferase, direct and total bilirubin content, creatine, urea, total protein levels, and astrocyte-specific protein S100b was examined. The level of alanine aminotransferase in the men from the warzone was 23.9 ± 3.9 μmol/l, compared with the healthy men – 31.2 ± 0.7 μmol/l. The level of aspartate aminotransferase in the group of men at the neurological department was similar to that in the healthy group. The content of direct bilirubin in the patients at the neurological department increased by 28.0%, compared with the healthy men. The concentration of creatinine, total protein, urea, and glucose levels did not significantly change, compared with healthy volunteers and were in the range of the standard reference data. The content of astrocyte-specific protein, S100b, in the blood of the patients was 0.011 μg/ml, 36.0% more than in the conditionally healthy men – 0.007 μg/ml. Prolonged stress could, therefore, induce profound changes in blood brain barrier and registration of neurospecific protein in the blood of men in warzones. These changes could become chronic if or when these men develop other social and economic problems that can be monitored with the specific marker for S100b.

Keywords: stress; aminotransferases; bilirubin; creatinine; urea; total protein; glucose

Introduction

The first-ever metabolic vital signs are classic values of biochemical blood tests, among which are parameters of total protein, glucose, activity of aminotransaminases, and end products of metabolism, namely, bilirubin, creatinine, and urea. The activity of two of the main liver enzymes, alanine aminotransferase (ALT, CF 2.6.1.2) and aspartate aminotransferase (AST, KF 2.6.1.1) are markers of the functional state of this organ (Zhang et al., 2015; Ferrannini et al., 2017), which is key to ensuring the metabolism of blood proteins. Substances that provide gluconeogenesis are formed as a result of the metabolism of aspartate aminotransferase. Chronic stress can lead to the release of excess concentrations of cortisol in the blood, which suppresses the enzymes of gluconeogenesis, enhancing the effect of adrenaline and glucagon on the liver and stimulating proteolysis in the muscles. Aminotransferases catalyze the transamination reactions between amino and α-ketoacids, thus taking part in the synthesis and degradation of cellular proteins. The activity of aminotransferases is insignificant in the blood of healthy people. Under standard conditions, the highest activity of aspartate aminotransferase occurs in the liver, nervous tissue, skeletal muscle, and myocardium. Alanine aminotransferase is also present in many organs, with the highest activity determined in the liver, pancreas, skeletal muscle, myo-

cardium, and kidneys. An increase in the activity of these aminotransferases is observed during pathological processes in which the corresponding organs are involved (Sookoian et al., 2016).

The splitting of proteins containing heme (hemoglobin, myoglobin, and cytochrome) ensures the flow of all aerobic processes in organs and tissues, which leads to the production of an intermediate metabolite – bilirubin. In small physiological doses, bilirubin acts as a natural antioxidant that inhibits the development of oxidative stress in various pathologies (Oda & Aizawa, 2013; Haeusler, 2016; Nano et al., 2016). However, if the physiological concentration is exceeded, it becomes toxic, sparking the development of jaundice and disorders of the liver and other organs. Creatinine, formed from creatine phosphate, which is involved in the energy supply of muscle and other tissues of the human body, is also one of the terminal metabolites of amino acid-protein metabolism. Creatine phosphate is a macroergic compound that is used for rapid ATP resynthesis during muscle activity. In the cells of the nerve tissue, creatine phosphate maintains the vitality of the cells that lack oxygen. The concentration of creatinine in the blood is the primary indicator of impaired muscle tissue, brain, and filtration capacity of the kidneys (Oda & Aizawa, 2013).

Urea is the ultimate metabolic protein and is utilized in combination with urine. This is one of the main components of the residual nitrogen of blood, accounting for about 50.0%.

The basic biochemical parameters of blood that characterize the general metabolism have a potential clinical benefit in diagnostics, prognosis, and therapeutic decision-making. They can be used to find out the pathophysiology of secondary trauma (Wang et al., 2018). However, these indicators are not classical markers for determining the state of the nervous system and cannot reflect the mental state of a patient. If significant damage is inflicted upon organs and tissues, a probable change in the features of the basic biochemical parameters of blood is observed. On the other hand, it is very difficult to determine any change in feature with minor injuries or at the initial stage of the development of the neurological state. Therefore, monitoring the state of the nervous system in today's diagnostics requires a specific biochemical marker that could indicate any form of damage to the central nervous system, irrespective of the severity of the damage.

Currently, the calcium-binding protein, S100b is used as a marker for injuries in craniocerebral traumas and infections (Di Battista, 2018). It was showed that, under conditions of experimental chronic hepatitis, the excessive elevation of the level of S-100b occurs in all parts of the brain of a rat. This phenomenon is accompanied by a deterioration in locomotor and orientational search activity of animals, increasing their susceptibility to stress (Ushakova et al., 2011). Also, S100b is a sensitive biomarker for assessing the integrity of the blood-brain barrier (BBB) (Li et al., 2014; Golmohammadi et al., 2018).

S100b is a representative of the S100 Ca^{2+}-containing protein superfamily and is an acidic protein with a molecular weight of 21 kDa. It exists in the form of a homodimer consisting of two β subunits. It can be secreted by astrocytes, glial cells, and the Schwann cells. Astrocytes and glial cells produce S100b in a complex system that involves changes in the intracellular Ca^{2+}-concentration. S100b can accumulate in the intercellular space and enter the bloodstream and cerebrospinal fluid as a result of its secretion or release from damaged cells. This protein is involved in the Ca^{2+}-dependent regulation of various intracellular processes, such as phosphorylation of proteins, enzyme activity, cell proliferation and differentiation, cytoskeleton component dynamics, structural organization of membranes, intracellular Ca^{2+}-homeostasis, inflammation, and protection against oxidative cell damage. Intracellular S100b regulates the dynamics of the cytoskeleton through the disorganization of the tubulin of the filaments, the protein of the type III intermediate filament, and the binding of fibrillary proteins, such as the capping protein of actin (CapZ) or inhibition of the phosphorylation of glial fibrillary acidic protein (GFAP) by stimulation with cyclic adenosine monophosphate (tsAMP) or calcium/calmodulin. S100b interacts with the cytoplasmic domain of myelin-associated glycoproteins and inhibits its phosphorylation with protein kinase. In the cell, the cadmium-binding protein interacts with a relatively large number of signal proteins, thus regulating their functions.

S100b is considered the most specific for neural tissue and reacts unidirectionally in the form of an increase in its level in response to mechanical, hypoxic, ischemic, biochemical, and other neurotoxic factors (Koh & Lee, 2014). The role of S100b protein as a marker of brain tissue damage was demonstrated initially in the study of liquor disease. High concentrations of S100b were later detected in brain injuries, brain tumors, neurodegenerative diseases, stroke, meningitis, encephalitis, perinatal asphyxia (the level of S100b at birth and in the first 2 to 3 days of life is one of the criteria for the prediction of adaptation in preterm and assessing the severity of damage to the central nervous system), and others. In addition, an elevated serum S100b level was found during cardiac arrest, after cardiac reanimation, in cardiac surgery with cardiopulmonary bypass. Thus, this astrocyte-specific protein, S100b, is currently considered one of the nodal molecular components of complex intracellular systems that provide functional homeostasis of the brain cells by combining and integrating various calcium-dependent metabolic processes (one of the processes is the activation of creatine kinase) (Woodbury-Fariña, 2014). The contents of S100b could, hence, act as an indicator of the correlation between the classical and specific methods for diagnosing the state of the nervous system.

The problem of stress is still of paramount importance today (Gianaros & Wager, 2015; Fox et al., 2018). Stress reaction is the most important link during the adaptation of an organism to environmental

factors (Buckner et al., 2017). But recently, the strength and number of stressful situations that can affect the condition of tissues and organs have increased (Ginty et al., 2017; Kraynak et al., 2018). A spasm of the vessels, stomach, and pancreas is observed in the case of excessively intense or inadequately long-term exposure to stress factors (Kornicka et al., 2018; Tahtacı et al., 2018). Due to the high frequency of stress and an increase in its duration, these disorders increase their effect and stability, resulting in hyperglycemia (Perez de la Hoz et al., 2018). In turn, an increase in insulin concentration is observed in chronic stress. Hyperinsulinemia, as a compensatory response to insulin resistance, increases the activity of the sympathetic nervous system, which further enhances stress-response, and has a negative impact on the course and prognosis of cardiovascular diseases (Oppenheimer & Cechetto, 2016; Brindle et al., 2017; Carroll et al., 2017).

The close relationship between organs and systems of the organism leads to violations, which, having fallen into a vicious circle, attract even more violations to it. The daily stress-induced changes to glucose levels and hyperglycemia causes excessive glycation and inactivation of antioxidants. The depletion of the antioxidant enzyme systems contributes to the development and progression of the complications of type 2 diabetes (Siddiqui et al., 2015; Gonzalez et al., 2018; Yaribeygi et al., 2018). A large number of metabolic mechanisms are activated against the background of hyperglycemia, the result of which is the formation of the active forms of oxygen and oxidative stress.

All that said, the purpose of this work is to analyze the ratio of the indices of the general biochemical analysis of blood total metabolites (total protein and glucose), key enzymes of protein metabolism (alanine aminotransferase and aspartate aminotransferase), end metabolites (direct and total bilirubin, creatinine, and urea), and astrocyte-specific protein, S100b, in the blood of men stationed for a long time in the war-torn territory of the ATU and admitted to the Department of Neurology of the I. Mechnikov Regional Clinical Hospital after military operations.

Material and methods

We selected 20 men aged 25 to 45 years old for our study. Blood was drawn from the men on an empty stomach in the morning. The men were divided into two groups: a group of conditionally healthy men and a group of men from the warzone admitted to the Department of Neurology of the I. Mechnikov Regional Clinical Hospital after military operations (from the war-torn territory of the ATO).

The study of the general metabolism was conducted at the base of clinical trials at the I. I. Mechnikov Dnipropetrovsk Regional Clinical Hospital (The authors express their sincere gratitude to the manager of the clinical and diagnostic laboratory E. V. Philippova for her cooperation). The general biochemical analysis of blood was performed with standard spectrophotometric methods using the test kits of PrAT Reagent (Dnipro), according to the manufacturer's instructions for use of the kits and the protocol for the measurement of all indicators.

The total protein level was determined using a biuret reaction (Code: BP 00.5.03), in which the proteins reacted in an alkaline medium with copper tartrate (II) to form compounds of purple colouration (biuret reaction). The concentration of glucose was determined using the glucose oxidase method (PK 24.10.06), in which glucose, in the presence of glucose oxidase enzyme, is oxidized to gluconic acid and hydrogen peroxide. The latter, under the influence of peroxidase, reacts with phenol and aminophenazone to form a coloured quinonine, which is determined photometrically.

The activity of each of aspartate aminotransferase and alanine aminotransferase enzymes was determined using the Rietman-Frenkel method (AF 002-01 and AF 001-01). The principle of the method is that aspartate transferase catalyzes the reaction between L-aspartate and 2-oxoglutarate to produce L-glutamate and oxaloacetate. This definition is based on the measurement of the optical density of the hydrazones of 2-oxoglutaric and pyruvic acids in alkaline media. The hydrazone of pyruvic acid, which occurs during the spontaneous decarboxylation of oxaloacetate, has a higher optical density. Alanine aminotransferase catalyzes the reaction between L-alanine and 2-oxoglutarate, resulting in their conversion to L-glutamate and pyruvic acid salt.

The content of direct and total bilirubin was determined by the Jedrashik-Klechhorn-Grof method (BP 002-03), which is based on the fact that diazophenylsulfonic acid is formed during the interaction between sulfanilic acid and sodium nitrate, producing a pinkish violet colouration of the blood serum and direct bilirubin. The intensity of the colour is determined by the amount of direct bilirubin. When added to the blood serum of a caffeine reagent, indirect bilirubin is converted into a direct bilirubin and is quantitatively determined by a diazo-reactive. The concentration of total bilirubin in serum is determined by the intensity of the colouration.

The concentration of creatinine is determined by means of the method of deproteinization of Jaffe-Popper (BH 018-04), which is based on the effects of trichloroacetic acid which precipitates proteins and pseudo creatinine chromogens. Creatinine picrate is formed as a result of the interaction of picric acid with creatinine in alkaline medium. The intensity of the colour is proportional to the concentration of creatinine.

To determine the level of urea, an enzymatic (urease) method (BH 023.1-04) was used, in which urea is hydrolyzed under the action of urease to form ammonia and carbon dioxide. Nitroprusid in an alkaline medium catalyzes the reaction of ammonia with salicylate and sodium hypochlorite. The size of the absorption of the analyzed samples determines the content of urea.

The content of S100b protein in serum was determined by direct solid-phase enzyme-linked immunosorbent assay using primary monospecific polyclonal antibodies against S100b, secondary anti-rabbit antibodies labeled with horseradish peroxidase, and purified protein S100b as a standard. All reagents were purchased from Sigma (USA). Measurements of the results were taken at a wavelength of 492 nm with an Anthos 2010 ELISA reader (Finland).

The statistical processing of the results was performed using the StatWin program via a ANOVA. The results were considered significant at $P < 0.05$.

Results

According to the general biochemical analysis of the blood, the total protein in the serum of the men from the warzone admitted to the neurology department was 73.4 ± 1.1 g/l, which was not significantly different from that of the group of conditionally healthy men (Fig. 1). The reference data for the total protein level is 66.0–87.0 g/l.
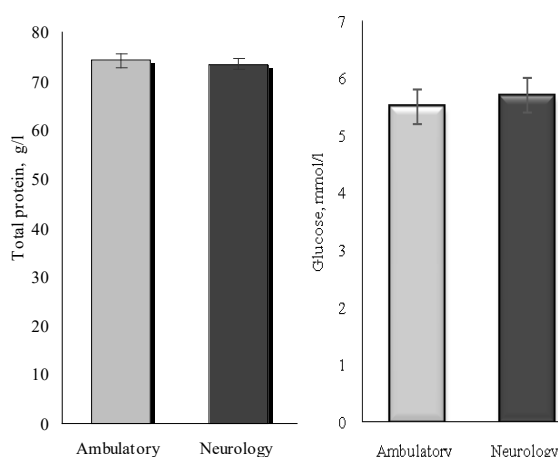

Fig. 1. The level of total protein and glucose in the blood serum of conditionally healthy men and patients at the neurology department: $x \pm SE$, n = 10

The level of glucose of the military men from the war-torn territory of the ATU was within the range of the reference norm, 5.1–5.7 mmol/l on average (Fig. 1). The activity of aminotransferases, alanine aminotransferase and aspartate aminotransferase, in the men who donated blood for analysis at the ambulatory and neurology departments was examined. In the blood serum of the men at the Department of Neurology, the activity of alanine aminotransferase was 23.9 ± 3.9 µmol/l (Fig. 2), compared with that of the conditionally healthy men (31.2 ±

0.7 µmol/l with a standard reference point of 42.0 µmol/l). This indicator decreased significantly, by 24.0%, compared with the conditionally healthy men. Aspartate aminotransferase activity in the men at the neurological department (24.1 ± 2.7 IU/l) was similar to that of the group of conditionally healthy men (24.5 ± 0.9 IU/l), well within the range of the standard reference (up to 37.0 IU/l).
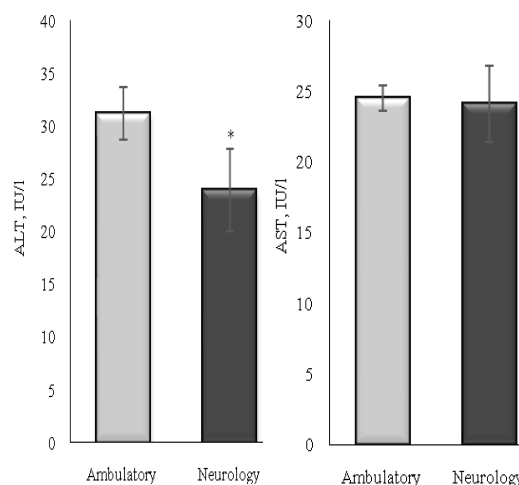

Fig. 2. The activity of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) in the serum of conditionally healthy men and patients of the Department of Neurology: * – P < 0.05 in comparison with the group of conditionally healthy men; m ± SE, n = 10

The content of direct and total bilirubin in the men who donated blood for analysis at the ambulatory department and the Department of Neurology was examined. The indicator of direct bilirubin in the group of men from the warzone was 5.1 ± 0.7 µmol/l (Fig. 3), which falls within the range of reference values (up to 5.4 µmol/l). However, in comparison with the conditionally healthy men, where the index was 4.0 ± 0.3 µmol/l, a significant increase, by 28.0%, in this indicator was observed in the patients.
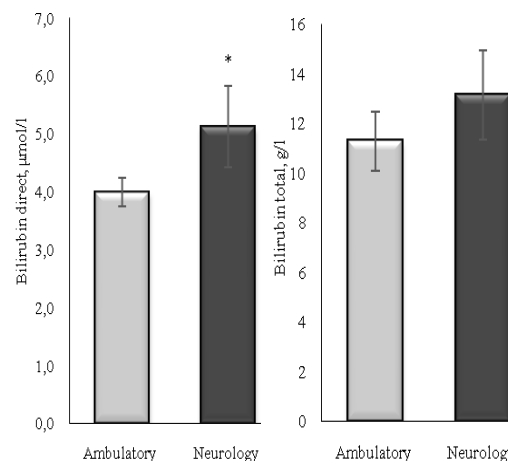

Fig. 3. The content of direct and total bilirubin in the blood of conditionally healthy men and patients at the Department of Neurology: * – P < 0.05 a significant difference, compared with the group of conditionally healthy men; m ± SE, n = 10

The total bilirubin content, which reflects the total content of direct bilirubin in conjunction with glucuronic acid and the indirect, which is formed by hemoglobin splitting, in the blood of patients in the Department of Neurology was 13.1 ± 1.8 g/l. The reference rate of this indicator was up to 21.0 g/l. The concentration of creatinine in the blood of the men in the Department of Neurology was 74.4 ± 5.1 µmol/l, which did not significycantly change compared with that of the conditionally healthy men (Fig. 4). The standard reference of this indicator ranges from 55.0 to 110.0 µmol/l. The level of urea in the men at the neurology department

also remained at the same level as that of the conditionally healthy group, amounting to 5.4 ± 0.5 mmol/l. The acceptable level of urea is 2.5–8.3 mmol/l.

The content of the astrocyte-specific protein, S100b, in the blood of the conditionally healthy men was 0.007 μg/ml. For patients at the neurology department, this indicator was 0.011 μg/ml, which is 36% higher than that of the conditionally healthy men (Fig. 5).
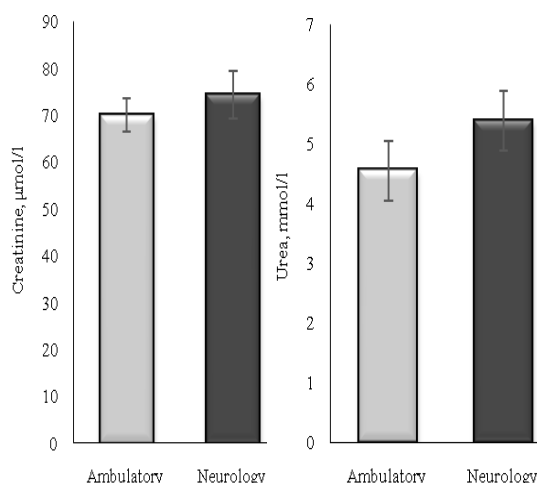

Fig. 4. The concentration of creatinine and urea in the blood of conditionally healthy men and patients at the Department of Neurology: $m \pm SE$, $n = 10$
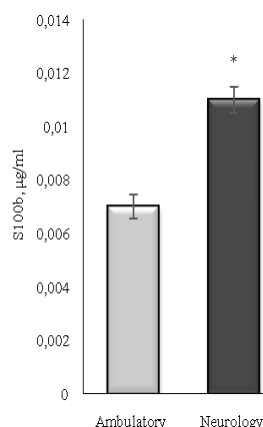

Fig. 5. The content of S100b in the blood of conditionally healthy men and patients at the Department of Neurology: * – P <0.001 a significant difference, compared with the group of conditionally healthy men; $m \pm SE$, $n = 10$

Discussion

The exchange of proteins represents a crucial stage during total metabolism. In the serum of patients at the Department of Neurology, changes in the total protein level were not observed, compared with the conditionally healthy men, and there were no deviations from the reference data (Hayden et al., 2001; Palmfeldt et al., 2016). No development of hyperglycemia was detected in our study, as evidenced by a stable level of glucose in the blood of the patients. However, in some studies, there is an effect of stress on the development of hyperglycemia (Marik, 2013). Particularly, glucose has been shown to increase in patients with brain trauma (Jeon et al., 2012; Plummer et al., 2016; Rau et al., 2017). The key indicators of protein metabolism in stress are the activity of aminotransferases, as they take part in catabolic and anabolic processes involving amino acids. A 2–3 fold change in the activity of alanine aminotransferase and aspartate aminotransferase in comparison with the norm can indicate the presence of pathologies in violation of the structures and functions of the liver cells and mitochondria under the influence

of oxidative stress (Sookoian et al., 2016; Ferrannini et al., 2017). In the blood serum of the men at the Department of Neurology, the activity of alanine aminotransferase was 23.9 ± 3.9 μmol/l, well within the range of the standard reference of 42.0 μmol/l, but significant smaller, by 24.0%, compared with that of the conditionally healthy men. A decrease in the activity of aminotransferases is observed during the insufficient intake of protein-containing food or during a decrease in the rate of metabolism as a result of vascular spasm, hypoxia, etc. The total protein in the patients remaining within the norm indicates a disturbance in the liver, where the synthesis of this enzyme occurs (Hayden et al., 2001; Zhang et al., 2014).

Direct bilirubin is formed by destroying hemoglobin in the liver, where it is directed with blood flow and is disintegrated by binding to glucuronic acid. For normal physiological values, it is excreted from the body, but with insufficient oxygen, violations of various levels are observed. Also, bilirubin is one of the important natural antioxidants that inhibit the development of oxidative stress in various pathologies (Oda & Aizawa, 2013; Haeusler et al., 2016; Nano et al., 2016). But, with increasing concentration, it becomes toxic, which leads to jaundice and damages to the liver and other organs. In the study of the content of direct bilirubin in the men at the Department of Neurology, there was a significant increase, 28.0%, compared with the content in the provisionally healthy men. We can assume that insignificant damages occurred in the liver.

In quantitative terms, proteins form the most important group of macromolecules. Compared with proteins, the proportion of other nitrogen-containing substances in the body is negligible. Therefore, the balance of nitrogen in the body is determined by the metabolism of proteins. The metabolism of nitrogen is generally balanced, that is, the amount of received and allocated nitrogen is approximately equal. Studies of blood urea in the men stationed in the war zone showed no damages compared with the conditionally healthy men. This was confirmed by the indicator of total protein in the serum of the patients, which also did not significantly change compared to that of the group of conditionally healthy men.

Creatinine, as the final product of the disintegration of creatine phosphate, is formed by the spontaneous cyclization of creatine and is the main metabolite of energy metabolism of muscle tissues of the human body. The concentration of creatinine in the blood of patients did not change significantly, remaining within the range of the standard reference, which indicates normal provision of muscles with the necessary energy.

The astrocyte-specific protein, S100b, is a cytosolic protein with a molecular weight of about 21 kDa. It can easily penetrate the BBB, making it a sensitive biomarker in assessing the integrity of the BBB (Golmohammadi et al., 2018). Li et al., in their study, showed that military training, which causes a significant level of stress, depression, and anxiety among soldiers, is accompanied by an increased permeability of the BBB (Li et al., 2014). Koh & Lee also revealed an increase in S100b content after exercise. Scientists suggest that the factors influencing the level of S100b may be muscular insufficiency, training levels, and oxidative stress. It is also assumed that an increase in the S100b level depends on the intensity of the exercises and training (Koh & Lee, 2014). Many studies have shown the dependence of changes in the serum protein S100b level on the level of brain injury (Kleindienst & Ross Bullock, 2006; Mendes Arent et al., 2014; Kabadi et al., 2015; Roh et al., 2017; Wang, 2018). Additionally, preclinical studies have been presented that demonstrate an increase in protein S100b in animals after modeling stress and depressive behavior (Rajkowska & Stockmeier, 2013).

The participation of S100 group proteins in the formation of basic forms of congenital behavior and in the mechanisms of memory and learning has been proven experimentally (Hermann et al., 2012; Li et al., 2013; Baptista et al., 2017; Delgado-Moreno et al., 2017). Zhang et al. (2009) reported a deterioration of cognitive characteristics in patients with depression and increased protein S100b, backed by Li et al. (2014), who demonstrated that the S100b protein acts as a modulator of the inflammatory response of the hippocampus and plays a key role in reducing cognitive properties. In the blood of the warzone patients in our study, the content of astrocyte-specific protein S100b increased significantly by 36.0%, compared with that of the conditionally healthy men, indicating an increase in permeability of the BBB.

Stress during military operations has, not only obvious immediate consequences, but also remote outcomes that cannot be foreseen (Steele et al., 2017). It can develop into a chronic issue, as a person faces other social and economic problems that exacerbate the effects of stress on the body after the cessation of hostilities (Kirkpatrick et al., 2014). Military monitoring in rehab centers or clinics can provide more focused information on the state of the central nervous system of the patient by determining the level of S100b in the blood.

Conclusions

Long-term stay in the war zone did not lead to significant changes in the biochemical parameters of the blood. Indicators of total protein, glucose, and end products of metabolism are within the reference range, indicating a balanced protein metabolism. A significant decrease in alanine aminotransferase activity, an increase in the content of direct bilirubin, and increased hemoglobin splitting suggests minor adaptation in the liver. The increased content of the astrocyte-specific protein, S100b, in the blood indicates the development of psychological stress that can increase the permeability of the BBB, which can be used to monitor the state of the central nervous system of soldiers. A more in-depth study is needed to include a wider range of neuro-specific markers of the BBB disturbance.

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