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# Free Radical Scavenging Activity of Benfotiamine in Nitrite-induced Hemoglobin Oxidation and Membrane Fragility Models

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**ABSTRACT**: Free radical formation in heme proteins is recognized as a factor in mediating the toxicity of many chemicals. The present study was designed to evaluate the dose-response relationship of the free radical scavenging properties of benfotiamine in nitrite-induced Hb oxidation. Different concentrations of benfotiamine were added at different time intervals of Hb oxidation in erythrocytes lysate, and formation of methemoglobin (MetHb) was monitored spectrophotometrically; the same approach was utilized to evaluate the effect of benfotiamine on the integrity of erythrocytes after induction of hemolysis with sodium nitrite. Moreover, the most effective dose of tested compound was administered in rats before challenge with toxic dose of sodium nitrite. The results showed that in both *in vitro* and *in vivo* models, benfotiamine successfully attenuates Hb oxidation after challenge with sodium nitrite; this protective effect was found to be not related to the catalytic stage of Hb oxidation, though such effect was reported to be more prominent when the compound was administered before nitrite. In conclusion, benfotiamine can effectively, in concentration-dependent pattern, attenuate sodium nitrite-induced Hb oxidation and maintain integrity of red blood cells both *in vitro* and in *vivo*.

**Key words**: benfotiamine, hemoglobin oxidation, membrane fragility, radical scavenging

## INTRODUCTION

Reactive oxygen species (ROS) including free radicals such as superoxide anion radicals ($O_2^{-}$), hydroxyl radicals (HO·), hydrogen peroxide ($H_2O_2$) and peroxyl radicals (RCOO·) are active oxygen components that are often generated during aerobic conditions [1]. ROS are usually highly reactive and short-lived, known to cause damage to cellular components including lipid, DNA, protein, carbohydrate, and other biological molecules. They consequently lead to many pathological processes such as cancer, cardiovascular diseases, diabetes, inflammation and neurodegenerative diseases [2,3,4,5]. In recent years, antioxidants have been subjected to many epidemiological studies that have related their consumption to a reduction in the incidence of oxidative damage related diseases. Therefore, much attention has been focused on the use of antioxidants (specially natural antioxidants) for improvement of human health [7,8]. Benfotiamine is a lipid-soluble thiamine precursor having much higher bioavailability than genuine thiamine [9,10]. Growing body of evidence revealed that benfotiamine alleviates the severity of diabetic complications such as neuropathy, nephropathy and retinopathy by inhibiting the formation of advanced glycation end products (AGEs) [11,12]. Moreover, benfotiamine has been shown to reduce oxidative stress in a mechanism unrelated to its anti-AGE property [10]. It also has direct antioxidant activity through prevention of experimentally-induced DNA damage *in vitro* [13]. However, as far as we know there are no reports on the estimation for the radical scavenging activity of benfotiamine in other experimental models like red blood cell (RBC) and also its protective effect on their plasma membrane *in vitro*. Therefore, the present study evaluates the radical scavenging activity and protection of plasma membrane integrity of benfotiamine using sodium nitrite-induced hemoglobin oxidation and membrane fragility models.

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## MATERIALS AND METHODS

### Chemicals

Benfotiamine (Polpharma, Poland), Sodium nitrite (Analar BDH, Ltd., Poole, England). All other chemicals were purchased from Sigma–Aldrich.

### In vitro study

#### *Blood sample collection and preparation of lysate*

Blood samples were obtained from healthy individuals by vein-puncture, and kept in Ethylene diamine tetraacetic acid (EDTA) containing tubes; then centrifuged at 2500 rpm and 4C$^{o}$ for 10 minutes to remove plasma and the buffy coat of white cells. The erythrocytes were washed thrice with Phosphate Buffer Saline (PBS, pH 7.4) and lased by suspending in 20 volumes of 20mM Phosphate Buffer (PB, pH 7.4) to yield the required hemolysate concentration of 1:20 [14].

#### *Preparation of benfotiamine solution*

Different concentrations of benfotiamine were prepared by dissolving the required quantity in phosphate buffer (pH 7.4) to prepare stock solution (250µM), from which serial dilutions were made to give concentrations of 200µM, 100µM, 50µM and 25µM.

#### *Effect of different benfotiamine concentrations on the time course of nitrite-induced oxidation of hemoglobin*

*In vitro* model for oxidation of hemoglobin with sodium nitrite was utilized for production of methemoglobin (MetHb). To 1.5 ml freshly prepared hemolysate, 1.0 ml of different concentrations of benfotiamine (200µM, 100µM, 50µM and 25µM) each time were added concomitantly with 0.1 ml sodium nitrite (final concentration 6.0 mM), and the formation of MetHb was monitored spectrophotometrically at 631 nm for 50 minutes using computerized UV-visible spectrophotometer. Then to 1.5 ml freshly prepared hemolysate, 1.0 ml of the highly effective concentration of benfotiamine was added either 10 minutes before, or at 10 and 20 minutes after the addition of sodium nitrite to the hemolysate solution, and the formation of MetHb was monitored as previously mentioned.

#### *Effect of benfotiamine on the nitrite-induced osmotic fragility of red blood cells*

Erythrocytes suspension was prepared by mixing a volume of fresh blood with 20 volumes of phosphate buffered saline (PBS, pH 7.4); Aliquots (0.2 ml) of erythrocyte suspension (2.5% hematocrit) were added to 1.8 ml of buffered saline solutions of decreasing concentrations, pH 7.4, (NaCl concentration range of 9.0–1.0 g/L). Different concentrations of benfotiamine (200µM, 100µM, 50µM and 25µM) and 0.1 ml sodium nitrite (final concentration 6.0 mM) were incubated with the suspensions. The suspensions were allowed to stand for 30 minutes at room temperature, mixed again and then centrifuged for 5 minutes at 1200 rpm. The supernatants were obtained and the level of lysis was determined spectrophotometrically at 540 nm. The percentage of hemolysis was calculated from the ratios of the absorbance [15].

### In vivo study

#### *Experimental animals and treatment schedule*

Seven to eight-weeks old female Wistar Albino rats, weighing 160-280g were purchased from the animal house of the College of Pharmacy/ Hawler Medical University. The animals were housed in the animal house of the College of Pharmacy/University of Sulaimani in well ventilated plastic cages at 24±2C$^{o}$ and 50±10 relative humidity, and subjected to 12hr light/12hr dark cycle. They were acclimatized for 1 week before starting the experiments, during which they had free access to standard commercial diet purchased from (Iraqi Center for Cancer Research and Medical Genetics, Baghdad) and tap water *ad libitum*. After acclimatization of animals with the environment of the animal house, they were randomly allocated into 3 groups, each with six rats and treated as follows: first group, positive control group; in which 1.0 ml of normal saline (0.9% sodium chloride) was given orally; 1 hour later, sodium nitrite (100 mg/kg) was given orally, after 45 minutes the animals were sacrificed by inhalation of high dose of anesthetic diethyl ether, and intra-cardiac blood samples were taken for measurement of MetHb level. Second and third groups: Long- and short-term benfotiamine exposure; in the former, benfotiamine (70 mg/kg) once daily was given orally by gavage tube for 7 days; on the seventh day sodium nitrite (100 mg/kg) was given orally, 45 minutes later the animals were sacrificed by inhalation of high dose of anesthetic diethyl ether, and intra-cardiac blood samples were taken for measurement of MetHb level. In the third group, benfotiamine (70 mg/kg) was given as a single oral dose; 1 hour later, sodium nitrite (100 mg/kg) was given orally, after 45 minutes the animals were sacrificed by inhalation of high dose of anesthetic diethyl ether, and intra-cardiac blood samples were taken for measurement of MetHb level [15].

#### *Statistical Analysis*

Data are expressed as the means ± SD. All the data were analyzed using Student's test and Microsoft Excel software. $P<0.05$ was considered statistically significant.

## RASULTS

### *Effect of different concentrations of benfotiamine on the time-course of nitrite-induced oxidation of hemoglobin*

In the presence of different concentrations of benfotiamine (25, 50, 100 and 200 µM) the time-course of oxidation of hemoglobin shows a slow increase in light absorbance related to reduced rate of Hb oxidation and inhibition of methemoglobin formation. The linear relationship was reported between benfotiamine concentrations and inhibition of MetHb formation (62%, 66%, 71%, and 75% respectively, Figure 1, Table 1), indicating a delay in the oxidation process in a concentration-dependent manner. The time required to convert 50% of the available hemoglobin in the erythrocyte lysate to methemoglobin was 25min in the absence of benfotiamine (control), whereas it was delayed to 66, 76, 88 and 100 min in the presence of 25, 50, 100 and 200 µM of benfotiamine respectively (Table 3-3). Addition of the highly effective concentration of benfotiamine (200 µM) to the hemolysate mixture at different time intervals (10 min before nitrite, 10 min after and 20 min after nitrite addition i.e. during autocatalytic phase) decreases absorbance of light attributed to methemoglobin formation, which is an index for protection of Hb oxidation due to the addition of sodium nitrite to the lysate (79.4%, 78% and 75% respectively, Figure 2, Table 2). The time required to convert 50% of the available hemoglobin to methemoglobin was 25 min in the absence of benfotiamine (control), whereas it was delayed to 122, 114 and 101 min when benfotiamine (200 µM) was added 10 min before nitrite, 10 min after and 20 min after nitrite addition respectively as shown in table 2.

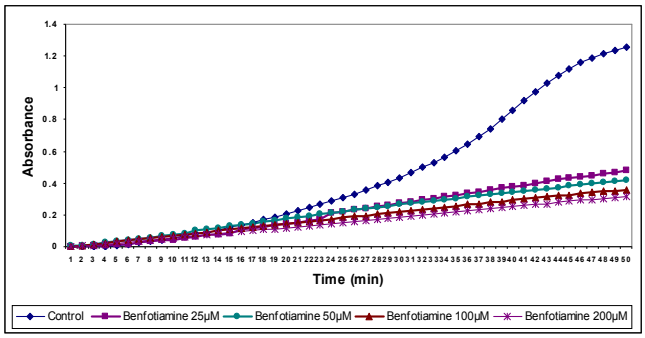


Fig. 1. Effect of different concentrations of benfotiamine (25, 50, 100, 200 µM) on the time-course of nitrite-induced oxidation of hemoglobin and methemoglobin formation in erythrocyte lysate

Table 1. Effect of different concentrations of benfotiamine (25, 50, 100, 200 µM) on the time required for oxidation of hemoglobin and formation of 50% methemoglobin with sodium nitrite in erythrocyte lysate

| Benfotiamine Concentrations | % formation of MetHb | % inhibition of MetHb | Time to form 50% MetHb ($t_{½}$)(min) |
|---|---|---|---|
| **Control** | 100 | 0 | 25 |
| **Benfotiamine 25µM** | 38 | 62 | 66 |
| **Benfotiamine 50µM** | 34 | 66 | 76 |
| **Benfotiamine 100µM** | 29 | 71 | 88 |
| **Benfotiamine 200µM** | 25 | 75 | 100 |

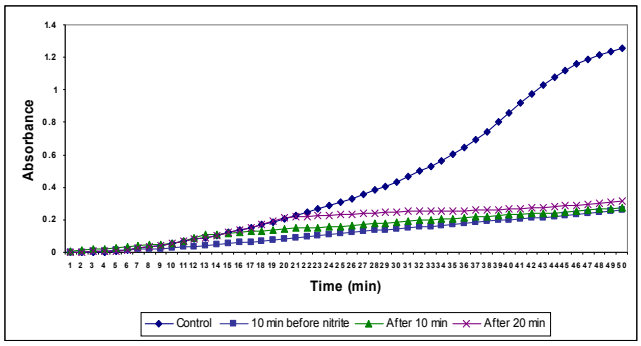


Fig 2. Effect of addition of benfotiamine (200 µM) at different time intervals (10 min before, 10min after, 20min after nitrite addition) on the oxidation of hemoglobin and formation of methemoglobin in erythrocyte lysate.

Table 2. Effect of addition of benfotiamine 200 µM at different time intervals (10 min before, 10min after, 20min after nitrite addition) on the oxidation of hemoglobin and formation of methemoglobin in erythrocyte lysate.

| Time-course of addition of 200µM benfotiamine | % formation of MetHb | % Inhibition of MetHb | Time to form 50% MetHb($t_{½}$)(min) |
|---|---|---|---|
| **Control** | 100 | 0 | 25 |
| **Incubation before 10 min** | 20.6 | 79.4 | 121.5 |
| **Addition after 10 min** | 22 | 78 | 114 |
| **Addition after 20 min** | 24.9 | 75.1 | 101 |

Values represent mean of 3 experiments.

### *Effect of different concentrations of benfotiamine on osmotic fragility of red blood cells*

The resistance of erythrocytes to lysis by diluted buffer saline solutions of decreasing concentrations (NaCl range of 9.0-1.0 g/l) was assayed. As shown in figure 3, 50% RBC hemolysis occurred with 0.49% NaCl buffer saline solution when RBCs were treated with sodium nitrite alone; whereas a 0.485 %, 0.475%, 0.465% and 0.425% buffered saline solutions were needed to cause 50% lysis of RBCs when solutions of different concentrations of benfotiamine (25, 50, 100 and 200 µM respectively) were added to the incubation mixture in addition to sodium nitrite. The figure shows the difference in susceptibility of human RBCs to osmotic lysis when subjected to increasing hypotonicity with and without

addition of benfotiamine, which slightly shifts the curve toward that of control RBCs which are not challenged by sodium nitrite.

### *Effect of benfotiamine on nitrite-induced MetHb formation in rats*

Pre-treatment of animals with benfotiamine (70mg/kg), both in long-term approach (single daily dose for 7 days before administration of sodium nitrite) and short-term approach (single dose 1 hour before administration of sodium nitrite) decreases the level of MetHb formation (40.1% and 35.6% respectively) compared to saline treated only group ($P$<0.05, table 3).

Table 3. Effects of single and multiple doses of Benfotiamine (70mg/kg) on nitrite-induced MetHb formation in rats.

| Type of treatment | n | % MetHb formation |
|---|---|---|
| | | Benfotiamine (70mg/kg) |
| Saline treated only | 6 | 55.6 ± 6.0 |
| Pre-treatment for 7 days | 6 | 33.3 ± 11.1*[a] |
| Treatment for one hour | 6 | 35.8 ± 16.4 *[a] |

Each value represents mean ± SD; n= number of animals; * significantly different compared to saline treated group ($P$<0.05); values with non-identical superscripts (a,b) were considered significantly different within the same group ($P$<0.05)

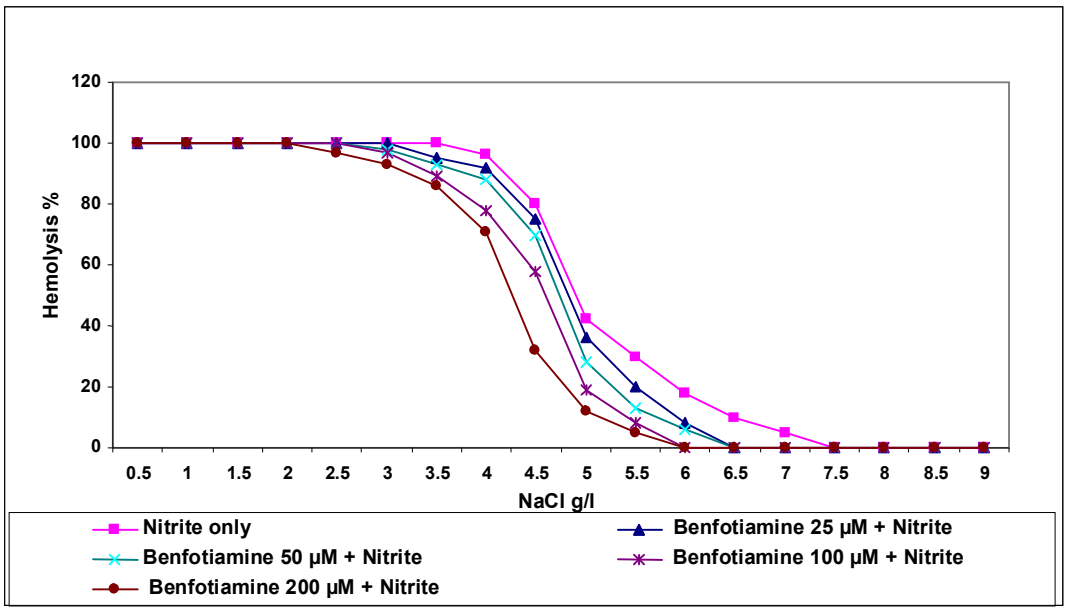


Fig 3. Effects of different concentrations of benfotiamine on the osmotic fragility of red blood cell challenged with sodium nitrite *in vitro*.

## DISCUSSION

This study for the first time shows that benfotiamine, a vitamin B1 analogue, protects hemoglobin and the plasma membrane of the erythrocytes against oxidation induced by addition of sodium nitrite in a concentration dependent pattern. Benfotiamine as well as thiamin penetrates human red cell membrane slowly, while S-benzolythiamine (SBT) penetrates RBC membrane very rapidly; thus SBT was suspected to be an actual form in the absorption of benfotiamine from intestine after dephosphorylation by alkaline phosphatase at the intestinal mucosa. The penetration of SBT into red cells proceeds through two steps, the first one includes passive diffusion of SBT through the cell membrane, and the second depends on rapid decomposition of SBT to thiamin and the resultant accumulation in the cell [16]. Whatever the metabolic fate and degradation product of benfotiamine, the reported results of the present study showed concentration-dependent protection of RBCs against the oxidative effect of sodium nitrite; a mechanism that takes place at the cellular level and might be associated with the reduction in oxidative stress; this seems to be consistent with the reported protective effect of benfotiamine on myocardial dysfunction [17]. Additionally, a recent study has shown that at high concentration (300µM) of benfotiamine exerts a direct antioxidant effect independent of its transformation into thiamin and increased transketolase activity [13]. The results of the present study might be partly explained by direct antioxidant activity of benfotiamine and partial dephosphorylation to S-benzoyl thiamin. Recently, a study on erythrocyte fragility in type II diabetic patients demonstrated that osmotic fragility of erythrocytes increases with the increase in the level of glycosylated hemoglobin in those diabetic patients [18], which makes benfotiamine a proper

candidate for erythrocyte protection and might be important finding that open vista of further investigations to add another novel therapeutic potentials of benfotiamine in prevention and/or treatment of diabetic complications associated with glycosylated Hb, AGEs and generation of ROS. Moreover, lipid peroxidation involves the metal-dependent reaction associated with various oxygen radical generations [19], and the metal chelating activity of TDP inhibits generation of peroxyl radical and hydroperoxide radical [20]. Although more detailed studies for radical scavenging activity of benfotiamine are required, the reported data in the present study might give a new biochemical or pharmacological aspect of this prodrug. Oral administration of benfotiamine leads to significant increase in thiamin, thiamin monophosphate and TDP level in blood [21], the suggested mechanism of absorption and the metabolic fate of benfotiamine is dephosphorylation to S-benzolylthiamine by ecto-alkaline phosphatase presented in the brush border of intestinal mucosal cells. A significant part of S-benzoyl thiamine is captured by erythrocyte [16, 22] and converted to free thiamin through a slow-non enzymatic transfer of the S-benzoyl group to SH groups of glutathione [21]. Recently, a new study showed that benfotiamine reduces superoxide and hydroxyl radical levels in diabetic heart by inducing the activation of pentose phosphate pathway, which regenerates the antioxidant NADPH [23]. Significant reduction in MetHb% in both short- and long-term benfotiamine approaches followed in the present study might be explained by radical scavenging property of benfotiamine, because benfotiamine exhibited antioxidant effect by reducing the oxidative stress and genomic damage caused by mitogenic model compounds; such effect is found to be related to its direct antioxidant capacity [13]. Furthermore, we can not exclude the other mechanisms of actions of benfotiamine and thiamin associated with the improvement in transketolase activity of erythrocytes [24,25], reducing superoxide overproduction through directing advanced glycation and lipoxidation end products substrate to the pentose phosphate pathway [9], decreasing the level of malondialdehyde and the increase in the levels of reduced glutathione and vitamin E levels, as reported in studies with acute ethanol and $CCl_4$ intoxication [26,27]. In conclusion, benfotiamine protects hemoglobin and erythrocyte plasma membrane against the oxidative damage induced by sodium nitrite, which may be attributed to direct radical scavenging properties.

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