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Research Article

Free Radical Scavenging Activity of Boron and Vitamin C in Nitrite-Induced Hemoglobin Oxidation Model: *In vitro* and *in vivo* Studies

Hozan Jaza Hama Salh [1], Tavga Ahmed Aziz [2*], Zheen Aorahman Ahmed[2], Dlivan Fattah Aziz[3]

[1]*Department of Clinical Pharmacy, College of Pharmacy, University of Sulaimani, Kurdistan Region, Iraq;*
[2]*Department of Pharmacology and Toxicology, College of Pharmacy, University of Sulaimani, Kurdistan Region, Iraq;*
[3]*Department of Pharmaceutics, College of Pharmacy, University of Sulaimani, Kurdistan Region, Iraq*

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Abstract

Objective: To investigate the dose-response relationship of the free radical-scavenging activities of boron and vitamin C in nitrite-induced hemoglobin oxidation *in vitro* and *in vivo*. ***Method***: Different concentrations of boron and vitamin C were added to a hemolysate challenged with nitrite to induce methemoglobinemia (MetHb), and the most effective dose of boron and vitamin C was used before and after different intervals of inducing Hb oxidation, and the production of MetHb was monitored using a spectrophotometer. The effective doses of boron and vitamin C, alone and in combination, were administered to rats before challenging them with an oral dose of 100 mg/kg sodium nitrite. ***Results***: In vitro results indicated that different concentrations of boron and vitamin C attenuated MetHb formation, with the maximum effect achieved by 0.08mg/L and 10mg/L, respectively. Moreover, when these doses were used at different time intervals, a maximum effect was achieved when added 10 min before nitrite. The in vivo results demonstrated a significant reduction in methemoglobin formation in rats treated with boron and vitamin C alone. The hematological markers were not changed except for the platelet levels that increased in the boron-treated and combination groups. The monocyte-to-lymphocyte ratio decreased significantly in all treatment groups compared with the positive control group. ***Conclusion***: Boron protects against Hb oxidation induced by nitrite, and a potentiated effect has been achieved with the combination of vitamin C.

Keywords: Boron, Methemoglobinemia, Nitrite, Oxidative stress, Vitamin C.

نشاط كسح الجذور الحرة للبورون وفيتامين سي في نماذج أكسدة الهيموجلوبين التي يسببها النتريت: دراسات في المختبر وفي الجسم الحي

الخلاصة

ألهدف: صممت الدراسة الحالية لتقييم العلاقة بين الجرعة والاستجابة لأنشطة إزالة الجذور الحرة للبورون وفيتامين C في أكسدة الهيموجلوبين الناجم عن النتريت في المختبر ولتقييم تأثيرها في النموذج الحي لميتيموغلوبين الدم. **الطرق:** تمت إضافة تراكيز مختلفة من كل من البورون وفيتامين C إلى الهيمولات المصابة بالنتريت الناجم عن ميتهيموغلوبين الدم (MetHb)، واستخدمت الجرعة الأكثر فاعلية قبل وبعد فترات مختلفة من تحفيز أكسدة الهيموغلوبين في محللات كرات الدم الحمراء وتم رصد تكوين الميثيموغلوبين. قياس الطيف الضوئي. علاوة على ذلك، تم إعطاء جرعة فعالة من كل من البورون وفيتامين C بمفردهما ومشتركين في الفئران قبل مواجهة جرعة سامة من نتريت الصوديوم، وتم رصد تكوين MetHb بطريقة طيفية. **النتائج:** أشارت نتائج الدراسة في المختبر إلى أن إعطاء تراكيز مختلفة من البورون وفيتامين C كان قادراً على تحسين أكسدة الهيموجلوبين المستحث تجريبياً بواسطة نتريت الصوديوم بأقصى تأثير محقق بـ (0.08 مجم/لتر) و (10 مجم/لتر)، على التوالي. علاوة على ذلك، هذه الجرعات عند استخدامها في فترات زمنية مختلفة؛ تم تحقيق أقصى تأثير عند إضافة 10 دقائق قبل التحدي مع النتريت. تدعم نتائج الدراسة المجراة بشكل كبير تلك النتائج في المختبر، حيث تم تسجيل انخفاض كبير في تكوين الميثيموغلوبين في الفئران المعالجة بالبورون وفيتامين سي لوحده. علاوة على ذلك، تم إنتاج المزيد من التأثير من قبل المجموعة المركبة مقارنة بالمجموعة المعالجة بنتريت الصوديوم. فيما يتعلق بعلامات الدم، لم يلاحظ أي تغيرات مهمة باستثناء مستويات الصفائح الدموية التي زادت في البورون والمجموعات المركبة. انخفضت نسبة الخلايا الأحادية إلى الخلايا الليمفاوية معنويا في جميع المجموعات العلاجية مقارنة مع مجموعة السيطرة الإيجابية. **الاستنتاجات:** تشير النتائج إلى أن البورون يعمل كمضاد للأكسدة مثل فيتامين C ويمارس تأثير وقائي ضد أكسدة الهيموجلوبين الناجم عن النتريت في كل من النماذج المختبرية والحيوية من ميتهيموغلوبين الدم وقد تم تحقيق تأثير إضافي في هذا الصدد مع المجموعة المركبة.

* ***Corresponding author***: Tavga A. Aziz, Department of Pharmacology and Toxicology, College of Pharmacy, University of Sulaimani, Kurdistan Region, Iraq; Email: tavga.aziz@univsul.edu.iq

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INTRODUCTION

The damaging effects of oxidants on cells outweigh the protective effects of antioxidants in a state known as oxidative stress [1]. Reactive species made of oxygen and nitrogen are involved in the chemistry of oxidative stress. They are a group of small, reactive molecules with varying half-lives, reactivity, and compartmentalization within cells [2]. These include free radicals like the hydroxyl radical (•OH) and the superoxide radical anion ($O•_2^-$), as well as non-radical species like singlet oxygen ($^1O_2$) and hydrogen peroxide (H_2O_2) [3]. Cells can control normal biological processes, including protein synthesis, DNA mutagenesis, gene transcription, and antibacterial action, when they maintain low intracellular ROS levels. High ROS levels, or oxidative stress, are known to cause a number of significant illnesses, including diabetes, cancer, neurological disorders, and cardiovascular and cancer diseases [4]. In patients with sickle cell anemia, b-thalassemia, glucose-6-phosphate dehydrogenase deficiency, and other hemoglobinopathies, reactive oxygen species have been linked to erythrocyte destruction. Reactive nitrogen species (RNS), which can harm cells through nitrosative stress, is another significant class of chemically reactive species. Nitric oxide, nitroxyl, nitrogen dioxide, and peroxynitrite are examples of RNS [5–6]. Free radicals cause protein oxidation and lipid peroxidation, which results in the creation of hemolytic holes in erythrocytes under oxidative stress. This alters cellular proteins and plasma membranes [7]. Antioxidants, both natural and synthetic, have been employed to halt free radical chain reactions and stop their harmful effects on membrane lipids. Antioxidant enzymes in the cell closely control the antioxidant redox equilibrium; they have a pivotal role in scavenging free radicals [8–9]. Many mechanisms are proposed for the protection against oxidative damage facing erythrocytes; these include the protection offered by the NADH-dependent reductase enzyme, glutathione, catalase, and SOD [10]. Oxidation of Hb continuously generates a small amount of methemoglobin (MetHb). MetHb production is linked to the production of $O•_2^-$; to avoid oxidative damage to Hb and other essential erythrocyte components, $O•_2^-$ must be detoxified. Under physiological circumstances, SOD changes $O•_2^-$ into H_2O_2, which is then broken down by glutathione peroxidase and catalase to H_2O [11]. Methemoglobinemia is produced as a result of the oxidation of iron in the Hb from a ferrous state to a ferric state, which is unable to carry oxygen [12]. MetHb can also be brought on by hereditary or acquired conditions. The acquired versions are the most prevalent, mostly as a result of exposure to chemicals that either directly or indirectly oxidize hemoglobin (Hb). Methemoglobinemia is caused by drugs or dangerous situations that make it easier for hemoglobin to change from the ferrous to the ferric state. Methemoglobinemia can be caused by a number of drugs, including phenazopyridine, sulfamethoxazole, dapsone, aniline, paraquat/monolinuron, nitrate, nitroglycerin, amyl nitrite, isobutyl nitrite, sodium nitrite, benzocaine, prilocaine, methylene blue, and chloramine [13]. Tissue hypoperfusion and/or hypoxia may develop when methemoglobin levels are increased in the blood. When the level of MetHb increases, it may cause ischemia, cyanosis, and irreparable tissue damage. These effects may contribute to an increase in mortality [14]. Methylene blue is now advised as a treatment for severe Met-Hb. Studies showed that ascorbic acid (Vitamin C), blood transfusions, and hyperbaric oxygen have all been documented to have positive effects in the management of MetHb [15]. Numerous epidemiological studies conducted in recent years on antioxidants have linked their use to a decline in the occurrence of illnesses linked to oxidative damage. As a result, the use of antioxidants, especially natural antioxidants, for the benefit of human health has received a lot of attention [16–18]. The fifth element on the periodic table, boron, is a substance that naturally occurs. Borates are the result of the combination of boron with oxygen and other elements in the atmosphere. Borates are extensively distributed in nature and may be found in soil, rocks, and seas. There are a number of borates that are economically significant, including borax, colemanite, and ulexite [19]. In addition to its traditional usage in healthcare, boron is frequently employed in pharmacological, industrial, agricultural, and cosmetic applications [20–22]. In many studies, boron has been shown to exert antioxidant, hepatoprotective [20], antigenotoxic, antimicrobial, and anti-inflammatory effects [23], in addition to anticancer [24] and wound healing effects [25]. The free radical scavenging activity of boron [26] renders it a good candidate to be tested in other models of oxidative damage. Accordingly, the current study was designed to test the antioxidant activity of boron alone or in combination with vitamin C in Hb oxidation induced by nitrite.

METHODS

Blood collection and preparation of lysate: in vitro study

Ten milliliters of venous blood samples were obtained by vein puncture from the antecubital vein of healthy individuals and using ethylene diamine tetraacetic acid (EDTA)-containing tubes to keep blood; the plasma and the buffy coat of white cells were then removed by centrifugation at 2500 rpm and 4 °C for 10 min. The erythrocytes were washed three times with phosphate buffered saline (PBS, pH 7.4) and lysed by suspending in 20 volumes of 20 mM phosphate buffer (PB, pH 7.4) to yield the required hemolysate concentration of 1:20 [5,6].

Preparation of vitamin C solution

To prepare different concentrations of vitamin C (ALPHA CHEMIKA, India), the required quantity of vitamin C was dissolved in distilled water. After a stock solution (100mg/100ml) was prepared, serial dilutions of the stock solution were performed to obtain concentrations of 10, 20, 30, 40, 50, 60 mg/L.

Effect of different concentrations of vitamin C on Hb oxidation induced by nitrite

In an *in vitro* model, sodium nitrite was added to oxidate hemoglobin to produce methemoglobin (MetHb) [27]. 1.0 ml of freshly prepared hemolysate was mixed with 1.0 ml of each concentration of vitamin C (10, 20, 30, 40, 50, and 60 mg/L) and 1.0 ml of sodium nitrite (Sigma-Aldrich, Germany) (final concentration 1.0 mM). The formation of MetHb was then monitored spectrophotometrically at 631 nm for 60 minutes using the UV-VIS Spectrophotometer Shimadzu 1700. 1.0 ml of the highly effective concentration of vitamin C was then added to 1.0 ml of freshly prepared hemolysate either 10 min before or 10 and 20 min after the addition of sodium nitrite to the hemolysate solution. Also, the formation of MetHb was monitored as previously mentioned [5,6].

Preparation of boron solution

To prepare different concentrations of boron (disodium tetrahydroborate; Riedel-de Haen AG, Hannover, Germany), the required quantity of boron was dissolved in distilled water. After a stock solution (100 mg/100 mL) was prepared, serial dilutions of the stock solution were performed to obtain concentrations of 0.02, 0.04, 0.08, 0.16, 0.32, and 0.64 mg/L.

Effect of different concentrations of boron on Hb oxidation induced by nitrite

In an *in vitro* model, sodium nitrite was added to oxidize hemoglobin to methemoglobin (MetHb) [27]. 1.0 ml of freshly prepared hemolysate was mixed with 1.0 ml of each concentration of boron (0.02, 0.04, 0.08, 0.16, 0.32, and 0.64 mg/L) and 1.0 ml of sodium nitrite (Sigma-Aldrich, Germany) (final concentration 1.0 mM). The formation of MetHb was then monitored spectrophotometrically at 631 nm for 60 minutes using the UV-Vis Spectrophotometer Shimadzu 1700. 1.0 ml of the highly effective concentration of boron was then added to 1.0 ml of freshly prepared hemolysate either 10 min before or 10 and 20 min after the addition of sodium nitrite to the hemolysate solution [5,6]. Also, the formation of MetHb was monitored, as previously mentioned.

In vivo studies: Experimental animal and treatment schedule

Male Wistar Albino rats (90–140 g)) were used. The rats were placed in the animal house of the Pharmacy College, University of Sulaimani, in well-ventilated plastic cages at 24±2 °C and 50±10 relative humidity. They were then subjected to a 12-hour light and 12-hour dark cycle and were acclimatized for one week prior to the beginning of the experiments. During this process, the rats were free to access diet and tap water ad libitum. The experimental protocols met the Guidelines for Animal Experimentation and were approved by the Ethical Committee of the University of Sulaimani, College of Pharmacy (Certificate No. PH67-22), following the Institutional Animal Ethics Committee. The study was performed according to the guidelines of the Canadian Council on Animal Care (CCAC). After the rats were acclimated to the environment of the animal house, they were randomly assigned into four groups, five rats per group, and treated as follows: The positive control group was given 1.0 ml of normal saline (0.9% sodium chloride) orally; the vitamin C-treated group (100 mg/kg) was orally administered as a single dose; the boron-treated group (6 mg/kg) was orally administered as a single dose; and the combination of boron (6 mg/kg) with vitamin C (100 mg/kg) was orally administered as a single dose. All treatment groups orally received sodium nitrite (100 mg/kg) 1 hour after the treatment schedule. After 45 min, rats were sacrificed using a high dose of anesthetic chloroform, and intracardiac blood samples were collected for the measurement of MetHb level [13] and analysis of the complete blood count (CBC).

Statistical Analysis

The data were statistically analyzed using GraphPad Prism8. The values of the assessed parameters were presented as mean±standard deviation (S.D.). One-way analysis of variance (ANOVA) was used for comparisons between different groups, and Tukey's test was performed to compare each group with the positive control group. A p-value < 0.05 was considered statistically significant.

RESULTS

As shown in Figure 1, 10 mg/L of vitamin C was the most effective dose for attenuating the rate of Hb oxidation and MetHb formation. The percent inhibition of different doses of vitamin C (10 mg/l, 20 mg/l, 30 mg/l, 40 mg/l, 50 mg/l, and 60 mg/l) was as follows: (91.8%, 90.9%, 91.7%, 89.7%, 91.5%, and 91.7%), respectively.

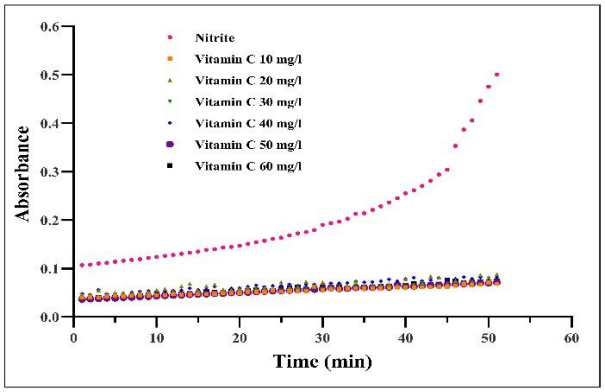

Figure 1: Effect of different concentrations of Vitamin C (10 mg/l, 20 mg/l, 30 mg/l, 40 mg/l, 50 mg/l, and 60 mg/l) on the time course of Hb oxidation induced by nitrite and MetHb formation in erythrocyte lysate.

In the absence of vitamin C (control), the time required to convert 50% of the available Hb to MetHb was 51 min, while it was slowing to (405, 365, 400, 322, 389, and 400 min), respectively, in the presence of vitamin C (Table 1).

Table 1: Effect of different concentrations of Vitamin C (10mg/l, 20mg/l, 30mg/l, 40mg/l, 50mg/l, and 60 mg/l) on the time course of Hb oxidation induced by nitrite and MetHb formation in erythrocyte lysate

Concentration	% Formation of MetHb	% Inhibition of MetHb	Time for 50% MetHb production (min)
Nitrite	100	0	51
Vit C 10mg/l	8.2	91.8	405
Vit C 20mg/l	9.1	90.9	365
Vit C 30mg/l	8.3	91.7	400
Vit C 40mg/l	10.3	89.7	322
Vit C 50mg/l	8.5	91.5	389
Vit C 60mg/l	8.3	91.7	400

Values represent the mean of three experiments.

Figure 2 showed that 0.08 mg/L boron was the most effective dose for attenuating the rate of Hb oxidation and MetHb formation. The percent inhibition of different doses of boron (0.02 mg/l, 0.04 mg/l, 0.08 mg/l, 0.16 mg/l, 0.32 mg/l, and 0.64 mg/l) was as follows: (89.12%, 88.34%, 91.23%, 89%, 89%, and 88.89%), respectively. In the absence of boron (control), the time required to convert 50% of the available Hb to MetHb was 51 min, while it was slowing to 306, 300, 379, 303, 303, and 300 min, respectively, in the presence of boron (Table 2). Figure 3 from the current study showed that adding the highly effective concentration of vitamin C (10 mg/l), which was found in the previous experiment, to the hemolysate either 10 minutes before nitrite, 10 minutes after nitrite, or 20 minutes after nitrite (during the autocatalytic phase) significantly decreased MetHb formation compared to the nitrite-treated group (p<0.0001) for 10 minutes before and 10 minutes after nitrite administration.

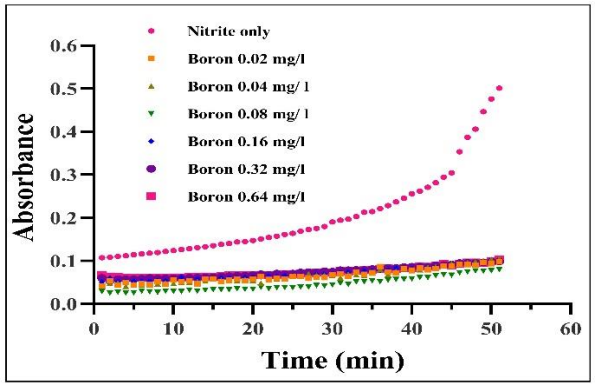

Figure 2: Effect of different concentrations of Boron (0.02 mg/l, 0.04 mg/l, 0.08 mg/l, 0.16 mg/l, 0.32 mg/l, and 0.64 mg/l) on the time course of Hb oxidation induced by nitrite and MetHb formation in erythrocyte lysate.

Table 2: Effect of different concentrations of Boron (0.02 mg/l, 0.04 mg/l, 0.08 mg/l, 0.16 mg/l, 0.32 mg/l, and 0.64 mg/l) on the time-course of Hb oxidation induced by nitrite and MetHb formation in erythrocyte lysate

Concentration	% Formation of MetHb	% Inhibition of MetHb	Time for 50% MetHb production (min)
Nitrite	100	0	51
Boron 0.02mg/l	10.88	89.12	306
Boron 0.04mg/l	11.66	88.34	300
Boron 0.08mg/l	8.77	91.23	379
Boron 0.16mg/l	11	89	303
Boron 0.32mg/l	11	89	303
Boron 0.64mg/l	11.11	88.89	300

Values represent the mean of three experiments.

However, the addition of vitamin C 10 min before induction was the most effective when compared to 10 min later (p<0.0001) and 20 min later (p<0.0001). The addition of vitamin C 20 minutes after nitrite produced only 12.7% protection. Furthermore, it attenuated the time required to produce 50% of MetHb to 40 min (Table 3).

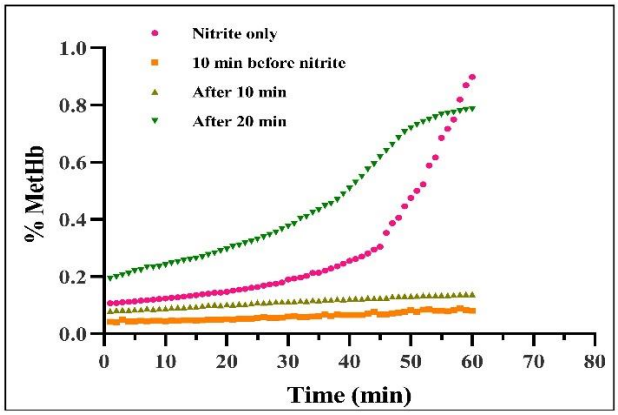

Figure 3: Effect of Vitamin C (10mg/l) on the time-course of Hb oxidation induced by nitrite and MetHb formation in erythrocyte lysate at different time intervals.

Table 3: Effect of incubation with Vitamin C (10 mg/l) at different time intervals 10 min before, 10, and 20 min after nitrite on the time course of Hb oxidation induced by nitrite and MetHb formation in erythrocyte lysate

Time course for the addition of 10 mg/l Vitamin C	% Formation of MetHb	% Inhibition of MetHb	Time for 50% MetHb production (min)
Control	100	0.0	51
Incubation before 10 min	9.0	91	270
Administration after 10 min	15.3	84.7	217
Administration after 20 min	87.3	12.7	40

Values represent the mean of three experiments.

In the current study, Figure 4 showed that adding the highly effective concentration of boron (0.08 mg/l), which was found in the previous experiment, to the hemolysate at different times, either 10 minutes before nitrite, 10 minutes after nitrite, or 20 minutes after nitrite (during the autocatalytic phase), led to a significant decrease in MetHb formation compared to the nitrite-treated group (p<0.0001) for 10 minutes before nitrite However, the addition of boron 10 min before the induction was the most effective when compared to 10 min later (p<0.0001) and 20 min later (p<0.0001). The addition of boron 20 min after nitrite produced only 6% protection and attenuated the time required to produce 50% of MetHb to 36 min (Table 4).

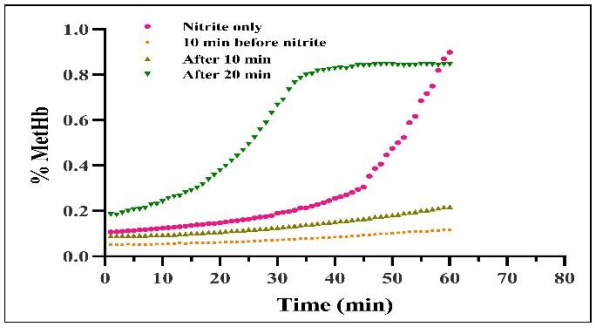

Figure 4: Effect of boron (0.08 mg/l) on the time-course of Hb oxidation induced by nitrite and MetHb formation in erythrocyte lysate at different time intervals.

Table 4: Effect of incubation with boron (0.08 mg/l) at different time intervals 10 min before, 10, and 20 min after nitrite on the time course of Hb oxidation in erythrocyte lysate

Time-course for the addition of 0.08 mg/l boron	% Formation of MetHb	% Inhibition of MetHb	Time for 50% MetHb production (min)
Control	100	0	51
Incubation before 10 min	12.8	88.2	260
Administration after 10 min	24.2	75.8	137
Administration after 20 min	94	6.0	36

Values represent the mean of three experiments.

Results presented in Figure 5 showed the percent MetHb formation reduced significantly in rats treated with boron alone and vitamin C alone, compared to the control group (p=0.021) and (p<0.027), respectively. The combination group also statistically reduced the percent of MetHb compared to the control group (p<0.01). Regarding the percent of formation of MetHb, in the control group, the percent was 43.2%, and this decreased to 19% in the vitamin C-treated group and to 16.4% in the boron-treated group. The maximum protection was achieved by the combination group at 14.4% (Table 5). In the current study, all treatment groups resulted in a significant reduction in the monocyte-to-lymphocyte ratio (MLR) in comparison with the positive control group (vitamin C, p=0.015), (Boron, p=0.014), and (vitamin C + Boron, p=0.04), (Figure 6). Meanwhile, no significant difference was observed in the platelet-to-lymphocyte ratio (PLR).

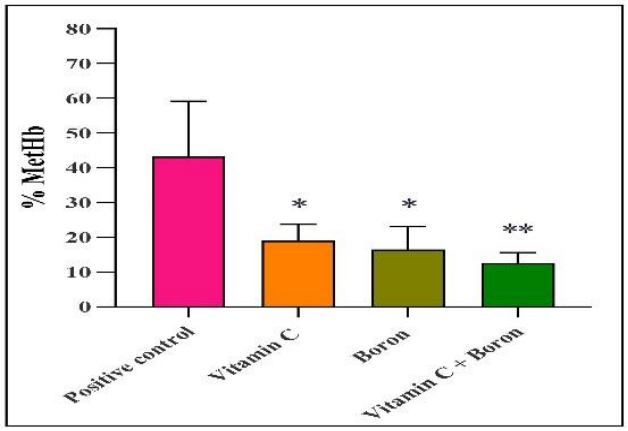

Figure 5: Effects of a single oral dose of vitamin C and boron alone and in combination on nitrite-induced MetHb formation in rats. * (p<0.05), and ** (p< 0.01), significantly different when compared with the positive control group using one-way ANOVA, for multiple comparisons, ANOVA followed by Tukey's test was used.

Table 5: Effects of a single oral dose of vitamin C and boron alone and in combination on nitrite-induced MetHb formation in rats

Treated groups	% Formation of MetHb	% Inhibition of MetHb
Control	43.2^{A}	56.7^{A}
Vit C (100 mg/kg)	19.0^{B}	81.0^{B}
Boron (6 mg/kg)	16.4^{B}	83.6^{B}
Vit C (100mg/kg)+Boron (6mg/kg)	14.4^{B}	85.6^{B}

Each value represents the mean percentage (n=5). Statistical comparison between groups: Mean values with different superscripts (A, B) are significantly different (p<0.05).

The hematological markers revealed no significant differences except for the platelets (Table 6).

DISCUSSION

The primary cell types in the blood are red blood cells; when exposed to environmental toxins, xenobiotics, and pesticides, the membrane structure and composition of erythrocytes change, which affects their characteristics and functions [28].

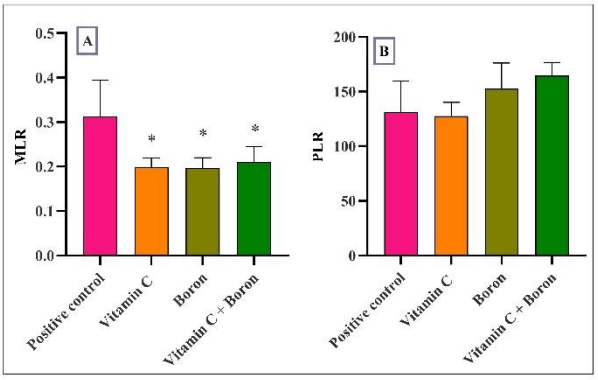

Figure 6: Effects of a single oral dose of vitamin C and boron alone and in combination on nitrite-induced blood inflammatory markers in rats (A) MLR and (B) PLR. * (p<0.05), significantly different when compared with the positive control group using one-way ANOVA, for multiple comparisons, ANOVA followed by Tukey's test was used.

MetHb is one of the mechanisms of toxicity in RBC produced by many drugs and toxicants, such as nitrite. MetHb occurs by the oxidation of the ferrous ion to the ferric state, and this process is usually accompanied by the generation of several ROS [29–31]. The sulfhydryl groups of the lipid bilayer and protein constituents of the erythrocyte membrane are thought to react with the nitrite ion, its metabolites, and lipid peroxidation products to affect the structure of the membrane [29]. Our results have revealed that sodium nitrite induces methemoglobin in both *in vitro* and *in vivo* models, and this is in tune with other studies [6,34].

Table 6: Effects of a single oral dose of vitamin C and boron alone and in combination on hematological markers in nitrite-induced MetHb in rats

Parameter	Positive Control	Vit C (100mg/kg)	Boron 6 mg/kg	Vit C+Boron
Hb (g/dl)	13.9±0.64	13.15±0.63	12.7±0.98	12.8±1.0
Hct %	34.4±1.0	35±1.4	34±2.7	32.9±2.9
RBC (×106 cells/µL)	6.97±0.15	7.13±0.36	7.16±0.58	6.7±0.7
WBC (×103 cells/µL)	10.4±3.0	8.85±3.2	6.3±1.0	8.0±1.0
Platelet (x103 cell/µL)	789±115^{a}	968±83^{a,c}	1127±130^{b}	1150±158^{c}

Each value represents the mean percentage (n=5). Statistical comparison between groups: Values with different superscripts (a,b,c) are significantly different (p<0.05).

During nitrite-induced MetHb, phospholipases are activated by nitrite-promoted Ca^{2+} influx in blood cells, which raises the amount of rigidly structured phospholipids in the membrane [29,31]. One of the powerful antioxidants that scavenges ROS and shields tissues from free radical damage is vitamin C. The ability of SOD and CAT to break down superoxide anions and H2O2 leads to a reduction in oxidative stress, which is the most efficient method of protecting cells from the deleterious effects of these radicals. Small variations in physiological concentrations of these antioxidant enzymes may have a significant impact on the resistance of cellular lipids, proteins, and DNA to oxidative damage. These enzymes cooperate to remove active oxygen radicals [35]. Superoxides, hydroxyl radicals, singlet oxygen, and hydrogen peroxide can all be inactivated by vitamin C. It has the ability to scavenge radicals that cause lipid peroxidation, protecting the membrane from oxidative injury [36–37]. In the current study, it has been observed that different concentrations of vitamin C in vitro were able to ameliorate the experimentally induced Hb-oxidation by sodium nitrite in hemolysate. Additionally, the results displayed that the addition of vitamin C 10 min before the addition of sodium nitrite and 10 min after nitrite addition (i.e., during the autocatalytic phase) can significantly prevent Hb oxidation by sodium nitrite. However, it did not reverse the MetHb formation after 10 minutes of the addition of nitrite. This effect is attributed to the free radical scavenging activity and not to the decrease of MetHb to Hb. Moreover, the addition of vitamin C 20 minutes after nitrite addition was not able to prevent Hb oxidation by sodium nitrite. A study reported that methemoglobin formation is significantly reduced by high concentrations of ascorbic acid [38]. Moreover, a study conducted by Hussain *et al.* (2018) observed that 30 min of pre-incubation with vitamin C followed by 4 hours of incubation with deltamethrin prevents MetHb formation in the hemolysate [28]. Another study showed that the incubation of erythrocytes in rats and healthy humans with sodium nitrite and ascorbic acid in a dose-dependent manner was significantly able to decrease MetHb formation [39]. The results of the in vivo study indicated that the group treated with vitamin C alone was able to decrease MetHb formation significantly compared to the control group. In tune with the current finding, a study by Kang *et al.* (2018) indicated that using vitamin C via tail vein injection significantly reduced MetHb formation in dapsone-induced methemoglobinemia in rats [14]. Previous studies showed that vitamin C decreases oxidative stress and lipid peroxidation, thereby preventing many damaging processes in cells [28]. Administration of vitamin C with sodium nitrite compared to the control group significantly reduced MLR. However, no significant difference was observed in the platelet-to-lymphocyte ratio. The hematological markers also showed no significant differences except

for the platelets. In contrast to the present study, another study reported that the administration of ascorbic acid at 200 mg/kg concurrent with sodium nitrite at 30 mg/kg daily to rats for 5 weeks significantly increased the RBC count, Hb, PCV, and MCV compared to the nitrite-treated group [40]. Additionally, Amin *et al*. (2016) found that administration of ascorbic acid at 500 ppm concurrent with sodium nitrite at 125 ppm daily to rats for 30 days significantly increased RBC count, HGB, WBCs, MCV, MCH, MCHC, lymphocytes, and monocytes compared to the sodium nitrite-treated group [41]. The difference in the findings of these two studies and the current study could be attributed to the dose and the duration of administration. Animals and humans both appear to require boron as a micronutrient, and according to the World Health Organization, boron is "probably necessary" for humans [42]. Studies have indicated that boric acid possesses antigenotoxic, hematoprotective, hepatoprotective, and renoprotective properties [43]. In the *in vitro* part of the current study, we found that different concentrations of boron were able to protect against the experimentally induced Hb-oxidation by the addition of sodium nitrite and attenuate the formation of several ROS in hemolysate. Furthermore, regarding the effect of different time intervals on the antioxidant activities of boron, the present study has displayed that the addition of boron early to the incubation mixture, 10 min before the addition of sodium nitrite, and 10 min after nitrite addition (i.e., during the autocatalytic phase), can significantly prevent Hb oxidation by sodium nitrite. However, it did not reverse the MetHb formation after 10 minutes of the addition of nitrite. This effect could be due to its radical scavenging activity and not to the decrease of MetHb to Hb. Moreover, the addition of boron 20 minutes after nitrite addition did not protect against Hb oxidation by sodium nitrite. The animal study greatly supports the in vitro finding, where a significant reduction in MetHb formation was observed in rats treated with boron alone and in combination with vitamin C in comparison to the sodium nitrite-treated group. Boron has a pivotal role in maintaining the activity of the cell membrane [43]. The free radical scavenging activity of boron is mainly due to its ability to boost the levels of antioxidant enzymes in the blood and cells, such as glutathione peroxidase, SOD, and catalase [44]. In one study, boron demonstrated great wound healing activity by attenuating the production of ROS, suggesting that boron may have similar activities to SOD, protecting the cells from oxidative damage [45]. Additionally, many other studies proved the antioxidant effects of boron via ameliorating lipid peroxidation and boosting total antioxidant capacity [35,46]. Furthermore, a previous study reported that boron supplementation contributed to enhancing the hepatic expression of SOD isozymes [47]. Another protective mechanism of boron is attributed to diminishing intracellular calcium, which plays a crucial role in reducing cancer cell proliferation, indicating that boron may have an indirect inhibitory impact on apoptosis and enhance tissue regeneration [48]. The antiapoptotic effect of boron occurs by lowering TNF-α and free radical scavenging activities [49]. Furthermore, a study conducted by Hu *et al.* (2014) showed that boron enhanced the level of antioxidant enzymes [50]. The results of the present study indicated that the administration of boron alone or in combination with vitamin C, when compared to the control group, significantly reduced MLR. In accordance with the previous findings, an experimental study done on rats revealed a significant decrease in lymphocyte and monocyte levels in the group treated with borax by adding borax to drinking water for 28 days [51]. Furthermore, a study conducted by Drmus *et al.* (2018) observed that using high boron significantly reduced leucocyte count. It was also observed that boron supplementation had no protective effect on leucocyte counts reduced by gentamicin [52]. In this study, no significant difference in the platelet-to-lymphocyte ratio has been detected. The hematological markers also showed no significant differences except for the platelets. These findings are in disagreement with Keklik *et al.* (2016), who reported that using borax for a period of 28 days causes a significant decrease in the WBC, HGB, Htc, RBC, and PLT counts in the borax-treated group [51]. The discrepancy between these two findings may be related to the duration of use of boron. Furthermore, a study done by Iztleuov *et al.* (2017) revealed that the administration of boric acid for 10 days improved blood rheological properties and stimulated erythropoiesis and hemoglobin synthesis against chromium-induced hemorrhology disorders in rats [53].

Conclusion

The findings of the current study revealed the antioxidant capacity of boron in both in vitro and in vivo models of methemoglobinemia, and boron was more effective than vitamin C in preventing the formation of MetHb in rats challenged with sodium nitrite. Additionally, the combination of vitamin C and boron produced more protection than each alone, and this could be attributed to the potentiated antioxidant activities of both.

Conflicts of interest

There are no conflicts of interest.

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Data sharing statement

Supplementary data can be shared with the corresponding author upon reasonable request.

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