Betaine (N,N,N-trimethylglycine) averts photochemically-induced thrombosis in pial microvessels *in vivo* and platelet aggregation *in vitro*

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Abstract

Betaine (N,N,N-trimethylglycine) is an important food component with established health benefits through its homocysteine-lowering effects, and is used to lower total homocysteine concentration in plasma of patients with homocystinuria. It is well established that hyperhomocysteinemia is an established risk factor for cardiovascular disease and stroke. However, the possible protective effect of betaine on coagulation events *in vivo* and *in vitro* has thus far not been studied. Betaine was given to mice at oral doses of either 10 mg/kg ($n=6$) or 40 mg/kg ($n=6$) for seven consecutive days, and control mice ($n=6$) received water only. The thrombotic occlusion time in photochemically induced thrombosis in pial arterioles was significantly delayed in mice pretreated with betaine at doses of 10 mg/kg ($P < 0.001$) and 40 mg/kg ($P < 0.01$). Similar effects were observed in pial venules with 10 mg/kg ($P < 0.05$) and 40 mg/kg ($P < 0.05$) betaine. *In vitro*, in whole blood samples collected from untreated mice ($n=3-5$), betaine (0.01–1 mg/mL) significantly reversed platelet aggregation induced by adenosine diphosphate (5 μ M). The number of circulating platelets and plasma concentration of fibrinogen *in vivo* were not significantly affected by betaine pretreatment compared with the control group. Lipid peroxidation (LPO) in mice pretreated with betaine was significantly reduced compared with the control group. Moreover, betaine (0.01–1 mg/mL) caused a dose-dependent and significant prolongation of PT ($n=5$) and aPTT ($n=4-6$). In conclusion, our data show that betaine protected against coagulation events *in vivo* and *in vitro* and decreased LPO in plasma.

Keywords: Betaine, platelets, thrombosis, mice, lipid peroxidation

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Introduction

Betaine (N,N,N-trimethylglycine) is either found in the diet or generated in the liver and kidney from choline through the sequential action of choline oxidase and betaine-aldehyde dehydrogenase.¹ It serves as a methyl donor in a reaction converting homocysteine to methionine, catalyzed by the enzyme betaine-homocysteine methyltransferase.¹ Betaine is an important food component that possesses homocysteine-lowering effects.² Betaine is found in microorganisms, plants, and animals and is a significant component of many foods, including wheat, shellfish, spinach, and sugar beets.³ Intake of betaine shows no consistent relation to either cancer or cardiovascular risk or risk factors, whereas an unfavorable cardiovascular risk factor profile was associated with low betaine concentrations in plasma.⁴ Epidemiologic studies have shown that elevated

plasma levels of homocysteine are independently associated with cardiovascular diseases, including venous thromboembolism and atherosclerotic arterial diseases, such as myocardial infarction and stroke.⁵

Betaine has been reported to prevent carbon tetrachloride-induced nephrotoxicity of rats.⁶ It has also been demonstrated that betaine reduces serum uric acid levels and improves kidney function in hyperuricemic mice.⁷ However, as far as we are aware, no study has investigated the possible protective effects of betaine on photochemically-induced thrombosis *in vivo* and coagulation *in vitro*.

Therefore, the aim of this study was to investigate whether and to what extent betaine pretreatment given orally (10 and 40 mg/kg) for seven days can mitigate photochemically induced thrombosis in pial arterioles and venules in mice *in vivo*, platelet aggregation in whole blood

in vitro, and prothrombin time (PT) and activated partial thromboplastin time (aPTT) *in vitro*.

Materials and methods

Animals and treatments

This project was reviewed and approved by the Institutional Review Board of the United Arab Emirates University, College of Medicine and Health Sciences, and experiments were performed in accordance with protocols approved by the Institutional Animal Care and Research Advisory Committee.

Male C57BL/6 mice (18–23 g, Jackson Laboratory, Bar Harbor, ME, USA) were housed in light (12-h light: 12-h dark cycle) and temperature-controlled ($22 \pm 1^\circ\text{C}$) rooms. They had free access to commercial laboratory chow and were provided tap water *ad libitum*.

Either betaine (Sigma-Aldrich Co., St Louis, MO, USA) 10 mg/kg ($n=6$) or 40 mg/kg ($n=6$) suspended in 200 μL of water, or 200 μL of water alone (control, $n=6$) was administered by oral gavage to mice for seven consecutive days, and various parameters were evaluated at the end of the treatment period.

Experimental pial arteriolar and venular thrombosis model

In vivo photochemically-induced thrombosis in mouse pial arterioles and venules was assessed at the end of the pre-treatment period with betaine according to a previously described technique.^{8–11} Briefly, the trachea was intubated after induction of anesthesia with urethane (1 mg/g body weight, i.p.), and a 2F venous catheter (Portex, Hythe, UK) was inserted in the right jugular vein for the administration of fluorescein (Sigma, St. Louis, MO, USA). After that, a craniotomy was first performed on the left side, using a microdrill, and the dura was stripped open. Only untraumatized preparations were used, and those showing trauma to either microvessels or underlying brain tissue were discarded. The animals were then placed on the stage of a fluorescence microscope (Olympus, Melville, NY, USA) attached to a camera and DVD recorder. A heating mat was placed under the mice and the body temperature was raised to 37°C , as monitored by a rectal thermoprobe connected to a temperature reader (Physitemp Instruments, NJ, USA). The cranial preparation was moistened continuously with artificial cerebrospinal fluid of the following composition (mM): NaCl 124, KCl 5, NaH_2PO_4 3, CaCl_2 2.5, MgSO_4 4, NaHCO_3 23 and glucose 10, pH 7.3–7.4. A field containing either venules or arterioles of 15–20 μm in diameter was chosen. Such a field was taped prior to and during the photochemical insult, which was carried out by injecting fluorescein (0.1 mL/mouse of 5% solution) via the jugular vein, which was allowed to circulate for 30–40 s. The cranial preparation was then exposed to stabilized mercury light. The combination produces endothelium injury of the arterioles and venules. This in turn causes platelets to adhere at the site of endothelial damage and then aggregate. Platelet aggregates and thrombus formation grow in size until complete vascular

occlusion. The time from the photochemical injury until full vascular occlusion (time to flow stop) in arterioles and venules was measured in seconds. At the end of the experiments, the animals were euthanized by an overdose of urethane.

Blood collection

Blood was collected from separate animals ($n=6$ in each group). Mice were anesthetized intraperitoneally with sodium pentobarbital (45 mg/kg), and then blood was drawn from the inferior vena cava in EDTA (4%). A sample was used for platelet counts using an ABX VET ABC HEMATOLOGY ANALYZER with a mouse card (ABX Diagnostics, Montpellier, France). The remaining blood was centrifuged at 4°C for 15 min at 900g and the plasma samples were stored at -80°C until further analysis for fibrinogen using commercially available ELISA kit (Molecular Innovation, Southfield, MI, USA) and NADPH-dependent membrane lipid peroxidation (LPO) using a kit that measures thiobarbituric acid reactive substances (Cayman Chemical Company, Ann Arbor, MI, USA).

Platelet aggregation in mouse whole blood

The platelet aggregation assay in whole blood was performed with slight modification as described earlier.^{11,12} After anesthesia, blood from separate animals was withdrawn from the inferior vena cava and placed in citrate (3.8%), and 100- μL aliquots were added to the well of a Merlin coagulometer MC 1 VET (Merlin, Lemgo, Germany). The blood samples were incubated *in vitro* at 37°C with various concentrations of betaine [0.01 ($n=4$), 0.1 ($n=3$) and 1 ($n=3$) mg/mL] in saline (0.9%) or saline alone ($n=5$) for 3 min and then adenosine diphosphate (5 μM) was added and incubated for 3 min. Each sample was then stirred for another 3 min. At the end of this period, 25- μL samples were removed and fixed on ice in 225 mL cellFix (Becton Dickinson, Franklin Lakes, NJ, USA). After fixation, single platelets were counted in a VET ABX Micros with mouse card (ABX, Montpellier, France). Platelet aggregation was quantified by measuring the fall in single platelets counted due to aggregation induced by 5 μM ADP. The degree of platelet aggregation was expressed as a percentage of that obtained in untreated (without ADP but with saline) whole blood obtained from mice not administered with betaine.

In vitro measurement of PT and aPTT in plasma *in vitro*

The PT was measured^{13–15} on freshly collected, platelet-poor plasma with human relipidated recombinant thromboplastin (Recombiplastin; Instrumentation Laboratory, Orangeburg, NY, USA) in combination with a Merlin coagulometer [MC 1 VET (Merlin, Lemgo, Germany)]. Briefly, the plasma was incubated for 3 min at 37°C , and then various concentrations of betaine [0.01 ($n=5$), 0.1 ($n=5$) and 1 ($n=5$) mg/mL] in saline (0.9%) or saline alone (control, $n=5$) were added for another 3 min and then PT was measured as previously

described.^{13–15} aPTT was measured^{13–15} with automated aPTT reagent [bioMerieux (Durham, NC, USA) using a Merlin coagulometer the MC 1 VET (Merlin, Lemgo, Germany)]. Briefly, the plasma was incubated for 3 min at 37°C, and then various concentrations of betaine [0.01 ($n=4$), 0.1 ($n=4$) and 1 ($n=4$) mg/mL] in saline (0.9%) or saline alone (control, $n=6$) were added for another 3 min and then aPTT was assessed as previously described.^{13–15} Normal plasma used as reference for both the PT and aPTT was prepared by pooling equal portions of platelet-poor plasmas from the blood of six untreated mice.

Statistics

All statistical analyses were performed with GraphPad Prism Software version 5. To determine whether parameters were normally distributed, the Kolmogorov–Smirnov statistic normality test was applied. Normally distributed data were analyzed using one-way analysis of variance (ANOVA) followed by Bonferroni multiple-range tests, whereas non-normally distributed ones (thrombosis in pial venules) were analyzed using Kruskal–Wallis test followed by Dunn’s multiple comparison test. P values less than 0.05 were considered significant. All the data in figures were reported as mean $\pm$ SEM.

Results

Effect of betaine on photochemically-induced thrombosis in pial arterioles and venules of mouse *in vivo*

Figure 1 illustrates the effect of betaine given orally for seven days at two doses (10 and 40 mg/kg) on photochemically-induced thrombosis in mouse pial microvessels *in vivo*. Compared with the control group, betaine pretreatment caused a marked delay in the thrombotic occlusion time in pial arterioles. The effect was statistically significant at 10 mg/kg ($P < 0.001$) and 40 mg/kg ($P < 0.01$) compared with the control group (Figure 1a). Likewise, the administration of betaine induced a significant prolongation of thrombotic occlusion time in pial venules at 10 mg/kg ($P < 0.05$) and 40 mg/kg ($P < 0.05$) compared with the control group (Figure 2b).

Effect of betaine on platelet aggregation in whole blood *in vitro*

Figure 2 exemplifies the effect of betaine on platelet aggregation in whole blood. Whole blood obtained from untreated mice and exposed to ADP caused a significant decrease in counted single platelets compared with the control group, indicating the occurrence of platelet aggregation. The pretreatment of whole blood *in vitro* with betaine at various concentrations caused a significant reversal of the proaggregatory effect of ADP. The effect was statistically significant at 0.01 mg/mL ($P < 0.01$), 0.1 mg/mL ($P < 0.001$) and 1 mg/mL ($P < 0.001$) compared with the ADP group. Interestingly, no statistical difference was observed between the control group and the highest concentration of betaine (1 mg/mL), indicating that betaine has robustly reversed platelet aggregation *in vitro*.

Figure 1 Thrombotic occlusion time in pial arterioles (a) and venules (b) in mice either orally administered betaine (10 and 40 mg/kg) in water or water-only (control). Data are mean $\pm$ SEM ($n=6$). * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$ compared with the corresponding control group

Figure 2 *In vitro* platelet aggregation in whole blood obtained from untreated mice. The blood samples were incubated at 37°C with adenosine diphosphate (ADP, 5 μ M) for 3 min, and then stirred for another 3 min. Platelet aggregation was quantified by measuring the fall in single platelets counted due to aggregation induced by 5 μ M ADP. The degree of platelet aggregation was expressed as the percentage of that obtained in untreated (without ADP but with saline) whole blood obtained from untreated mice. Data are mean $\pm$ SEM ($n=3-5$). Δ $P < 0.01$ and ΔΔ $P < 0.001$ compared with the corresponding ADP group. * $P < 0.001$ compared with the corresponding control group

Effect of betaine on the number of circulating platelets and plasma concentrations of LPO and fibrinogen

Figure 3 illustrates the effects of betaine on the number of circulating platelets and plasma concentration of LPO and

Figure 3 Circulating platelet numbers (a) and plasma concentrations of lipid peroxidation (LPO) (b) and fibrinogen in plasma (c) in mice either orally administered betaine (10 and 40 mg/kg) in water or water-only (control). Data are mean $\pm$ SEM ($n = 6$). * $P < 0.05$ and ** $P < 0.01$ compared with the corresponding control group

fibrinogen. The number of circulating platelets was not affected by betaine pretreatment (Figure 3a). Similarly, the numbers of erythrocytes and leukocytes were not affected by betaine administration (not shown). The plasma concentration of LPO, a marker of cell membrane lipid peroxidation *in vivo*, was significantly decreased by betaine pretreatment at 10 mg/kg ($P < 0.05$) and 40 mg/kg ($P < 0.01$) (Figure 3b). The fibrinogen concentration in plasma of betaine-treated mice did not significantly differ from that of control group (Figure 3c).

Effect of betaine on PT and aPTT *in vitro*

Figure 4 exemplifies the effect of betaine on PT and aPTT *in vitro*. Compared with control group, betaine

Figure 4 Prothrombin time (PT; a) and activated partial thromboplastin time (aPTT; b) *in vitro*. Data are mean $\pm$ SEM ($n = 4-6$). * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$ compared with the corresponding control group

induced a significant and dose-dependent prolongation of PT and aPTT.

Discussion

In this work, we showed that betaine pretreatment exerts a protective effect on photochemically-induced thrombosis in pial arterioles and venules *in vivo* and platelet aggregation in whole blood *in vitro*. Betaine caused a dose-dependent and significant prolongation of PT and aPTT *in vitro*. We also observed that betaine pretreatment decreased LPO in plasma.

Here, we assessed the effect of seven days pretreatment with two doses of betaine on coagulation *in vivo* and *in vitro*. The duration of pretreatment and the doses administered in the current study were selected from a previous publication which showed the effectiveness of betaine in reducing serum uric acid levels and improving kidney function in hyperuricemic mice.⁷

Hyperhomocysteinemia is an established risk factor for myocardial infarction, stroke and thrombosis.⁵ The experimental role of hyperhomocysteinemia in thrombosis has been recently investigated in mice.¹⁶ In spite of severe metabolic disturbance, hyperhomocysteinemic mice did not display any propensity toward accelerated thrombosis.¹⁶

The goal of the present study was to assess the possible protective effect of betaine on thrombosis *in vivo* and *in vitro*, instead of studying the relationship between hyperhomocysteinemia and thrombosis. Such an effect has not been, as far as we are aware, investigated before.

In our model, platelet aggregation in pial microvessels was induced photochemically, by activation of circulating sodium fluorescein with mercury light.^{8–11} Platelet aggregates and thrombus formation grow in size until complete vascular occlusion.^{8–11} Our data show that betaine pretreatment significantly delayed the thrombotic occlusion time in pial microvessels, indicating that betaine exerts an antithrombotic effects. To verify whether and to what extent betaine exhibits similar effect *in vitro*, blood from untreated mice was pretreated with various concentrations of betaine, and then exposed to ADP. Our data show that betaine pretreatment induced a significant reversal of the proaggregatory effect of ADP. This *in vitro* finding confirms the *in vivo* antithrombotic effect of betaine that we observed using photochemically-induced thrombosis in pial microvessels. Neither the number of circulating platelets nor the concentration of fibrinogen was affected by betaine pretreatment. Likewise, the erythrocyte and leukocyte numbers did not change in betaine-treated mice compared with the control group (not shown).

Formation of free radicals and reactive oxygen species is a normal consequence of a variety of biochemical reactions. These free radicals can cause oxidative damage to the tissues through lipid peroxidation.¹⁷ Our data show that the plasma concentrations of LPO in mice administered with betaine were significantly lower compared with the control group. This effect could be ascribed to the lipotropic effect of betaine and its contribution to the transmethylation pathway, the formation of S-adenosylmethionine and phospholipid synthesis.¹⁸ Betaine has been reported to normalize the activities of superoxide dismutase and glutathione peroxidase and to protect against nephrotoxicity induced by CCl₄ in rats.⁶

We also found that betaine causes a significant and dose-dependent prolongation of PT and aPTT *in vitro*. PT measures the formation of the fibrin clot through the activity of the extrinsic and common coagulation pathways, which involve the interaction of tissue factor and activated factor VII, in addition to factor X, factor V, prothrombin, and fibrinogen. aPTT, in contrast to PT, measures the activity of the intrinsic and common pathways of coagulation. The significant prolongation of PT and aPTT reflects the anticoagulatory effect of betaine.

In conclusion, our data show that betaine protects against coagulation events *in vivo* and *in vitro*. We also established that betaine pretreatment decreased LPO in plasma. Further detailed experiments are warranted to uncover the mechanisms behind our present findings. Additional confirmatory studies assessing the protective effects of betaine on thrombotic complications induced by pollutants such as diesel exhaust particles and tobacco smoke will be conducted.

Authors' contributions: All authors have read and approved the manuscript. AN designed, planned, supervised all the experiments, and wrote the article. PY and SB performed the experiments. BHA contributed to the design of the study and wrote the article.

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