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Methylene blue elicits non-genotoxic H_2O_2 production and protects brain mitochondria from rotenone toxicity

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Abstract
Methylene blue (MB) is a promising compound with a broad range of neuroprotective activity. One of therapeutic effects is the activation of mitochondrial biogenesis via Nrf2/ARE signaling cascade. Probably, mild oxidative stress caused by MB-depended H_2O_2 production is a trigger for activation of this signaling cascade. So mechanistically, MB can be regarded as prooxidant. We investigated the dose-dependent H_2O_2 production in intact brain mitochondria and showed the increase in the H_2O_2 production after adding as little as 50 nM MB. We have not found genotoxic effect of therapeutic concentration of MB to mitochondrial genome. 100 μM MB selectively damaged fragments of mitochondrial DNA, which correlated with the number of purine-T-G-purine (RTGR)-sequences in studied fragments. Furthermore, 20 μM MB combined with the red light caused the formation of singlet oxygen, which strongly damaged mitochondrial DNA in all studied fragments. We did not observe mitochondrial DNA lesions in brain after single intraperitoneal injection of MB in the concentration of 50 mg/kg. Furthermore, we showed the neuroprotective properties of MB pretreatments after rotenone injection. Therefore, we suggest that MB-induced mild oxidative stress does not have genotoxic effect on mitochondrial DNA.

Keywords: DNA damage; Methylene blue; mtDNA; Neuroprotection; Rotenone; Toxicity

Highlights:
- Methylene blue promotes a hydrogen peroxide production.
- Methylene blue in therapeutic concentrations induces production of the hydrogen peroxide, which does not damage mtDNA.
- Rotenone selectively damages the ventral midbrain mitochondria, but not the frontal cortex.
- Methylene blue can protect mitochondria of ventral midbrain from the rotenone poisoning.

Introduction

Methylene blue (MB) is an organic basic thiazine dye widely used as a medicine for malaria, methemoglobinemia and cyanide poisoning (Schirmer et al., 2011). During the last two decades MB has been considered one of the most promising therapeutic agents for neurodegenerative diseases. That is possible thanks to its high lipophilicity, which allows it to penetrate the blood-brain barrier and the membranes of mitochondria (Rojas et al., 2012). Once MB enters the brain, it exhibits the properties of redox ETC (electron transport chain) component, since it can oxidize NADH and transfer the electrons to the downstream components of ETC. This effect is known as alternative electron transport (Wen et al., 2011). MB can act as a neuroprotector thanks to its ability to regulate the mitochondrial metabolism and homeostasis of ROS (reactive oxygen species), which play an important role in neurodegenerative disorder genesis and are involved in the aging process (Harman, 2009).

However, the question of the antioxidant properties of MB is still open to debate. On one hand, there is a number of studies, which demonstrate the antioxidant effect of MB in cell cultures exposed to strong oxidizing agents, such as exogenous H_2O_2 or rotenone (Atamna et al., 2008; Poteet et al., 2012; Wen et al., 2011). On the other hand, other studies show that MB exhibits the prooxidant characteristics. Those studies are generally focusing on the photosensitizer properties of MB, which lead to singlet oxygen generation (Oliveira et al., 2011). Also, it was shown that MB in concentrations of 1 μM and during the chronic drug administration contributes to the increase in the production of H_2O_2 in isolated brain mitochondria (Gureev et al., 2016; Tretter et al., 2014).

A number of researchers emphasize the hormetic effect of MB and indicate that the optimal MB concentration in the injections ranges from 1 to 4 mg/kg/day (Bruchey and Gonzalez-Lima, 2008; Rojas et al., 2012). Administering high concentrations of MB (more than 10 mg/kg/day) nullifies the positive effects and using the extremely high concentration (more than 50 mg/kg/day) has an adverse effect and causes the in-

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hibition of psychomotor activities of the animals (Riha et al., 2005). Pharmacokinetics of MB is somewhat different when it is administered orally since a significant portion is excreted in the urine (Peter et al., 2000). Using the 5 mg/kg/day MB does not induce any significant therapeutic effect (Gureev et al., 2016) and the optimal results for this type of administration are shown to be at concentrations ranging from 15 to 40 mg/kg/day (Eroğlu and Cağlayan, 1997; Gureev et al., 2016; Stack et al., 2014). In any way MB is characterized by an inverted U-shaped curve typical of hormesis (Rojas et al., 2012).

It is worth mentioning that in our previous studies (Gureev et al., 2016) and in the studies of Vekaria et al. (2017) and Tretter et al. (2014), the prooxidant effect was detected using the optimal MB concentration and the positive bioenergetics and physiological reaction has been observed. It was hypothesized that the increase in the H_2O_2 generation rate can activate mitochondrial biogenesis through Nrf2/ARE signal cascade, thus improving the physiological parameters. H_2O_2 induced low-level oxidative stress can thus cause adaptive retrograde response that culminates in subsequently increasing stress resistance (Radak et al., 2005; Ristow and Zarse, 2010). Mitochondrial oxidative stress is in addition characterized by a hormetic effect, also known as "mitohormesis" (Ristow and Zarse, 2010). At the same time, the information about the genotoxicity of the MB caused by its prooxidant properties is absent. Consequently, the aim of this research is to investigate the impact of different concentrations of MB on the integrity of mtDNA, which is extremely sensitive to the oxidative damage. mtDNA is localized in the mitochondrial matrix and it is in the close proximity to ETC and MB, both of which produce H_2O_2. Our modification of the qPCR method used for detecting the mtDNA lesions can also show the regions of the mtDNA which are most sensitive to damage (Gureev et al., 2017).

Materials and methods

Laboratory animals

C57Bl/6 mice, 4-month-old males and females (distribution of 1 : 1) were used for the experiments. The animals were obtained from the Stolbovaya Nursery (Scientific Center for Biomedical Technology, Russia) and housed in plastic cages under standard conditions (25 °C; 12-h light/dark cycle; relative humidity >40%). Water and food (ssniff Spezialdiäten GmbH, Germany) were administered *ad libitum*. All experimental procedures with animals, such as maintenance, injections and sacrifice were performed strictly in accordance with the rules set by Institutional animal care and use committee of Voronezh State University.

Isolation of mitochondria from the brain

Brain tissue was homogenized with a Dounce tissue grinder in 25 ml of the mitochondria isolation buffer containing 220 mM mannitol, 100 mM sucrose, 1 mM EGTA, 20 mM HEPES, 2 mg/ml BSA, pH 7.4. The homogenate was centrifuged at 900 g for 5 min. The pellet was discarded and supernatant transferred to clean sample tube. Supernatant was then centrifuged at 14 000 g for 10 min. The resulting pellet was resuspended and layered on top of the 23% Percoll. The gradient centrifugation was performed at 23 000 g for 15 min. The top and the middle layer were removed. The bottom layer was resuspended in the mitochondria isolation buffer without BSA and centrifuged at 14 000 g for 10 min. The washing step was repeated twice, after which the final resuspension was done in 70 µl of the mitochondria isolation buffer without BSA.

The rate of H_2O_2 generation was measured on fluorescence spectrophotometer (Hitachi F-7000, UK) using AmplexUltraRed (Sigma, USA). At least three measurements were repeated for 6 mice (n = 6). Calibration and further measurements were done according to the procedure of Starkov (2010).

Initiation and the quantitative evaluation of the mtDNA lesions in vitro

Isolated mitochondria from 6 mice brains were aliquoted and incubated for 30 min at room temperature with respiratory substrates (10 mM malate + 10 mM glutamate) as a control and 1 µM rotenone (Sigma, USA) or 5 µM antimycin A (Sigma, USA) + 40 µM 2,4-Dinitrophenol (DNP) with the same respiratory substrates.

Isolated mitochondria from 6 mice brains were incubated with the MB in 3 different concentrations: 10 µM, 20 µM and 100 µM. In order to determine the genotoxicity of MB as a photosensitizer, mitochondria were incubated with 20 µM MB under the red LED with the wavelength of 630 nm, as described by Guan et al. (2014).

mtDNA isolation was performed using the Plasmid Miniprep Kit (Evrogen, Russia), as described in our earlier papers (Gureev et al., 2017). Number of lesions was measured for six mtDNA fragments. The 1st fragment corresponded to 12S and 16S rRNAs, the 2nd fragment corresponded to 16S rRNA and ND1, the 3rd fragment corresponded to ND1 and ND2, the 4th fragment corresponded to ND5, the 5th fragment corresponded to ND6 and CytB, the 6th fragment corresponded to D-loop. Primers for amplification of six mtDNA fragments used in this study were done according to the same protocol (Gureev et al., 2017). The extent of excessive mtDNA damage which was induced by rotenone, antimycin A and MB was estimated using the ΔΔCq method. The extent of mtDNA damage was estimated using the ΔΔCq method: ΔCq for the control and experimental long fragments was compared to ΔCq for the control and experimental short fragments. Since the amplified fragments differed in length, the number of mtDNA lesions was calculated per 10 kb of mtDNA using the equation (1).

$$\text{Lesions per 10 kb} = (1-2^{-(\Delta\text{long}-\Delta\text{shot})}) * 10000\text{bp} / \text{fragment, bp} \quad (1)$$

Assessment of MB toxicity in vivo

Acute drug toxicity of MB was tested on mice. The 50 mg/kg solution of MB in saline solution was administered intraperitoneally (n = 8). 0.2 ml of saline solution was administered in the same manner to the control group (n = 6).

Both the control and the experimental groups were sacrificed 24 hours later. The mtDNA was isolated from both the midbrain and the cerebral cortex according to the methods described earlier (Gureev et al., 2017).

Assessment of neuroprotective effect of MB in vivo

Three groups of 4-month-old mice were used for the study the neuroprotective properties of MB: (1) a control group that did not receive injections (n = 6); (2) a group that received a single intraperitoneal injection of rotenone (1 mg/kg) (n = 6); (3) a group that received a single intraperitoneal injection of rotenone, and previously treated with 15 mg/kg/day of MB for 7 days (n = 6). All mice groups were sacrificed 24 hours later. The mtDNA was isolated from both the midbrain and the cerebral cortex according to the methods described earlier (Gureev et al., 2017).

Assessment of diene conjugates concentration

Diene conjugates are primary products of lipid peroxidation (Beľskaya et al., 2016). To determine the concentration of

diene conjugates, the following procedure was used. A mixture containing a brain homogenate (125 µl), saline solution (125 µl), heptane (1.5 ml), isopropyl alcohol (1.5 ml) was centrifuged at 3000 g for 10 min. 1/10 volume of distilled water was added to supernatant and shaken. Then, the heptane phase was taken and 500 µl of ethanol was added. The measurement was carried out on a spectrophotometer Hitachi U-2900 at a wavelength of 233 nm. The concentration of diene conjugates was calculated using the equation (2).

$$C_{dk} = V_{total} \times D \times 10^6 / L \times E \times m \times V_{add} \quad (2)$$

Where, C_{dk} – concentration of diene conjugates (mol/g); V_{total} – the total volume of the sample (ml); D – optical density; L – optical path length; E – the molar extinction coefficient (2.2×10^5 $M^{-1}s^{-1}$); V_{add} – the volume of added sample (ml).

Statistical analysis

Statistical analysis was performed using statistical variance methods (Microsoft Excel software package). The results were expressed as means ± SEM. The significance of differences between groups was estimated with the Student's *t*-test; $p < 0.05$ was considered to be statistically significant.

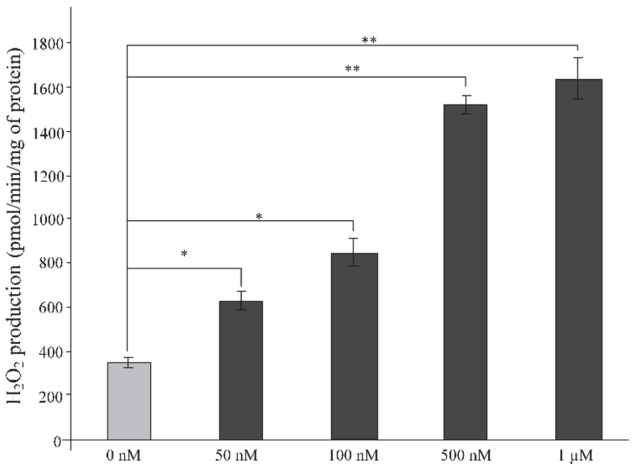

Fig. 1. Rate of H_2O_2 production after adding 50 nM, 100 nM, 500 nM, 1 µM MB to intact brain mitochondria compared with the basic rate of H_2O_2 production ($n = 6$); * $p < 0.05$; ** $p < 0.01$.

Results

The rate of H_2O_2 generation

MB increases the rate of H_2O_2 production. 50 nM MB caused its increase to 585 ± 36 pmol/min/mg of protein (compared to control, where the rate was 324 ± 24 pmol/min/mg of protein) in mitochondria respirated on 5 mM malate and 5 mM glutamate. Addition of 100 nM MB increased the generation rate of H_2O_2 to 790 ± 58 pmol/min/mg of protein, 500 nM MB to 1420 ± 41 pmol/min/mg of protein, and 1 µM of MB to 1526 ± 88 pmol/min/mg of protein (Fig. 1).

MB-induced mtDNA lesions in vitro

Incubating the mitochondria with 10 µM MB for 30 minutes did not cause damage to any of the mtDNA fragments. 20 µM MB did not affect the mtDNA integrity although a slight increase in lesion accumulation on the 6th fragment, which corresponds to D-loop (0.25 ± 0.4 lesions/10 kb), was observed. However, the data were not statistically significant. We have observed the damage only while using the 100 µM MB. Significant lesions were found in 1st fragment (1.19 ± 0.5 lesions/10 kb), 2nd fragment (1.14 ± 0.4 lesions/10 kb) and in 6th fragment (1.51 ± 0.8 lesions/10 kb). The 3rd fragment was shown to have 0.09 ± 0.2 lesions/10 kb and in 4th and 5th fragments the lesions had negative values (–0.32 ± 0.2 lesions/10 kb and 0.71 ± 0.4 lesions/10 kb respectively).

Additionally, in order to induce further damage, we have used 20 µM MB together with the 630 nm light. Under these conditions, the significant damage to all of the fragments was observed. In 1st fragment the amount of lesions was shown to be 0.63 ± 0.06 lesions/10 kb; in 2nd fragment 1.1 ± 0.3 lesions/10 kb; in 3rd fragment 0.82 ± 0.4 lesions/10 kb; in 4th fragment 0,94 ± 0.6 lesions/10 kb; in 5th fragment 0.73 ± 0.3 lesions/10 kb and in 6th fragment 2.18 ± 0.6 lesions/10 kb (Fig. 2).

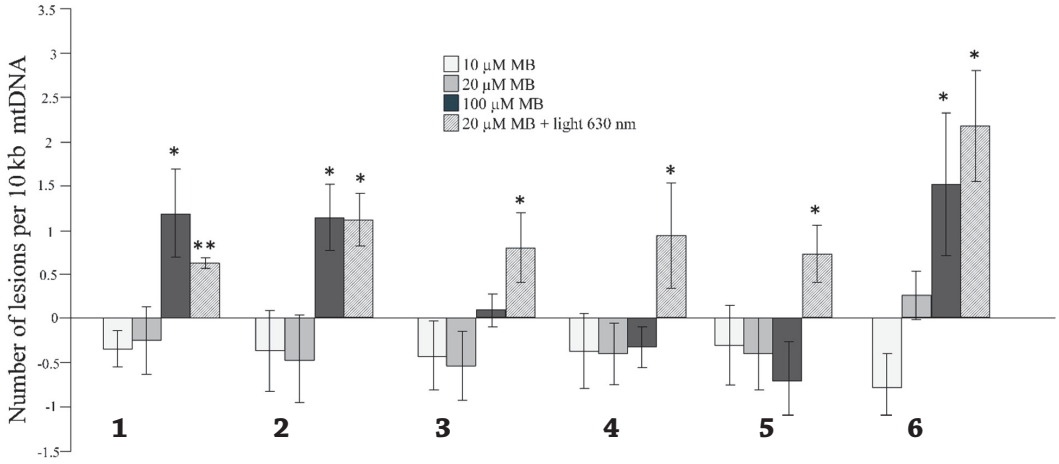

Fig. 2. Number of lesions in six mtDNA fragments induced by 10 µM MB, 20 µM MB, 100 µM MB and 20 µM MB + light 630 nm (for photodynamic therapy) in mitochondria isolated from 6 mice brains; * $p < 0.05$; ** $p < 0.01$ compared to control.

mtDNA damage induced by ETC inhibitors

The incubation of the mitochondria with rotenone for 30 minutes caused the damage in all of the six fragments. Average damage value was 2.51 ± 0.3 lesions/10 kb. It is important to note the significant variability in the lesion values in 6th fragment (1.78 ± 1.1 lesions/10 kb).

Incubating the mitochondria with antimycin and 40 μM 2,4-DNP has also caused the lesion accumulation (on average 2.19 ± 0.4 lesions/10 kb). A tendency towards a lower number of lesions was observed in 3rd fragment (1.12 ± 0.8 lesions/10 kb) and 6th fragment (1.41 ± 0.9 lesions/10 kb) (Fig. 3).

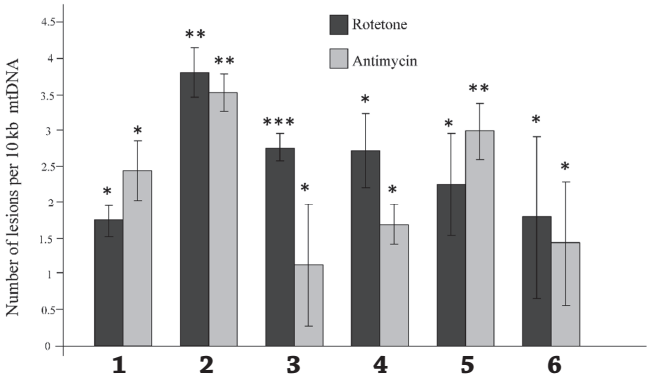

Fig. 3. Number of lesions in six mtDNA fragments induced by 1 μM rotenone (dark grey column) or 5 μM antimycin + 40 μM 2,4-DNP (light grey column) in mitochondria isolated from 6 mice brains; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ compared to control.

mtDNA damage after MB injection

50 mg/kg MB injections did not induce any mtDNA damage in the cortex and the midbrain of mice 24 hours after the administration. Although the tendency for a higher number of lesions was observed in the 6th fragment (0.49 ± 0.8 lesions/10 kb), we consider it to be the result of the D-loop structural polymorphism which can significantly impact the effectiveness of Encyclo-polymerase used for the quantitative determination of mtDNA lesions (Table 1).

Table 1. Number of lesions per 10 kb mtDNA induced by 50 mg/kg dose injection of MB in the ventral midbrain and frontal cortex ($n = 8$)

Fragment	Ventral midbrain	Frontal cortex
1	–0.15 ± 0.21	0.41 ± 0.24
2	–0.08 ± 0.19	–0.17 ± 0.39
3	0.04 ± 0.2	–0.34 ± 0.46
4	–0.27 ± 0.26	–0.21 ± 0.23
5	–0.3 ± 0.21	0.16 ± 0.35
6	–0.48 ± 0.67	0.45 ± 0.78

Rotenone in vivo *toxicity and MB neuroprotection*

The rotenone injection caused a significant increase in mtDNA lesions in the ventral midbrain for all six observed fragments. A significantly reduced number of the lesions was in 1st, 4th and 6th fragments ($p < 0.05$) in mice previously treated by MB (Table 2). The concentration of diene conjugates increased by 24% in mice receiving rotenone injections. The MB treatment reduced the amount of diene conjugates by 28% compared to the group receiving only rotenone injections (Fig. 4A).

In frontal cortex, there was an unequal distribution of the number of lesions. There were no injuries in 3rd and 4th fragment. MB treatment statistical significantly reduced number of lesions only in 6th fragment ($p < 0.05$) (Table 2). The number of diene conjugates in all three study groups did not differ significantly (Fig. 4B).

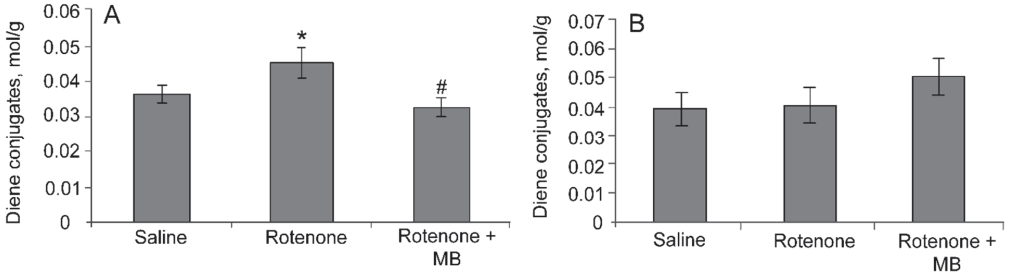

Fig. 4. The concentration of diene conjugates in a ventral midbrain (**A**) and frontal cortex (**B**) for mice that received saline injection ($n = 6$), rotenone injection ($n = 6$) and rotenone injection + MB treatment ($n = 6$); * $p < 0.05$ compared to saline group; # $p < 0.05$ compared to rotenone group.

Table 2. Number of lesions per 10 kb mtDNA induced by rotenone injection (n = 6) and rotenone injection + MB treatment (n = 6) in the ventral midbrain and frontal cortex

Fragment	Ventral midbrain		Frontal cortex	
	Rotenone	Rotenone + MB	Rotenone	Rotenone + MB
1	1.31 ± 0.19**	0.73 ± 0.21#	1.81 ± 0.15**	1.71 ± 0.39
2	1.45 ± 0.37*	0.96 ± 0.48	1.14 ± 0.25*	1.02 ± 0.39
3	0.65 ± 0.29*	0.85 ± 0.32	0.12 ± 0.34	0.64 ± 0.24
4	1.59 ± 0.47*	0.77 ± 1.16#	0.34 ± 0.54	0.29 ± 0.36
5	1.14 ± 0.26*	1.48 ± 0.31	1.85 ± 0.26**	1.32 ± 0.38
6	3.09 ± 0.54**	0.98 ± 0.29#	1.98 ± 0.28**	1.25 ± 0.24#

* $p < 0.05$; ** $p < 0.01$ compared to saline group; # $p < 0.05$ compared to rotenone group.

Discussion

Hormetic effect is a characteristic feature of a significant part of pharmaceuticals influencing the catabolic processes and the metabolism of ROS. MB is not an exception since a number of studies were dedicated to the research of its hormetic effect (Bruchey and Gonzalez-Lima, 2008; Riha et al., 2005; Rojas et al., 2012). Oz et al. (2011) have demonstrated that in preclinical studies *in vitro* the working MB concentrations ranged from 1 to 10 μM and the toxic effects were observed at the 100 μM concentration. In earlier research, it was shown that 5 μM MB causes mitochondrial swelling (Visarius et al., 1997) and that the concentration optimum lies between 100 nM and 1 μM (Atamna et al., 2008). Our study has confirmed that the significant increase in H_2O_2 production rate can be observed at 50 nM MB (Fig. 1). Tretter et al. (2014) have observed the prooxidant effect of MB at a concentration of 100 nM.

The studies mentioned above confirm that the prooxidant effect of MB occurs on a threshold of its optimal concentration. Apparently, the positive therapeutic effect of MB is caused not only by metabolism improvements but also by a low-level oxidative stress, which can be observed even at the low concentrations of the compound. It was shown repeatedly that mild oxidative stress induced by small doses of H_2O_2 is capable to activate the Nrf2/ARE signaling cascade (Erlank et al., 2011; Piantadosi et al., 2008). Nuclear translocation of Nrf2 induces transcription of genes, which trigger mitochondrial biogenesis. It is well known that the oxidative stress is a cause of damage to a variety of cell elements and that the component of the cell most susceptible to its impact is mtDNA. mtDNA is localized in the mitochondrial matrix and is therefore close to the site of ROS generation. Additionally, mitochondrial genome lacks the advanced DNA reparation system, compared to the nuclear one, so the mtDNA can be used as an accurate marker of oxidative damage (Yakes and Van Houten, 1997).

In our experiment the starting concentration of MB was 10 μM – the highest limit of MB concentration *in vitro* (Oz et al., 2011). 10 μM MB did not cause mtDNA lesions in any of the six fragments we have experimented on. The damage was also absent when using the 20 μM MB (Fig. 2). D-loop was the only mtDNA fragment in which the tendency towards lesions was observed (0.25 ± 0.4 lesions/10 kb). However, we do not consider it to be a marker of oxidative stress. First, the data is not statistically significant. Second, D-loop region is characterized by a complex triple-stranded structure and high variability (Pohjoismäki and Goffart, 2011). Those D-loop properties are confirmed by the fact that in all the research on the mtDNA damage, regardless of the damage inducing agent used, the significant variability of the results was observed.

The aim of this study was to define the extent of MB toxicity at the concentrations used in a variety of studies, so we do not consider the further gradual increase in MB concentration to be necessary. It was also intriguing to clarify the types of lesions induced by MB at the concentration significantly exceeding the normal physiological one. For this purpose, we have incubated isolated mitochondria with 100 μM MB. We have observed the mtDNA lesions on 1st, 2nd and 6th fragments (Fig. 2). 1st, 2nd and 6th regions were also the ones with the highest percentage of purine-T-G-purine (GTRG)-enriched sequences (1.2%; 1.3% and 1.1% respectively). These fragments are more susceptible to Fenton's reaction due to the specific reaction between Fe^{2+} and thymine (Henle et al., 1999). As a result of this reaction, the hydroxyl radical is formed from H_2O_2, produced by MB. Hydroxyl radical is the factor causing the damage to mtDNA. These data confirm the hypothesis of Tretter et al. (2014) which states that the first form of ROS, generated in a MB-mediated process, is namely H_2O_2. This explains why a number of researchers made a conclusion about ROS-scavenger properties of MB: since either the non-selective ROS sensor or Mitosox were used in the studies, the decrease in superoxide concentration was observed (Poteet et al., 2012; Wen et al., 2011). Using this approach, it was difficult to measure the production rate of H_2O_2 directly.

As a control, we have tested the compounds known to cause serious oxidative damage even at low concentrations. We have used rotenone, which inhibits the electron transfer from the mitochondrial complex I to coenzyme Q and increases the ROS generation rate (Kudin et al., 2004). We have also used antimycin in uncoupled mitochondria. Antimycin blocks the electron transfer from cyt b_h to semiquinone, while the second electron transfer continues down the ISP – cyt C1 – cyt C – COX – O_2 chain. The result of these events is an unstable semiquinone, which can transfer the electron to oxygen, thus forming the superoxide anion (Grigolava et al., 1980). The addition of the uncoupling agent further stimulates the ROS production. That effect was observed by Boveris and Chance (1973) but the underlying mechanism is not well understood. Additionally, blocking the ETC causes the overreduction of the mitochondrial NADH, which in turn also generates the ROS (Kushnareva et al., 2002).

For the reasons mentioned above, the serious damage was observed not only in RTGR-rich sequences, but in all the others as well (Fig. 3). MB had an opposite effect since it increases the NADH oxidation rate. That effect is confirmed by the increased NAD^+/NADH ratio 15 minutes after the addition of

MB to the cell cultures (Atamna et al., 2015). The increase of the reduced NADH can explain why 1 μM ETC inhibitor causes more mtDNA lesions than 100 μM MB. Also, inhibition of ETC was shown to cause more mtDNA damage than exogenous addition of 500 μM H_2O_2 in our past experiments (Gureev et al., 2017).

MB has caused a significant mtDNA damage when used as a photosensitizer. It is well known that, when combined with the light, MB produces singlet oxygen and it is therefore classified as a type II photosensitizer (Costa et al., 2016). Singlet oxygen causes a variety of mtDNA lesions in all fragments, which is shown in this study (Fig. 2). It is worth mentioning that the lesions' quantity in each of the fragments does not correlate to mtDNA GTRG content. That also confirms the hypothesis that the damage is caused by singlet oxygen and not by hydroxyl radicals, formed from H_2O_2 in the Fenton reaction.

Since MB used *in vitro* does not damage the mtDNA at the concentrations up to 20 μM, we have initially decided to use the higher dose in our experiments *in vivo*. The study of Riha et al. (2005) shows that the one-time 50 mg/kg intraperitoneal injection of MB causes the decreased running wheel behaviour. We have chosen that concentration for our acute experiment. The qPCR analysis did not show any lesions in neither the mtDNA fragments of cerebral cortex nor the mtDNA fragments of midbrain (Table 1). We have done a separate analysis of the cortex and the midbrain since the previous studies had shown that certain agents, such as rotenone, affect the mtDNA of different brain regions in a different manner. Midbrain was shown to be the most sensitive to the rotenone injections (Sanders et al., 2014). However, our experiment has demonstrated that the MB does not cause any genotoxic effect to mtDNA, independently of the brain region, even at the extremely high concentrations, which are not used in medical purposes.

Earlier studies had shown that the high doses of the intravenous injections of MB exhibit a wide range of neurotoxic effects, such as the increased levels of caspase-3 positive cells in the brain which initiate dendritic arbor retraction in cell culture (Vutskits et al., 2008). Our studies do not contradict those findings, but rather show that even at the high concentrations, MB does not have genotoxic properties. This is consistent with the study of Auerbach et al. (2010) on hybrid vigor lines mice and rats for broader array of responses to stresses. Authors had indeed shown that the cancerous processes develop in animals after 2 years of high-dose MB therapy (the difference between the control and experimental group was not statistically significant in all of the cases), but it had no effect on the survival rate of the rodents.

However, it is impossible to state unequivocally that the MB does not damage mitochondria in neurons. The mitochondria extraction method in Percoll gradient leads to an elimination of synaptosome (Kristian, 2010), so we used only non-synaptic mitochondria to perform measurement. Nevertheless, these mitochondria are less prone to the other factors, for example, an aging (Lores-Arnaiz et al., 2016) or brain trauma injury (Hill et al., 2018). Further studies are necessary to reveal the differential influence of MB on a synaptic and non-synaptic mitochondrion.

In addition, we have shown that MB may demonstrate a neuroprotective effect in case of rotenone poisoning. Injections of the rotenone, as it was shown earlier (Gureev et al., 2017; Sanders et al., 2014) cause significant damage in mtDNA. Preliminary therapy with MB for 7 days resulted in reduced mtDNA lesions (Table 2) and lipid peroxidation in the ventral midbrain (Fig. 4B).

These results are consistent with previous studies conducted on the retina of mice (Rojas et al., 2009; Zhang et al., 2006) and retinal ganglion cells (RGCs) in rats (Daudt et al., 2012). Poteet et al. (2012) showed that MB reduces the ROS production and provides neuroprotection in HT-22 cells against the rotenone toxicity. The subcutaneous infusion of MB reduced oxidative stress caused by repeated injections of the rotenone (Abdel-Salam et al., 2014). Some studies have found that the administration of MB significantly reduced mortality after cyanide poisons (Berling, 1970; Salaris et al., 1991; Sun et al., 2018, Wen et al., 2011), which inhibits IV complex of ETC.

As in the Sanders experiment (Sanders et al., 2014), we noted that the frontal cortex is less prone to rotenone-induced damage. It was shown that this applies not only to the measurement of mtDNA damage, but also to the measurement of the concentration of products of lipid peroxidation. Perhaps this is due to the different permeability of the rotenone through the blood-brain barrier in different brain compartments, but this issue needs further research.

Conclusions

Thus, summarizing the data obtained in this research, we can conclude that MB increases production of H_2O_2 due to bypassing of ETC components of brain mitochondria. MB contributes to activation of mitochondrial biogenesis through Nrf2 by stimulation of H_2O_2 production. At the same time MB-related H_2O_2 was produced in non-toxic volume that does not exert genotoxic effect on mtDNA, which is the most susceptible to oxidative damage. All above mentioned proves the possibility of using mild oxidative stress in the activation of compensatory reactions, which may have a therapeutic effect in the treatment of neurodegenerative age-depended diseases.

Conflict of interests

The authors confirm that the study causes no conflict of interests as related to funding, commercial activity, intellectual property rights, or other conceivable issues.

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