Mediation of the uncoupled eNOS pathway following oxidative stress using tetrahydrobiopterin and nitric oxide donor drugs to restore tetrahydrobiopterin concentration

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Abstract

The eNOS pathway, found in the endothelium of blood vessels, is a key regulator of nitric oxide levels in the circulatory system. The pathway is controlled through several positive and negative feedback loops [2]. The cofactor tetrahydrobiopterin (BH4) is a major control point in this pathway and under conditions of stress can be reduced into the dihydrobiopterin (BH2) [2,6,7,8,9]. When the reduced form is predominant, the pathway produces reactive oxygen species (ROS) rather than nitric oxide, causing stress and damage to the vessels [6,7,8,9]. In this study, different treatments were studied to determine which is most effective in restoring BH4 levels in the eNOS pathway of bovine aortic endothelial cells (BAECs). Nitric oxide supplementation was the main focus of this study and was tested as a stand-alone treatment and as a combined treatment along with a BH4 donor drug, sapropterin dihydrochloride. Following the two treatments, only the BAECs given the nitric oxide donor drug showed levels of BH4 higher than the untreated control cells. The cells treated with 25 μM nitric oxide donor drug and 2.5 μM BH4 donor drug showed levels of BH4 that were most similar to the untreated control cells with a concentration of approximately 27 mM BH4.

Introduction

According to the American Heart Association, in 2016, nearly 1 in every 3 deaths can be attributed to some form of cardiac dysfunction, making it a prime area of interest for researchers [1]. In recent years, nitric oxide (NO) deficiency has been identified as a key player in cardiac dysfunction [2]. NO acts as a neurotransmitter and promotes vasodilation, electrical stimulation of the pacemaker, and angiogenesis [3,4,5]. Deficiency of NO in the blood vessel endothelium can be largely attributed to uncoupling of the enodothelial nitric oxide synthase (eNOS) pathway [6,7]. The eNOS pathway converts L-arginine to L-citrulline and NO using the cofactor tetrahydrobiopterin (BH4) [2,6,7,8,9]. When the eNOS pathway is uncoupled, levels of

L-arginine, L-citrulline, and BH4 are reduced, and the O_2^- radical is produced [8]. As radical species accumulate in the blood vessel endothelium, NO is scavenged by the uncoupled eNOS to produce peroxynitrite ($ONOO^-$) [8]. High levels of $ONOO^-$ create further oxidative stress, leading to a cascade of cardiac stress [8]. One induction of oxidative stress can cause this cascade to be initiated, leading to low bioavailability of NO in the blood vessels and high concentrations of superoxide radicals [10]. A method to mediate this oxidative stress cascade in cardiac arrest cases is to supplement NO by inhalation or medication [11,12].

Cardiac arrest has been identified as a significant cause of oxidative stress in the blood vessel endothelium [8,11,12]. Therefore, in cases where NO supplementation was used as a treatment after cardiac arrest, many of the eNOS pathways may have been uncoupled due to ischemia beginning the cardiac cascade [8,11,12]. Uncoupling of the eNOS is not isolated to cardiac arrest and is associated with atherosclerosis, diabetes mellitus, ischemia-reperfusion (I/R) injury, hypertension, chronic flow overload, cardiac hypertrophy with ventricular remodeling, and diastolic heart failure [8]. New therapies have emerged which introduce BH4 into the blood vessel endothelium to recouple the eNOS pathways and upregulate NO synthesis [7]. Other studies have also expressed issues with NO introduction because of the instability of the gas and its disruption of hemoglobin function [13]. If BH4 therapy recouples eNOS pathways in a time efficient manner, it could prove to be a more suitable and efficient therapy for patients following cardiac arrest that need NO supplementation. This researcher proposes a combined therapy for optimal results where BH4 is introduced to shift the eNOS pathways into the correct position, followed with NO. It is possible that the combined treatments will allow nitric oxide levels to be increased to proper levels for a longer period of time than seen in previous studies.

In this study, bovine aortic endothelial cells (BAECs) were cultured and then introduced to oxidative stress in order to uncouple the eNOS pathway. An assay of biopterin was conducted using High Performance Liquid Chromatography (HPLC) to determine if the oxidized (BH4) or reduced form (BH2) of biopterin was present, which indicated the coupled and uncoupled forms of the eNOS pathway, respectively. Nitric oxide donor drugs were given to the BAECs following oxidative stress, to see if BH4 levels increased in the cells favoring the coupled conformation of the eNOS pathway. A combined treatment of sapropterin dihydrochloride and nitric oxide donor drugs were also given to the BAECs to determine if this is more effective in restoring the oxidized form of biopterin in the cell samples. This researcher hypothesized that BAECs given the combined treatment would produce higher levels of BH4 than the nitric oxide donor drugs alone.

Methods

Cell Culture

Bovine aortic endothelial cells were obtained through the Coriell Institute and incubated overnight as directed by the provider before being transferred to a T-75 culture flask using aseptic technique. BAECs were grown in Eagle's Minimum Essential Media (EMEM) that was supplemented with 10% fetal bovine serum (FBS) until early confluency. At 80% confluency, cells were sub-cultured using Trypsin/EDTA as the dissociation solution. The cells were spun down at 180 x g for 5 minutes and the supernatant was removed without disturbing the cell pellet. The cell pellet was resuspended in 2 to 8 mL of growth medium by pipetting gently up and down. Before further processing, 100 μL of cell suspension was removed for counting on the hemocytometer. Cells were then seeded at a density of 2,500 to 5,000 cells per cubic centimeter. Flasks were placed in an incubator set at 37 degrees Celsius and 5% CO_2 for at least 24 hrs

before the cells were further processed. Growth media was changed every 48 hrs between subculturing or when the media showed a significant pH change. All culturing was performed using aseptic technique in a laminar flow hood with a HEPA filter. Human embryonic kidney (HEK293) cells were used as a control cell type and were sourced from frozen stocks provided by Olivet Nazarene University's Biology Department. The HEK293 cells were cultured using the same protocol as described for the bovine aortic endothelial cells.

Induction of Oxidative Stress

Each cell type was plated on a 12-well plate and cytotoxicity treatments were done in triplicate using a serial dilution of hydrogen peroxide (H_2O_2) concentrations (200 μM, 100 μM, 50 μM and 25 μM). Results from the cytotoxicity tests were used to determine the concentration of H_2O_2 to be used for oxidative stress induction. Cells were seeded on 12-well plates and were treated at early confluence with 100 μL of 100 μM of H_2O_2 dissolved in 1X D-PBS to induce oxidative stress. Cells were incubated with the treatment for 24 hours before the media was removed by aspiration and replaced with serum-free media or treatment media.

NO and BH4 supplementation

BAECs and HEK293 cells were treated in triplicate with several concentrations of diethylamine NONOate diethylammonium salt, a slow releasing NO donor drug. This was done using a serial dilution method to give final concentrations of 25, 50, 100 and 200 μM. Cell viability counts, a nitrite assay, and HPLC analysis were completed to determine the approximate LD_{50} of the NO donor drug. The optimum concentration was determined to be at or below 100 μM of diethylamine NONOate diethylammonium salt. For the combined treatments, lower concentrations of NO donor drug were used to offset the additional treatment given to the cells. Sapropterin dihydrochloride, a precursor of BH4, was used as the BH4 donor drug in the

combined treatment. Low concentrations of this drug were used due to lack of information about approximate cellular concentrations and toxicity found in the literature. Concentrations of 2.5 and 1.25 μM of BH4 donor drug were given to the cells in triplicate in combination with 25 and 12.5 μM of nitric oxide donor drug, respectively. Cells were incubated with these treatments for 12 hours before being collected for HPLC analysis.

HPLC Quantification of BH4 and BH2

An isocratic method was used for quantification of BH2 and BH4 using the Hitachi Elite LaChrom HPLC. A four-point standard curve was created using (6R)-5,6,7,8-tetrahydrobiopterin dihydrochloride, a BH4 supplement drug, that had been incubated for an hour in the dark with an alkaline (0.02 M KI/I_2 in 0.1 M NaOH, for detection of BH2) or an acidic (0.02 M KI/I_2 in 0.1 M HCl, for detection of BH2 and BH4) solution. The oxidized alkaline or acidic samples were mixed with 10 μL 1M HCl or water (respectively) and centrifuged to retrieve supernatants. The supernatants were then mixed with 10 μL 0.2 M ascorbic acid and neutralized with 1 M phosphate buffer with pH 8. The final sample was filtered and 30 μL was injected into the HPLC and detection was run at $\lambda = 350$ nm and $\lambda = 450$ nm. This method was repeated using cell suspensions collected from the 12-well plates following oxidative stress and after nitric oxide and tetrahydrobiopterin supplementation. The alkaline area beneath the curve was subtracted from the acidic area for each treatment to determine the concentration of BH4 in each sample.

Results

In Figure 1, the standard curve made using known concentrations of BH4 is shown. This curve was used for later determination of BH4 concentration in the BAECs and HEK293 samples and for indication of the coupled and uncoupled conformation of the eNOS pathway in the cell samples before and after the induction of oxidative stress.

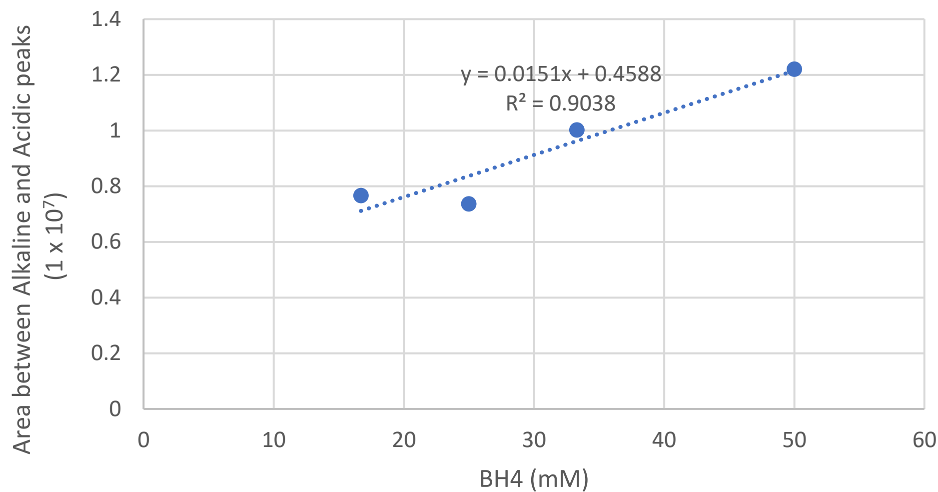

Figure 1. Standard curve of BH4 concentrations

Figure 2 shows the multichromatogram of a control BAEC sample run prior to the induction of oxidative stress. The sample was run incubated with the alkaline condition before being run on the HPLC. The alkaline area was subtracted from the acidic area to give the baseline concentration of BH4 in the BAECs. Several runs of control cell samples were compared with the standard curve multichromatograms to confirm that the double peak seen between two and three minutes of elution shows the elution of BH4 from the column. This double peak was used to calculate the area beneath the curve for the calculation of BH4 concentration. The solvent peak is shown at approximately one minute of elution.

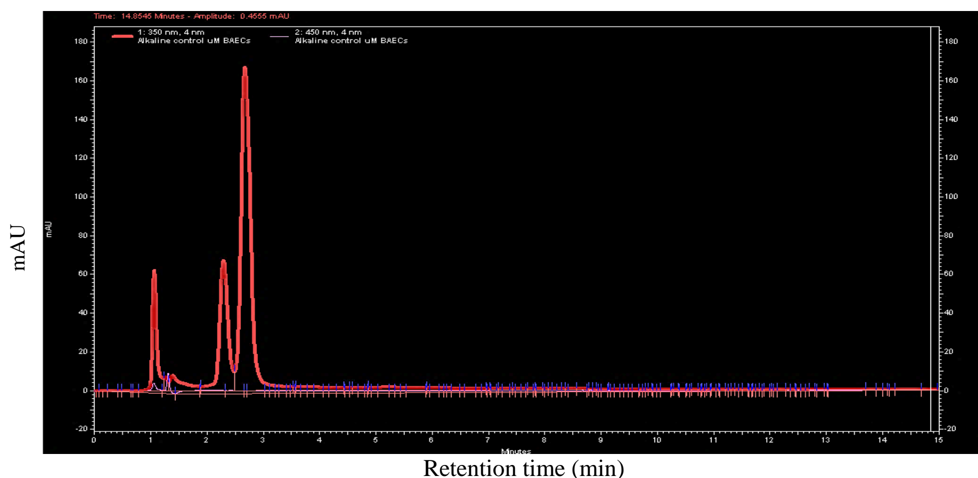

Figure 2. Multichromatogram of BAECs without oxidative stress run with alkaline conditions

Figure 3 shows the multichromatogram of a representative sample of BAECs that were treated with 100 μM H_2O_2 to induce oxidative stress. The BH4 concentration found in these samples was used to show that oxidative stress had converted the BH4 form of biopterin to the BH2 form in the cells.

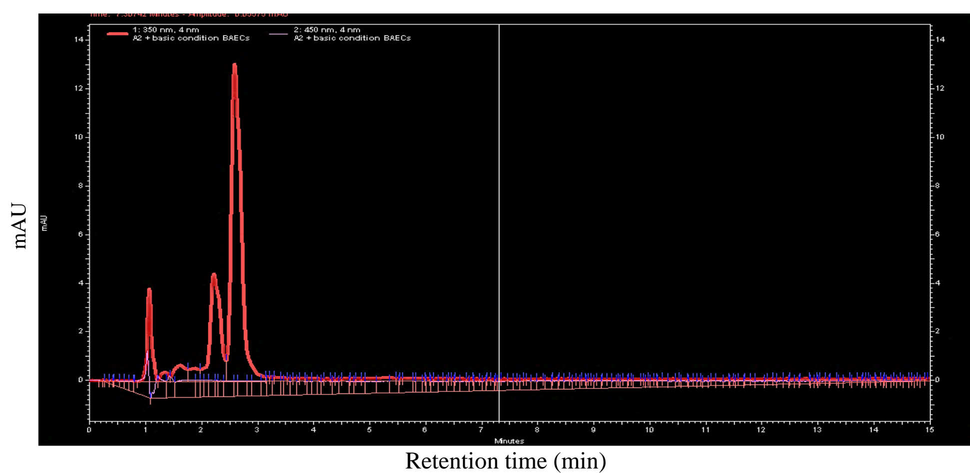

Figure 3. Multichromatogram of BAECs with 100 μM H_2O_2 run with the alkaline condition*
*Note that the range presented on vertical axis of each multichromatogram varies from sample to sample due to the figures being taken directly from the instrument.

In Figure 4, the BAECs given the hydrogen peroxide treatment and the 100 μM treatment

with nitric oxide donor drugs is shown under alkaline conditions. In this multichromatogram, the solvent peak (peak at approximately one minute of elution), is larger than the double peak. In Figures 2 and 3, the solvent peak was smaller than the BH4 peak by a considerable amount. Another variance in Figure 4 compared to the previous multichromatograms is that the first peak in the double peak is larger than the second, which is opposite in Figures 2 and 3.

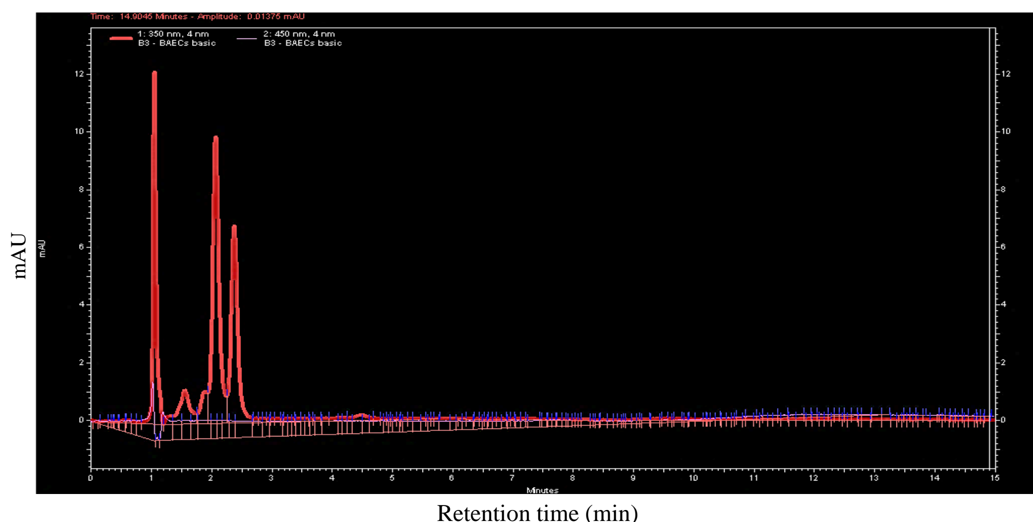

Figure 4. Multichromatogram of BAECs treated with 100 μM H_2O_2 and 100 μM NO donor drug under alkaline conditions.

In Figure 5, the cells given the combined treatment of 25 μM nitric oxide donor drug and 2.5 μM BH4 donor drug is shown under alkaline conditions. This multichromatogram shows a larger BH4 peak than seen in Figure 4, but the same inversion of the double peak in comparison to the control is still present.

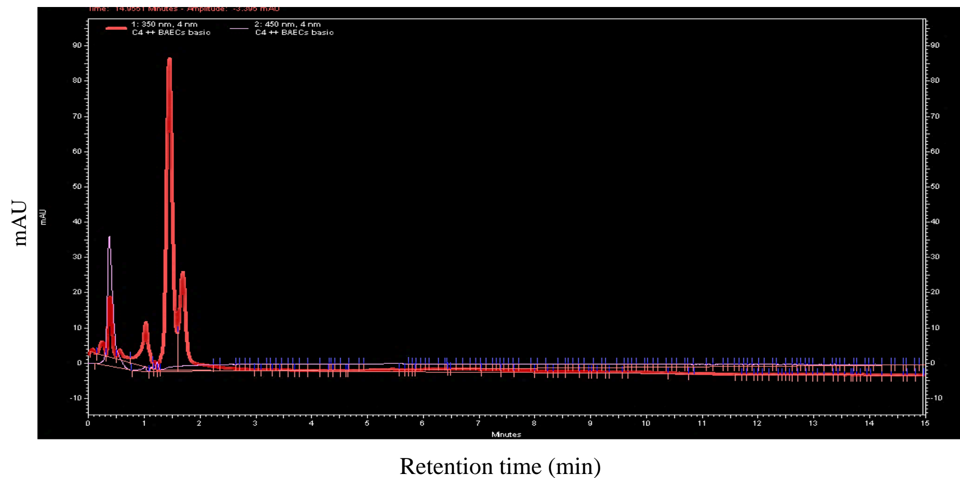

Figure 5. Multichromatogram of BAECs treated with 100 µM H_2O_2 and 25 µM NO donor drug and 2.5 µM BH4 donor drug under alkaline conditions.

In Figure 6, BH4 concentration is plotted for each treatment, conducted in triplicate and averaged. Standard deviations are also plotted for each of the treatments. The negative control received no treatment while the positive control underwent oxidative stress induced by 100 µM H_2O_2. The BH4 concentration of approximately 22 mM was considered the target concentration for BH4 restoration and was found as an average of three wells of untreated BAECs. A BH4 concentration of approximately 12 mM was considered to be uncoupled and was found as the average of three wells of BAECs cells following oxidative stress with 100 µM of H_2O_2.

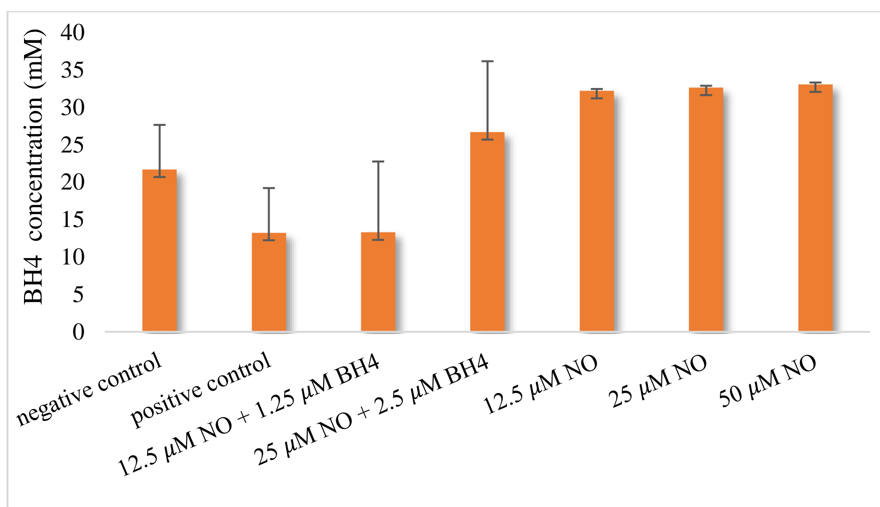

Figure 6. BH4 concentrations for various BAEC treatment conditions

Each concentration of nitric oxide donor drug alone resulted in a BH4 concentration of approximately 33 mM. The combined treatment of 12.5 µM nitric oxide and 1.25 µM BH4 donor drugs had a concentration of BH4 of approximately 12 mM. The higher concentration of combined treatment, 25 µM nitric oxide and 2.5 µM BH4 donor drugs, lead to a BH4 concentration of approximately 27 mM.

Discussion

The BAECs given the combined treatment of 25 µM nitric oxide and 2.5 µM BH4 donor drugs exhibited a BH4 concentration that was the closest to the untreated BAECs. However, the BAECs treated with only the nitric oxide donor drug showed the highest concentration of BH4 at a concentration of approximately 33 mM. The combined treatment of 12.5 µM nitric oxide and 1.25 µM BH4 was less effective than the higher concentration at increasing BH4 concentration. This treatment, when analyzed with the HPLC, indicated a concentration that was nearly the same as in the BAECs after undergoing oxidative stress. This suggests that there may be a threshold in which the sapropterin dihydrochloride becomes effective that is between a 2.5 µM to 1.25 µM. A greater range of concentrations should be tested to determine if this is the cause of the low concentration of BH4 in the BAECs given the combined treatment. Continued testing of a greater range of combined treatments would also help to determine if the sapropterin dihydrochloride works synergistically with the diethylamine NONOate diethylammonium salt to raise BH4 concentrations.

Previous studies have not defined a normal range of intracellular concentration for BH4, a circumstance that makes data interpretation difficult for this experiment. However, it has been suggested that a higher bioavailability of BH4 promotes angiogenesis and nitric oxide production and has not been found to have negative effects in high abundance [24]. This suggests that the

treatment of nitric oxide alone may be more effective than the combined treatment for the range of concentrations tested in this study. Another possibility is that while increased BH4 concentration is not harmful, it does reach a saturated level. At this point, it is no longer as able to be utilized by the cell. If this prediction is accurate, it may mean that the nitric oxide donor drug and the combined treatment of 25 μM nitric oxide and 2.5 μM BH4 donor drug are equally effective at restoring BH4 levels in the BAECs.

Another discrepancy in the data is seen when comparing the multichromatograms for the positive and negative control cell samples seen in Figures 2 and 3 and the cells given both treatments shown in Figures 4 and 5. The double peak seen between two and three minutes of elution changes in shape after the cells were given treatments. The first of the BH4 double peaks is larger in the samples analyzed after treatment. An explanation for this could be that the treatment elutes near the time expected for the BH4 to elute, causing the first peak to have a greater area than in the positive and negative control cells. Also, the double peak used to calculate BH4 concentration may not only include BH4. In order to calculate BH4 concentration, the area beneath the entire double peak was measured, and this could have led to considerable error in the calculations. The protocol for the acidic and alkaline preparations of the cell samples may also have led to error in the HPLC analysis. The protocol required many steps and reagents with short shelf-lives. These circumstances may have contributed to experimental error.

Because of the lack of clarity in these results, the hypothesis could not be supported. Additional studies should be conducted to assay other control points within the eNOS pathway to determine which treatment is more effective at restoring the coupled conformation of the pathway. Nitrite assays would be helpful in determining the effectiveness of each treatment.

Conclusion

The data collected in this experiment was not sufficient to support the effectiveness of either of the treatments in comparison to the other. The nitric oxide donor drug treatments yielded a concentration of 33 mM BH4 while the combined treatment of 25 μM nitric oxide and 2.5 μM BH4 donor drugs raised the BH4 concentration to approximately 27 mM. Each treatment type was sufficient at raising the concentration of BH4 in the cells to levels at or above those seen in the control BAECs. However, without data from additional assays for reactive oxygen species or angiogenesis rates, a conclusion cannot be made as to which treatment is more effective for recoupling the eNOS pathway.

Additionally, the data among the BAECs given the combined treatment was not consistent, suggesting that the optimal concentration of BH4 donor drug was likely not used in each of the treatments. Cytotoxicity studies should be done in greater depth before further experiments are conducted. The scope of this experiment also did not allow for the possible synergistic effects of the combined treatment to be evaluated. Modifications to the HPLC methodology in previous experiments may have led to inaccuracies in the integration of the area beneath each peak, leading to errors in the calculation of BH4 concentration. In future experiments, the method for the acidic and alkaline preparation of the cells and the HPLC protocol should be simplified to reduce potential error.

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