Concentration-Dependant Antioxidant Activity of Pentoxifylline in Nitrite-induced Hemoglobin Oxidation Model

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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 pentoxifylline in nitrite-induced Hb oxidation. Different concentrations of pentoxifylline were added at different time intervals of Hb oxidation in erythrocytes lysate, and formation of methemoglobin (MetHb) was monitored spectrophotometrically. The results showed that in this model, pentoxifylline 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 at the same time of induction of Hb oxidation with nitrite. In conclusion, pentoxifylline can effectively, in concentration-dependent pattern, attenuate sodium nitrite-induced Hb oxidation *in vitro*.

Key words: pentoxifylline, radical scavenging, hemoglobin oxidation

الخلاصة

تعتمد عملية تكوين الجذور الحرة في بروتين الدم من الظواهر المصاحبة لظاهرة التسمم بالعديد من المركبات الكيمياوية. تم تصميم هذه الدراسة لتقييم العلاقة بين التركيز والتأثير لفعالية مركب البنتوكسيفيللين في اقتناص الجذور الحرة في نموذج اكسدة الهمغلوبين المستحدث بنايترايت الصوديوم. تمت أضافة تراكيز مختلفة من مادة البنتوكسيفيللين وخلال فترات متعددة خلال أكسدة خضاب الدم في متحلل الخلايا الحمراء، وتمت متابعة تكون مادة الميثهمغلوبين بواسطة امتصاص الطيف الضوئي. أظهرت النتائج ان في هذا النموذج تمكن البنتوكسيفيللين بنجاح من تأخير عملية أكسدة خضاب الدم بعد أضافة نايترايت الصوديوم، ولوحظ بأن هذا التأثير كان أكثر وضوحا عندما تمت أضافة البنتوكسيفيللين في نفس الوقت الذي تم فيه استداث الأكسدة. وعليه يمكن الأستنتاج بأن للبنتوكسيفيللين ألقابلية على تأخير أكسدة خضاب الدم بواسطة نايترات الصوديوم وبشكل يتناسب مع تركيز المادة المضافة.

Introduction

Pentoxifylline (PTX) [1-(59-oxohexyl)-3, 7-dimethylxanthine] is widely used as a drug since long period of time [(1)]. It is used in the treatment of cerebrovascular and peripheral vascular diseases [(2)]. PTX is known to possess anti-inflammatory properties [(3)] which are probably related to its ability to suppress oxygen radical production or scavenge reactive oxygen species [(4)]. The ability of pentoxifylline to scavenge hydroxyl radicals has been demonstrated earlier [(5)]. PTX is known to become metabolized to its corresponding 8-oxo derivatives in the mammalian system [(6)]; these metabolites (methyluric acids) are known to inhibit lipid peroxidation in human erythrocyte membranes *in vitro* and function as free radical scavengers [(7,8)] suggesting their antioxidant effects *in vivo*. It has also been reported that PTX has inhibitory effect on the generation of superoxide anion and hydrogen peroxide in human leukocytes *in vitro* and *in vivo* [(9,10)]. However, the relationship between the concentration and radical scavenging activity of PTX is not completely explained in standard *in vitro* system. The present study was designed to evaluate the concentration-dependence radical scavenging and membrane stabilizing activities of PTX using *in vitro* model of nitrite-induced oxidation of hemoglobin.

Materials and Methods

Preparation of blood samples and hemolysate

Blood samples were obtained from healthy individuals by vein puncture and kept in (EDTA) containing tubes for isolation of erythrocytes and preparation of hemolysate. The blood samples were centrifuged at 2500 rpm and 4^{o}C 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 20 mM phosphate buffer (PB, pH 7.4) to yield the required hemolysate concentration of 1:20 [(11)].

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Preparation of pentoxifylline solutions
Stock solution of 80mM pentoxifylline was prepared, from which different concentrations series of 40 mM, 20 mM, 10 mM and 5mM were prepared.

Effect of different PTX concentrations on the time course of nitrite-induced oxidation of hemoglobin
To 1.5 ml of freshly prepared hemolysate, 1 ml of each concentration of pentoxifylline (5 mM, 10 mM, 20 mM, 40 mM and 80 mM) was added concomitantly with 0.1 ml sodium nitrite, and the formation of MetHb was monitored spectrophotometrically at 631 nm for 45 minutes using computerized UV-visible spectrophotometer. The experiments were performed 3 times for each concentration under controlled temperature (27-30^{o} C).

Effect of pentoxifylline on the time course of MetHb formation at various time intervals from nitrite addition
To 1.5 ml freshly prepared hemolysate, 1.0 ml of the highly effective concentration of pentoxifylline was added 10 minutes before, at time zero, 10 and 20 minutes after the addition of sodium nitrite to the hemolysate solution, and the formation of MetHb was monitored spectrophometrically at 631 nm.

Results

In the presence of different concentrations of PTX (5, 10, 20, 40 and 80 mM) 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 PTX concentrations and inhibition of MetHb formation (0%, 0%, 28%, 68.3 and 76.2% respectively, figure 1 and 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 22.5 min in the absence of PTX (control), whereas it was delayed to 32, 70.9 and 94.8 min in the presence of 20, 40 and 80 mM of PTX respectively (table 1). Addition of the highly effective concentration of PTX (80 mM) to the hemolysate mixture at different time intervals (10 min before nitrite, zero time, 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 against oxidation due to the addition of sodium nitrite to the lysate (57%, 76.2%, 57% and 56.6% respectively, figure 2). The time required to convert 50% of the available hemoglobin to methemoglobin was 22.5 min in the absence of PTX (control), whereas it was delayed to 52.3, 94.8, 51.8 and 46.1 min when PTX (80 mM) was added 10 min before nitrite, zero time, 10 min after and 20 min after nitrite addition respectively as shown in table 2.

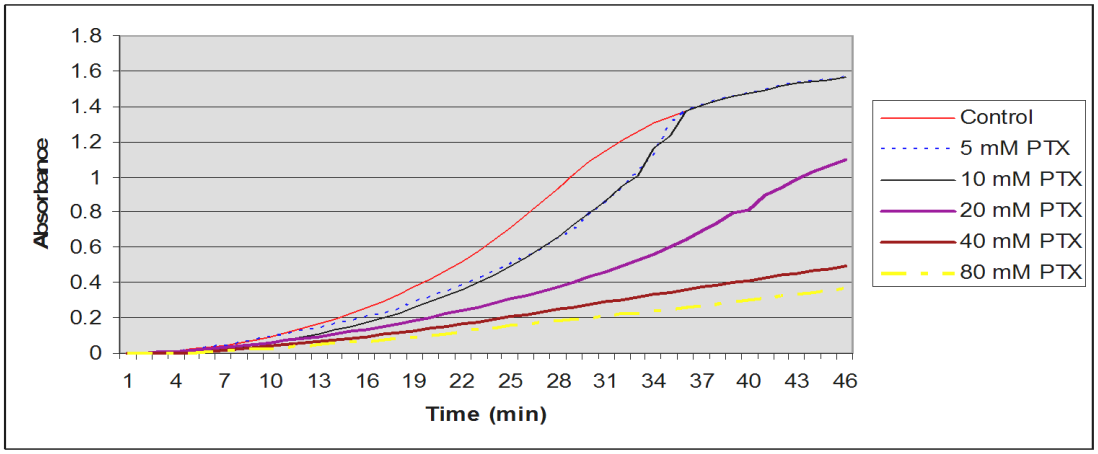

Figure 1. Concentration-effect relationship for the radical scavenging activity of pentoxifylline in nitrite-induced Hb oxidation *in vitro*.

Table 1. Effect of different concentration of PTX on the time-course of nitrite-induced oxidation of Hb and MetHb formation.

Concentration of PTX	% Formation of MetHb	% inhibition of MetHb	Time to form 50% MetHb (min)
5mM	100	0	22.5
10mM	100	0	22.5
20mM	72	28	32
40mM	31.7	68.3	70.9
80mM	23.7	76.2	94.8

All values represent the average of 3 experiments

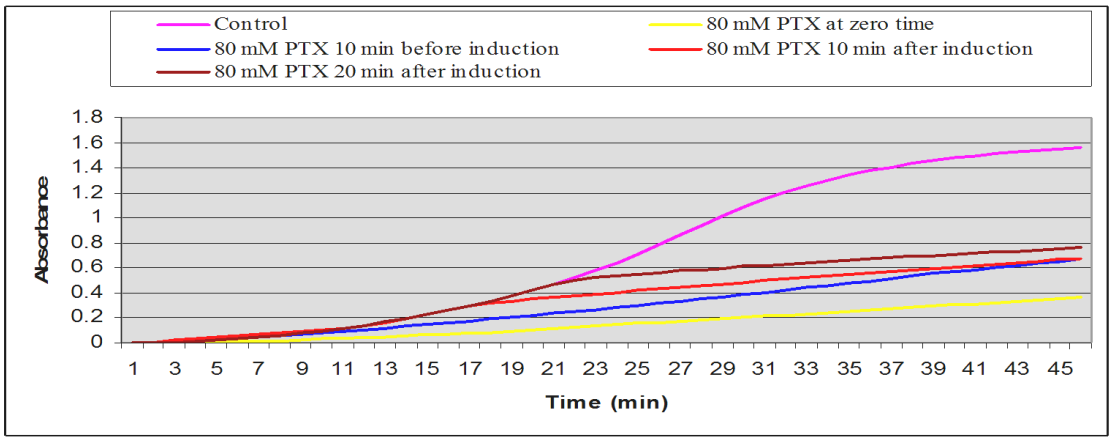

Figure 2. Effect of time course of PTX addition on its radical scavenging activity in nitrite-induced Hb oxidation *in vitro*.

Table 2. Effect of addition of PTX at different time intervals of nitrite addition on the time course of Hb oxidation and MetHb formation in erythrocyte lysate.

Time for addition of 80 mM PTX	% Formation of MetHb	% inhibition of MetHb	Time to form 50% MetHb (min)
Control	100	0	22.5
10 min before induction	43	57	52.3
At zero time	23.7	76.2	94.8
10 min after induction	43	57	51.8
20 min after induction	43.4	56.6	46.1

All values represent the average of 3 experiments

Discussion

In the present study, the data clearly showed that free radicals liberation due to incubation of erythrocyte lysate with sodium nitrite leads to oxidative damage and production of MetHb. Free radicals and their derivatives are known to damage red blood cells resulting in functional and structural alterations [12]. Decreased membrane fluidity, caused by an increase of lipid peroxidation, is a common consequence resulting from the influence of free radicals [13]. and functional aspects of free radical mediated damage to RBC include altered cation permeability and reduced RBC deformability [14,15]. In this *in vitro* study, we investigated the scavenger capacity of different concentrations of PTX; the results showed that this effect was concentration dependent and only predictable in relatively high concentrations. There is considerable evidence of the role of oxygen free radicals as important contributors to cell damage in many blood disorders [16], Acceptance of the free-radical hypothesis has contributed to an increasing interest in the use of free-radical scavengers as potential cytoprotective agents [17]. Horvath et al (2002) reported a mild antioxidant activity of high concentrations of pentoxifylline in an in vitro system [18], and the reported data in the present study are compatible with these finding. However, in the present study, PTX showed an antioxidant capacity; this effect became prominant only at very high concentration, which can be rarely reached in

the clinical practice. This result is concordant with previously reported data, which indicated that PTX had significant antioxidant capacity at very high concentration [17], In a study employing PTX in peripheral vascular disease [19], PTX was found to inhibit the generation of leukocyte-derived active oxygens (superpxide dismutase-inhibitable reduction of ferricytochrome) *in vivo*. Crouch and Fletcher (1992) reported superoxide anion production by polymorphonuclear cells (PMN), and a strong correlation between reduced PMN response to activated complement and plasma concentration of PTX metabolites [10], The same study also suggested that PTX reduced oxygen radical production and protected against unwanted tissue damage *in vivo* via the action of its metabolites. Thus, in addition to PTX alone, evaluation of the radical scavenging capacity of PTX metabolites in the nitrite-induced Hb oxidation system seems to be an option to reveal the exact role of PTX in this respect. In conclusion, in this *in vitro* study, pentoxifylline produced strong radical scavenger effect only in relatively high concentrations, which may be of value in the protection of erythrocytes against oxidative damage; and this idea merits further investigations.

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