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PHOTOBIOMODULATION THERAPY PROTECTS RED BLOOD CELLS AGAINST NITRATIVE STRESS DURING STREPTOZOTOCIN-INDUCED DIABETES

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Background. According to the International Diabetes Federation Diabetes Atlas, 10th edition, diabetes is responsible for 6.7 million deaths in 2021. Diabetes mellitus is a group of metabolic disorders characterized by hyperglycemia secondary to either resistance to insulin, insufficient insulin secretion, or both. Oxidative and nitrative stress is a vital part of the complex mechanism by which diabetes and its complications develop. It is known that Photobiomodulation therapy accelerates diabetic wound healing, treats relegated inflammation, and increases oxygen availability for cells. Although some basic molecular mechanisms caused by photobiomodulation therapy in different cell types are already known, they have not been studied in erythrocytes and are different due to the absence of central organelles such as nucleus and mitochondria. The aim of the study was to investigate the effect of photobiomodulation therapy on the development of nitrative stress in blood plasma and erythrocytes of rats from different experimental groups.

Materials and Methods. The study was performed on white outbred male rats weighing 130–180 g. The diabetes mellitus was induced by intraperitoneal injection of streptozotocin (60 mg/kg). Rats were exposed to photobiomodulation with light-emitting diodes at a wavelength of 630–660 nm daily for 10 days. The irradiation time was 5 minutes. The content of nitrite and nitrate anions, total NO synthase activity, as well as the activity of its endothelial and inducible isoforms in red blood cells of rats were determined spectrophotometrically.

Results and Discussion. Under streptozotocin-induced diabetes mellitus, the content of nitrite and nitrate anions and NO synthase activity increased in the rats' red blood cells, as well as in blood plasma. Moreover, we found an increase in inducible NO synthase activity and nitrate ion content in red blood cells of irradiated healthy rats. Also, there was an increase in nitrite and nitrate ion content after photobiomodulation

therapy in the blood plasma of healthy animals. On the other hand, irradiation caused a decrease in NO synthase activity with a parallel reduction in both nitrite and nitrate anions content in erythrocytes and blood plasma of rats with experimental diabetes.

Conclusion. Photobiomodulation therapy protects rats' red blood cells from nitrative stress during streptozotocin-induced diabetes mellitus.

Keywords: diabetes mellitus, photobiomodulation therapy, nitrative stress, red blood cells

INTRODUCTION

According to the International Diabetes Federation Diabetes Atlas, 10th edition, 537 million adults (20–79 years) lived with diabetes in 2021, and this number is predicted to rise to 643 million by 2030. Diabetes mellitus (DM) is the group of endocrine diseases characterized by elevated blood glucose concentrations secondary to either resistance to insulin, insufficient insulin secretion, or both conditions. The chronic hyperglycemia of DM is associated with long-term damage, dysfunction, and failure of various organs, especially the eyes, kidneys, nerves, heart, and blood vessels (Adane, Getaneh & Asrie, 2020).

DM is directly associated with several hematological changes that affect red blood cells (RBCs), white blood cells (WBCs), platelets and coagulation factors (Antwi-Baffour *et al.*, 2018). The reactive oxygen species (ROS) and reactive nitrogen species (RNS) disrupt the redox state of biological tissues/cells (e.g., RBCs, blood plasma) (Peng *et al.*, 2020). RBCs are important biological agents in ameliorating oxidative and nitrative stress (Peng *et al.*, 2020), but they are highly sensitive to ROS that oxidatively damages membrane macromolecules, ultimately compromising oxygen delivery and leading to cell damage (Wang & Zennadi, 2021). RBCs can also transport stable nitric oxide (II) (NO) metabolites such as nitrite, nitrate, nitrosylhemoglobin (HbNO), nitroso-species, and were defined as the "major storage of nitrite" in the circulation (Mahdi *et al.*, 2021). Hemoglobin (Hb) has a vast impact on the balance of NO formation and scavenging by RBCs. NO can be rapidly sequestered by hemes and eliminated in reactions with oxyHb (oxygenated Hb), forming metHb (methemoglobin) and nitrate. NO can also rapidly react with deoxyHb (deoxygenated Hb) forming HbNO under hypoxic conditions. Besides the ability to react with iron heme, NO can conjugate to cysteine (Cys) thiols in Hb to form an S-nitrosohemoglobin (SNO-Hb) (Su *et al.*, 2020). Given the abundance of O_2 within RBCs, inevitable interactions between NO and other free radicals may result in the formation of secondary reactive species such as peroxynitrite, which detrimentally impacts RBC mechanics. Thus, it is clear that the effect of NO on RBCs is complex (Grau *et al.*, 2021). Mature RBCs lack mitochondria, and the levels of expression of enzymes producing reactive species, including xanthine oxidoreductase (XO), NADPH oxidase (NOX), endothelial and inducible NO synthase, are low under physiological conditions (Mahdi *et al.*, 2021; Slivinska & Iskra, 2020). On the contrary, RBCs have a strong antioxidant capacity as they express a battery of antioxidant systems, including enzymes such as catalase (CAT), superoxide dismutase (SOD), glutathione peroxidase (GP), glutathione reductase (GR), and nonenzymatic antioxidants either produced intracellularly (glutathione (GSH)/glutathione disulfide (GSSG) and NADH/NADPH) or uptaken by the cells, such as ascorbate (in high concentration), α-tocopherol, and bioflavonoids. All of them can neutralize ROS overload, thus reducing oxidative and

nitrative stress (Wang & Zennadi, 2021; Mahdi *et al.*, 2021). As described previously, RBCs from patients with DM increased adherence to endothelial cells. Beyond this, the alterations caused by DM in the intracellular redox state and NO signaling in RBCs are the source of an increased vascular oxidative stress, which induces detrimental effects on the vascular and cardiac function (Mahdi *et al.*, 2021).

Photobiomodulation therapy (PBMT) is a therapeutic use of visible red light of the spectrum (630–660 nm). It has beneficial effects in a variety of diseases, including wound healing, hypoxic injury, cerebral degeneration, Alzheimer disease, retinal degeneration, and DM (Cheng *et al.*, 2018). It is thought that in cells, glycolysis and ATP production are promoted due to PBMT that stimulates electrons in chromophores to move from higher energy orbits, and then electron carriers (such as cytochrome *c* oxidase) deliver these electrons to their ultimate electron acceptors. Furthermore, various transcription factors are switched on by PBMT (Dompe *et al.*, 2020). Additionally, the ability of PBMT to increase the activity of antioxidant enzymes, including SOD (Sunemi *et al.*, 2021), CAT (Karkada *et al.*, 2021), and the total antioxidant capacity of cells (Chen *et al.*, 2021) is well known. Other authors have shown that PBMT improves glucose uptake in cells (Gong *et al.*, 2021). A study by Gong *et al.* (2020) revealed that PBMT improves insulin resistance by inhibiting lipolysis in adipose tissues in DM. An increase in energy supply in cells and a decrease in blood glucose concentration (Karmash *et al.*, 2018) after an improvement of glucose uptake (Maslakova, Liuta & Sybirna, 2021) and inhibition of lipolysis by PBMT, as can be expected, lead to a decrease in the production of ketones. In addition, there is evidence of inhibition of lactate dehydrogenase (LDH) activity due to PBMT exposure (Walski *et al.*, 2018), which correlates with a reduction in plasma lactate concentration (de Oliveira *et al.*, 2018). All these processes can cause an increase in blood pH (**Fig. 4**) and reduce diabetic ketoacidosis. Change in blood plasma pH by PBMT in diabetes is very important because nitrite uptake by RBCs is pH sensitive and is greater when pH is lower than the pH value under normal physiological conditions (Nolan *et al.*, 2015). Moreover, some research clearly showed that the action of PBMT on diabetic rats reduces oxidative and nitrative stress in leukocytes, which may influence RBCs, in particular, PBMT decreases total NO synthase activity and increases enzymatic antioxidant activity, including SOD and CAT (Karmash *et al.*, 2021; Karmash *et al.*, 2020). However, there is no study of the effect of PBMT on nitrative stress development in RBCs in diabetes mellitus. Notably, numerous studies showed the ability of PBMT to decrease RBCs sedimentation rate, reduce oxidative stress, and increase cell membrane mechanical resistance (Walski *et al.*, 2018; Elgendy *et al.*, 2020). Also, it has been shown that during RBCs exposure to red light the amount of metHb decreases and oxygen availability in humans increases, however the mechanism is unknown (Linares *et al.*, 2020; Walski *et al.*, 2018; Karmash *et al*., 2021).

Our study aimed to investigate the effect of PBMT on the development of nitrative stress in blood plasma and RBCs of rats from both healthy and diabetic groups. Our additional aim was to visualize a hypothetical mechanism of the effect of PBMT on nitrogen species and hemoglobin conversion in RBCs (**Fig. 4**).

MATERIALS AND METHODS

The object of research. The experiments were performed on white outbred male rats weighing 150±25 g, which were kept in the vivarium of Ivan Franko National University of Lviv in standard vivarium conditions with free access to food and water. All

manipulations with animals were performed according to the General Ethical Principles of Animal Experiments approved by the First National Congress for Bioethics (Kyiv, Ukraine, 2001), which conforms with the provisions of the European Convention for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes (Strasbourg, 1986), as well as approved by the Ethics Committee of Ivan Franko National University of Lviv, Ukraine (Protocol No. 18 of May 10, 2022).

Induction of experimental diabetes mellitus (EDM). DM was induced by intraperitoneal injection of streptozotocin ("Sigma", USA), diluted in 10 mM Na-citrate buffer (pH 4.5) calculated as 6.0 mg of streptozotocin per 100 g of rats' weight. The development of diabetes was controlled by measuring blood glucose concentration. Rats with blood glucose concentrations exceeding 12 mmol/L were used in the experiments. Glucose concentration was determined by glucose oxidase method using diagnostic kits "Philisit–Diagnostics" (Ukraine) (Lopez, 2012).

Design of experiment. Animals were divided into four groups: 1 – control rats; 2 – control rats exposed to irradiation; 3 – rats with EDM; 4 – rats with EDM exposed to irradiation. For ten days, the rats were daily exposed to photobiomodulation using a matrix of 30 ultra-bright light-emitting diodes of 630–660 nm wavelength and 150 mW power 14 days after induction of DM. The irradiation time was 5 minutes.

Blood sampling. Blood was collected by decapitation of the rats using ether anesthesia. In order to prevent blood clotting, samples were collected into test tubes with heparin (dilution of heparin to whole blood = 1:100). A portion of blood (2 mL) was centrifuged at 3000 rpm for 15 min to separate and obtain blood plasma. RBCs were resuspended into 0.9% NaCl and centrifuged at 3000 rpm for 5 min at 4 °C. This procedure was performed three times to wash the cells. RBCs and plasma were stored at 20 °C. RBCs were hemolyzed with distilled water at a ratio of 1:3.

Spectrophotometric determination of nitrate anions (NO_3^-) content (Miranda, Espey, & Wink, 2001)**.** The content of NO_3^- was determined in the samples of RBCs hemolysates and blood plasma. Deproteinization was performed by adding 96% ethanol to the samples with next centrifuging for 20 min at 5000 rpm at 20 °C. 70 µL of the obtained supernatant, 70 µL of 50 mM VCl_3 dissolved in 1 M HCl, and 70 µL of Griess reagent (0.05% N (1-naphthyl)-ethylenediamine and 1% sulphanilamide in 12% acetic acid in proportion 1:1) were added into microplate vials. The blank vial contained 70 µL of distilled water instead of the sample. Vials were incubated for 30 min at 37 °C, and the optical density at a wavelength of 540 nm was measured using a microplate reader spectrophotometer (Epoch, Biotek, USA). The result was calculated through a calibration curve drawn using standard solutions of NaNO3. The obtained results were expressed in µM.

Spectrophotometric determination of nitrite anions (NO_2^-) content (Miranda, Espey, & Wink, 2001)**.** The content of NO_2^- was determined in the samples of RBCs hemolysates and blood plasma. The method consists in determining the color intensity of the diazonium salt complex formed by the reaction of NO_2^- with sulfanilamide and N (1-naphthyl)-ethylenediamine in an acidic medium. Deproteinization was performed by adding 96% ethanol to the samples with next centrifuging for 20 min at 5000 rpm at 20 °C. 100 µL of the obtained supernatant and 100 µL of Griess reagent were added into microplate vials. The blank vial contained 100 µL of distilled water instead of the sample. Vials were incubated for 30 min at 37 °C, and the optical density at a wavelength of 540 nm was measured. The result was calculated through a calibration curve drawn using standard solutions of $NaNO_2$. The obtained results were expressed in µM.

Spectrophotometric determination of NO synthase (NOS) activity (Dawson & Knowles, 1998). The NOS activity was determined in the samples of RBCs hemolysates. To determine the total NOS activity, an incubation mixture consisting of 10 mM of HEPES buffer containing 1 M $MgCl_2$, 1 M $CaCl_2$, 3 mM L-arginine, and 0.1 mM NADPH(H^+) was added to the samples. Control samples were first prepared by deproteinizing the hemolysates' with 96% ethanol at a ratio of 1:3 and then adding the incubation mixture. After 30 minutes of samples incubation at 37 °C, the reaction in the test samples was stopped by adding 96% ethanol at a ratio of 1:3 with next centrifuging for 20 min at 5000 rpm at 20 °C. 100 µL of the obtained supernatant and 100 µL of Griess reagent were added into microplate vials, the mixture was incubated for 30 min at 37 °C, and the optical density at a wavelength of 540 nm was measured. The total NOS activity was expressed as nmol of the newly formed NO_2^- for 1 min per 1 mg of protein. The concentration of proteins was determined according to the Lowry method (Lowri *et al.*, 1951). **Determination of inducible NOS activity** is similar to the previously mentioned one, but to determine Ca^{2+}-independent NOS activity, 1 M EDTA was added to the incubation mixture instead of 1 M $CaCl_2$. **Determination of endothelial NOS activity** was calculated by subtracting the value of the inducible isoform activity from the total NOS activity, based on the fact that RBCs do not have neuronal NOS (Wallis, 2005).

Statistical analysis. The obtained results were statistically treated through the Microsoft Excel (2016) computer program using Student's *t*-test (p). The data are represented as the mean values (M) ± standard deviation (m). The value $P < 0.05$ is considered statistically significant.

RESULTS AND DISCUSSION

1. Effect of photobiomodulation therapy on the content of nitrite and nitrate anions in the blood plasma of rats. In our study, we aimed to investigate the nitrative part of oxidative/nitrative stress. Firstly, we analyzed the content of NO_2^- and NO_3^- anions (NO_2^- + NO_3^- = NOx) in the blood plasma of rats (**Fig. 1**). We revealed an increase in both NO_2^- (1.99-fold) and NO_3^- (1.59-fold) anions content (1.66-fold increase in NOx level) in the blood plasma of rats with streptozotocin-induced DM compared to the control group of animals. Besides, we observed that after PBMT of healthy animals, the blood plasma content of both NO_2^- (1.38-fold) and NO_3^- (1.15-fold) anions (1.19-fold increase in NOx level) was higher than that in non-irradiated rats. On the contrary, PBM treatment of rats with EDM caused a decrease in the blood plasma content of NO_2^- (1.41-fold) and NO_3^- (1.30-fold) anions (1.32-fold decrease in NOx level) compared to the group of rats with EDM.

Based on our results and literature, we created a scheme of the effect of PBMT on nitrogen species and hemoglobin conversion in RBCs of rats with EDM (**Fig. 4**). In diabetic rats, the formation of ROS increases, which affects the state of the body by stimulating the formation of pathologically high levels of NO (Bahadoran *et al.*, 2018). In addition to reducing NO by hemoglobin uptake, NO also reacts with plasma components to form nitrites (Ferents *et al.*, 2012), which explains why rats with streptozotocin-induced DM have a higher level of NO_2^-. Irradiation of control rats increases the formation of ROS in phagocytes, which is accompanied by higher consumption of oxygen required for the conversion of NO into nitrates that diffuse into the blood plasma. An intensive increase in the level of oxidative modification of proteins in control rats under the influence of PBMT may be a confirmation of the increased formation of ROS in leukocytes,

as shown by Karmash *et al.*, 2020. Also, the increased formation of ROS causes the enhancement of the iNOS activity in WBCs of irradiated control animals (Karmash *et al.,* 2021), which increases the generation of RNS. It is possible that in addition to reducing oxidative and nitrative stress and increasing the activity of antioxidant enzymes, irradiation of rats with DM may enhance the processes of non-enzymatic reduction of NO in blood plasma by interacting with reduced albumin-Cu^{2+} to Cu^{1+} (Mahdi *et al.*, 2021). This may contribute to vasodilation and reduce endothelial dysfunction (Opländer *et al.,* 2013). While more research is needed, we suggest that the possibility of the described effect of PBMT exists not only because of the higher activity of the enzymatic antioxidants, but also due to the fact that the maximum absorption peak of albumin bound to Cu^{2+} is 630–650 nm (Matsuura & Sugimoto, 2014). This means that albumin-Cu^{2+} is a photoacceptor in the range of our irradiation with a wavelength of 630–660 nm. On the other hand, PBMT is likely to reduce endothelial dysfunction due to ceruloplasmin, which plays a great role in the production of NO_2^- from NO in plasma and whose absorption peak is in the range of 550–650 nm (Musci *et al.*, 1996). Pacheco *et al.* (2019) mention the ability of ceruloplasmin to absorb red light. According to Opländer *et al.* (2013), ceruloplasmin catalyzes the nitrite reduction and the subsequent non-enzymatic NO generation simultaneously with the reduction of Cu^{2+} to Cu^{1+} by physiologically available copper reductants (i.e., ascorbate, glutathione, Fe^{2+}). These processes prevent the formation of $ONOO^-$ under oxidative and nitrative stress in DM and, accordingly, increase NO bioavailability (**Fig. 4**), which is necessary for vasodilation (Samuel & Gitlin, 2006).

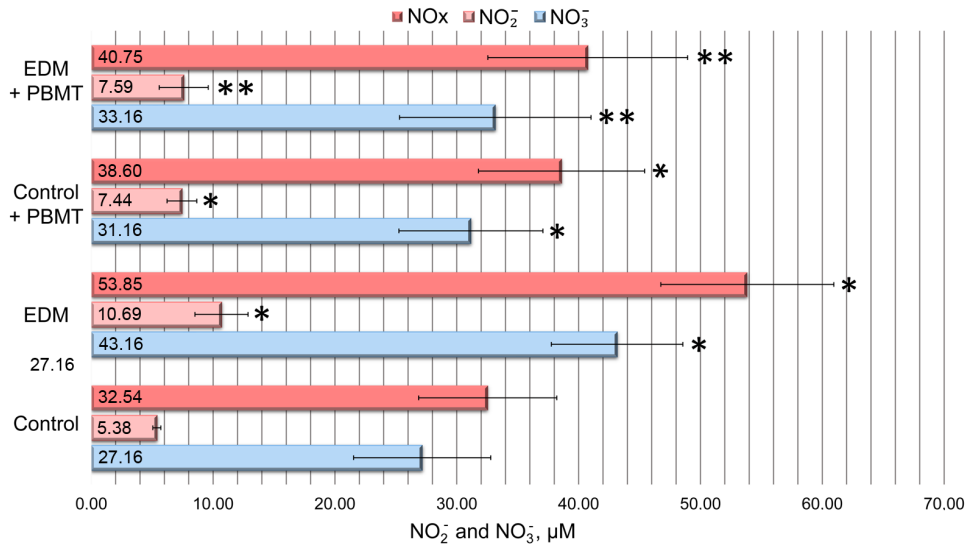

Fig. 1. The content of NO_2^- and NO_3^- anions in the blood plasma of rats (NO_2^- + NO_3^- = NOx), μM. * – significant difference compared with the control, $P < 0.05$; ** – significant difference compared with experimental diabetes mellitus (EDM), $P < 0.05$

An increase in NO_2^- anions plasma content in both diabetic rats and healthy animals exposed to PBMT causes a corresponding increase in NO_3^- content. At the same time, a decrease in the content of NO_2^- anions in the blood plasma of irradiated rats with EDM contributes to a lower NO_3^- content. We assume that irradiation of the control

group will not cause any deterioration of vasodilation. A significant contribution to the deterioration of vasodilation in DM is made not only by oxidative and nitrative stress, but also by changes in the activity and function of endothelial eNOS, which can be corrected under the influence of PBMT. In particular, some studies demonstrate the ability of PBMT to modulate the activity and expression of eNOS genes in vascular endothelial cells, which reduces endothelial dysfunction in diabetes (Colombo *et al.*, 2021). For example, Barolet, Litvinov, & Barolet (2021) and Stepanov *et al.* (2022) proposed that PBMT promoted extracellular Ca^{2+} influx and NO generation by eNOS in endothelial cells. Another way to reduce the content of NO_3^- anions is renal excretion, which is significantly enhanced by diabetes due to hyperglycemia (Francesconi *et al.*, 2001) and which can theoretically be reduced by therapy. It is well known that the effects of PBMT include, but are not limited to, a reduction in hyperglycemia (Marks, 2021) and hypotensive effect due to an increase in the bioavailability of NO (Oishi *et al.*, 2017) that may affect renal excretion (**Fig. 4**).

2. Effect of photobiomodulation therapy on the content of nitrite and nitrate anions in red blood cells hemolysates of rats. In the next stage of our research, we analyzed the effect of PBMT on the NO_2^- and NO_3^- anions content in RBCs hemolysates of rats (**Fig. 2**). We found that the content of NO_2^- (1.60-fold) and NO_3^- (2.68-fold) anions increased in RBCs hemolysates of rats with DM compared with the control group (2.20-fold increase in NOx level). In the case of irradiation of the control group of rats, the content of NO_3^- anions in RBCs hemolysates increased 1.57-fold compared with the same values of the intact group of animals. Besides, we did not find any significant changes in the content of NO_2^- anions in control animals after irradiation compared with intact ones, but the NOx level increased 1.43-fold. The effect of PBMT on rats with diabetes caused a decrease in the content of NO_2^- (1.31-fold) and NO_3^- (1.52-fold) anions (1.45-fold decrease in NOx level) in RBCs hemolysates compared to similar indicators in non-irradiated diabetic rats.

One of the characteristic features of DM is a decrease in the activity of enzymatic antioxidants, which enhances oxidative/nitrative stress and increases the formation of free-radical oxidation products, whereby causing a modification of proteins with a change in their functional activity, as well as triggering changes in phospholipid structure, membrane transport, and functional properties of membranes (Asmat *et al.*, 2016). Inflammatory processes occurring in diabetic animals cause a decrease in plasma pH (Marunaka, 2015), which is associated with an increased diffusion of NO_2^- into erythrocytes (Nolan *et al.*, 2015). It is well known that in RBCs (Mahdi *et al.*, 2021), leukocytes, and other cell types, the following enzymes of antioxidant protection: GR, GP, SOD, and CAT play a vital role in reducing oxidative and nitrative stress. As we described earlier, irradiation increases the activity of the above enzymes (Silva Macedo *et al.*, 2016), which causes a decrease in iNOS activity and oxidative/nitrative stress by reducing the production of ROS and RNS, which lessens inflammatory reactions and diffusion of NO_2^- into RBCs that correlates with lower pH. Such changes in pH can be corrected by reducing diabetic ketoacidosis under the influence of PBMT, as described in the introduction. On the contrary, irradiation of control animals promotes the activation of WBCs and increases ROS and RNS generation, which eventually amplifies the diffusion of NO_2^- into RBCs. Walski *et al.* (2022) associated local acidification of the membrane environment with a drop of pH after red/near-infrared light action. However, experiments by Walski *et al.* (2022) were conducted in vitro using venous blood from healthy pigs as research material; therefore, further study is needed.

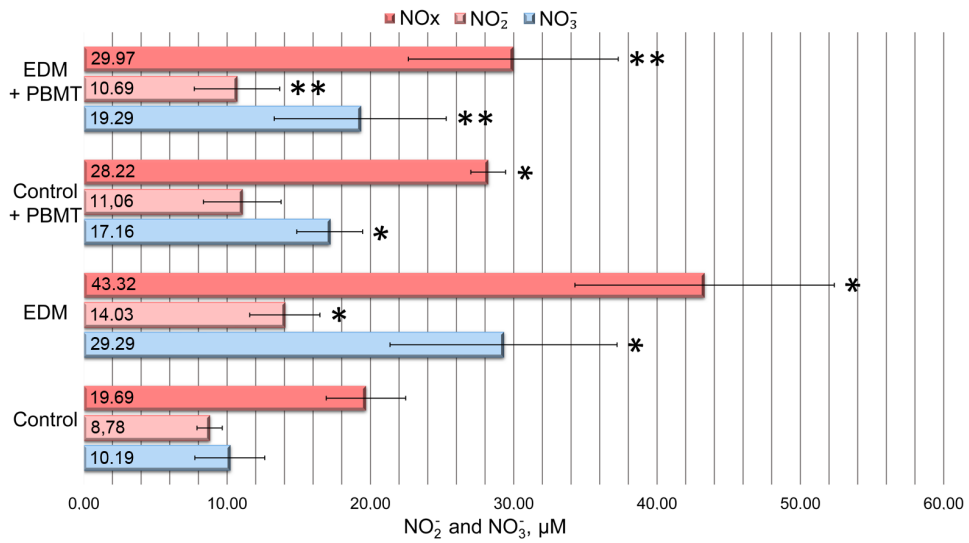

Fig. 2. The content of NO_2^- and NO_3^- anions in rat red blood cells (NO_2^- + NO_3^- = NOx), µM. * – significant difference compared with the control, P < 0.05; ** – significant difference compared with experimental diabetes mellitus (EDM), P < 0.05

In RBCs, NO_3^- and deoxyHb are formed as a result of reaction of NO_2^- with oxyHb, so the NO_3^- content is closely correlated with changes in NO_2^- and oxyHb content. Under oxidative/nitrate stress, a reduced ascorbate is depleted, leading to the reaction of NO_2^- with deoxyHb to form metHb and NO, so that in the next stage NO can react with $O_2^{\bullet}$ to form $ONOO^-$ (**Fig. 4**). Consequently, metHb levels increase in DM (James *et al.*, 2004), and NO's bioavailability for vasodilation reduces. NADPH-methemoglobin reductase, a cofactor of which is NADPH(H^+) formed in the pentose phosphate pathway (PPP), is of great importance for the formation of deoxyHb from metHb (Nolan *et al.*, 2015). Later, deoxyHb reacts with NO to form HbNO (Tejero *et al.*, 2012). In order for glucose to be included in PPP, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) must be inactive and bound to the cytoplasmic domain of Anion exchanger 1 (AE-1) – cdb3. In addition, there should be a cycle in which GAPDH is cleaved from cdb3 after an interaction with NO (GAPDH-SNO) and later interacts with GSH with the subsequent formation of active GAPDH (promotes glycolysis, synthesis of ATP and NADH) and S-Nitrosoglutathione (GSNO). Active GAPDH has the ability to bind rapidly to cdb3, which promotes upregulation of PPP and the corresponding formation of NADPH(H^+) (Zhao *et al.*, 2018). Increased SOD activity in RBCs under the influence of PBMT prevents depletion of ascorbate. On the other hand, reducing oxidative stress may increase GSH, which is not only required for ascorbate reduction by dehydroascorbic acid reductase (DHAR) and NADPH(H^+) (Smirnoff, 2018), but also promote GAPDH activation and maintaining its cycle (Zhao *et al.*, 2018). The described mechanisms are shown in **Fig. 4**. Thus, PBMT can help to maintain the cycle of glucose uptake into PPP and glycolysis, increasing the content of the following cofactors: NADPH(H^+) and NADH(H^+), which are necessary for the formation of HbNO. There is evidence of an increase in hemoglobin after exposure to PBMT in diabetic rats (Karmash *et al.*, 2018), which increases the likelihood of NO binding with minimal adverse effects on cells. Moreover, irradiation

of rats with DM helps to lower the content of NO_2^- anions in RBCs hemolysates and reduce metHb formation with increasing oxy- and deoxyHb, which is also confirmed by the results obtained by other authors (Walski *et al.*, 2018; Linares *et al.*, 2020; Karmash *et al*., 2021). Importantly, according to Allen, Stamler, & Piantadosi (2009), the formation of HbSNO (**Fig. 4**) can promote vasodilation after interaction with AE-1 and conversion to oxyHb. In addition, HbSNO can inhibit the binding of metHb to AE-1, which reduces hemolysis of RBCs (Arashiki *et al.*, 2013). Thus, the formation of HbSNO and reduction of metHb help to reduce the degree of RBC hemolysis, which was confirmed in studies by Walski *et al.* (2018).

3. Effect of photobiomodulation therapy on the total activity of NO synthase, as well as on the activity of its endothelial and inducible isoforms in rat red blood cells. In the last stage of our studies, we determined the effect of PBMT on the total activity of NOS, as well as on the activity of its endothelial and inducible isoforms in rat RBCs (**Fig. 3**). We found that the total activity of NOS in RBCs hemolysates of rats with EDM increased by 1.80 times compared to the same indicator in a healthy group of animals. A detailed analysis of the results of PBMT study revealed a 1.55-fold decrease in the total activity of NOS in RBCs hemolysates of irradiated rats with EDM in comparison with non-irradiated animals with streptozotocin-induced DM. In the study of the PBMT effect on iNOS and eNOS activity, we observed a 2.0-fold decrease in eNOS activity in irradiated diabetic rats compared with the non-irradiated and diseased group of animals. On the other hand, we found a 2.21-fold increase in the activity of iNOS in RBCs hemolysates of animals with EDM compared with the intact group. Irradiation of rats from the control and diabetic groups caused a decrease in iNOS activity in RBCs hemolysates by 1.17 and 1.82 times, respectively. Thus, PBMT has a protective effect on RBCs from nitrative stress during streptozotocin-induced DM.

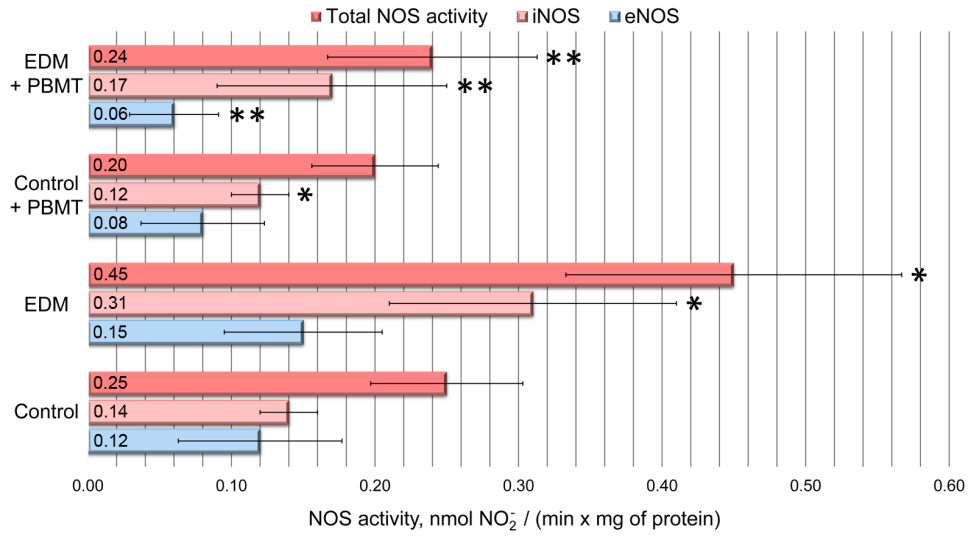

Fig. 3. Total activity of NO synthase (NOS), as well as the activity of its endothelial (eNOS) and inducible (iNOS) isoforms in rat red blood cells, nmol newly formed NO_2^-/ (min × mg of protein). * – significant difference compared with the control, $P < 0.05$; ** – significant difference compared with experimental diabetes mellitus (EDM), $P < 0.05$

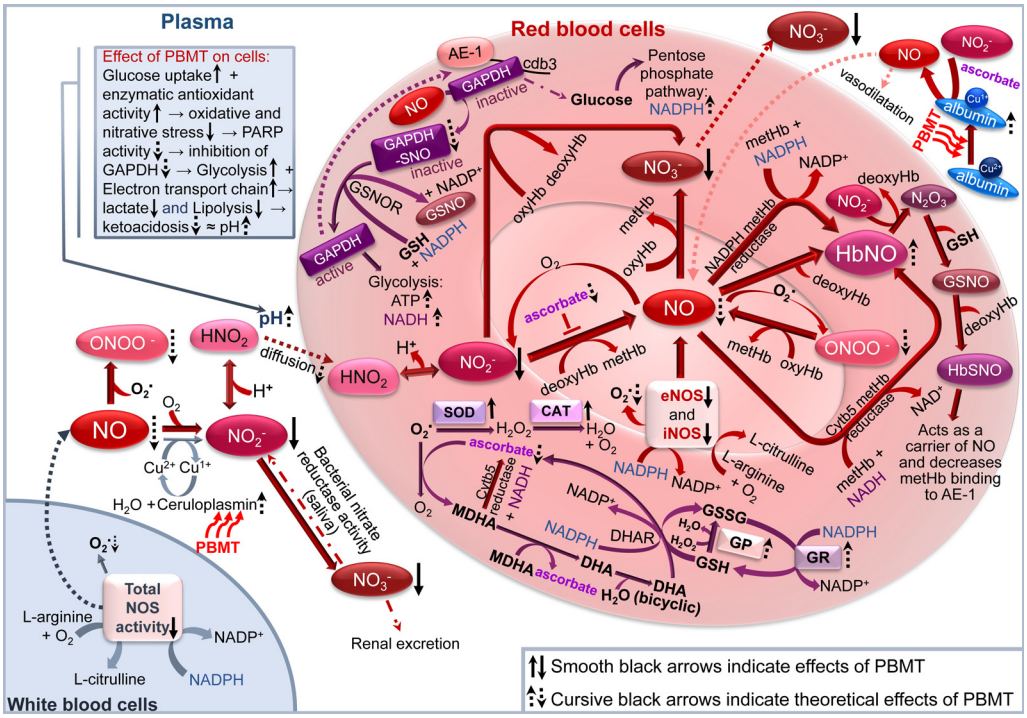

Fig. 4. Hypothetical mechanism of the effect of photobiomodulation therapy on nitrogen species and hemoglobin conversion in red blood cells of rats with experimental diabetes mellitus
Legend: AE-1 – Anion exchanger 1; CAT – Catalase; DeoxyHb – Deoxygenated Hb; DHA – Dehydroascorbic acid; DHAR – Dehydroascorbic acid reductase; GAPDH – Glyceraldehyde-3-phosphate dehydrogenase; GP – Glutathione peroxidase; GR – Glutathione reductase; GSH – Glutathione; GSNO – S-Nitrosoglutathione; GSSH – Glutathione-S-transferase; HbNO – Nitrosylhemoglobin; HbSNO – S-nitrosohemoglobin; MDHA – Monodehydro-ascorbate; MetHb – Methemoglobin; NOS – Nitric oxide synthase; OxyHb – Oxygenated Hb; PARP – Poly (ADP-ribose) polymerase; PBMT – Photobiomodulation therapy; SOD – Superoxide dismutase

According to Wallis (2005), eNOS and iNOS are present in RBCs of rats and humans, but not nNOS. Locally synthesized NO in RBCs can be used to both enhance microvascular permeability and activate macrophages (Jubelin & Gierman, 1996). DM has a pathologically high level of NO production by iNOS (Bahadoran *et al.*, 2018) and eNOS (Slivinska & Iskra, 2020; Förstermann, Xia, & Li, 2017), which is also evidenced in our results. Moreover, in diabetes, eNOS has a limited access to important cofactors, such as tetrahydrobiopterin, due to the high activity of iNOS (Förstermann *et al.*, 2017). This contributes to the disruption of normal enzymatic activity and generation of $O_2^{\cdot}$ instead of NO, which increases the formation of $ONOO^-$ (Dixon *et al.*, 2005) and is one of the causes of endothelial dysfunction in DM due to the reduced bioavailability of NO (Ferents *et al.*, 2012). In general, the intensity of NO synthesis in RBCs is vital for maintaining the balance between NO production and its binding to hemoglobin, which plays a significant role in regulating its bioavailability and metabolism. Our studies showed a decreased enzymatic activity of endothelial and inducible isoforms of NOS after exposure to PBMT in rats with streptozotocin-induced DM, which is probably due to a decrease in oxidative and nitrative stress in rat blood rather than a direct effect of

red light on enzymes. We can draw this conclusion based on the fact that the maximum absorption spectrum of NOS is 450 nm, which is not within the range of the irradiation wavelength we used for therapy (630–660 nm). In short, our research of the effect of PBMT on the nitrative part of oxidative/nitrative stress in diabetes showed preliminary evidence that the addition of red-light treatment has a positive corrective effect.

CONCLUSION

Based on our experimental results, we can conclude that photobiomodulation therapy has a positive corrective effect on the development of nitrative stress in red blood cells during streptozotocin-induced diabetes, in particular, photobiomodulation therapy causes a decrease in NO synthase activity of all isoforms with a parallel reduction in both nitrite and nitrate anions content in erythrocytes and blood plasma. We believe that further research on molecular and cellular mechanisms of the effect of photobiomodulation therapy on different types of cells would be appropriate and promising.

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COMPLIANCE WITH ETHICAL STANDARDS

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Human Rights: This article does not contain any studies with human subjects performed by the any of the authors.

Animal studies: All international, national and institutional guidelines for the care and use of laboratory animals were followed.

AUTHOR CONTRIBUTIONS

Conceptualization, [L.M.Ya]; methodology, [M.A.O.; L.M.Ya]; validation, [M.A.O.; L.M.Ya]; formal analysis, [M.A.O.; L.M.Ya]; investigation, [M.A.O.; L.M.Ya]; resources, [M.A.O.; L.M.Ya]; data curation, [M.A.O.; L.M.Ya]; writing – original draft preparation, [M.A.O.]; writing – review and editing, [L.M.Ya]; visualization, [M.A.O.]; supervision, [L.M.Ya]; project administration, [L.M.Ya]; funding acquisition, [-]. All authors have read and agreed to the published version of the manuscript.

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ФОТОБІОМОДУЛЯЦІЙНА ТЕРАПІЯ ЗАХИЩАЄ ЕРИТРОЦИТИ ВІД НІТРАТИВНОГО СТРЕСУ ПРОТЯГОМ СТРЕПТОЗОТОЦИН-ІНДУКОВАНОГО ДІАБЕТУ

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Вступ. За даними 10-го видання Міжнародної федерації діабету, цукровий діабет є причиною 6,7 млн смертей у 2021 році. Цукровий діабет – це група метаболічних розладів, для якої характерна гіперглікемія внаслідок резистентності до інсуліну, недостатньої секреції інсуліну або обох. Оксидативно-нітративний стрес є важливою частиною складного механізму розвитку цукрового діабету та його ускладнень. Відомо, що фотобіомодуляційна терапія прискорює загоєння діабетичних ран, зменшує запалення та підвищує доступність кисню для клітин. Хоча вже відомі деякі основні молекулярні механізми, які індуковані фотобіомодуляційною терапією у різних типах клітин, вони не досліджені в еритроцитах і відрізняються тому, що у зрілих червоних кров'яних тільцях немає центральних органел таких як ядро та мітохондрії. Метою дослідження було дослідити вплив фотобіомодуляційної терапії на розвиток нітративного стресу у плазмі крові й еритроцитах щурів різних дослідних груп.

Матеріали та методи. Дослідження проводили на білих безпородних щурах чоловічої статі масою 130–180 г. Цукровий діабет індукували внутрішньоочеревинним введенням стрептозотоцину (60 мг/кг). Щурів щодня протягом 10 днів піддавали фотобіомодуляції світлодіодами з довжиною хвилі 630–660 нм. Час опромінення становив 5 хв. Спектрофотометрично визначали вміст аніонів нітриту й нітрату, сумарну активність NO-синтази, а також активності її ендотеліальної та індуцибельної ізоформ в еритроцитах щурів.

Результати. Під час стрептозотоцин-індукованого цукрового діабету в еритроцитах щурів, як і у плазмі крові, підвищився вміст аніонів нітриту і нітрату, а також активність NO-синтази. Крім того, ми виявили підвищення активності індуцибельної NO-синтази та вмісту аніонів нітрату в еритроцитах опромінених здорових щурів. Також відбувалося збільшення вмісту аніонів нітриту й нітрату після фотобіомодуляційної терапії у плазмі крові здорових тварин. З іншого боку, опромінення зумовлювало зниження активності NO-синтази з паралельним зниженням вмісту аніонів нітриту й нітрату в еритроцитах і плазмі крові щурів з експериментальним цукровим діабетом.

Висновки. Фотобіомодуляційна терапія захищає еритроцити щурів від нітративного стресу протягом стрептозотоцин-індукованого цукрового діабету.

Ключові слова: цукровий діабет, фотобіомодуляційна терапія, нітративний стрес, еритроцити

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