Hesperidin Inhibits Nitrite-Induced Methemoglobin Formation

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Hesperidin protects hemoglobin from nitrite-induced oxidation to methemoglobin. The protection was not observed when hesperidin was added after the autocatalytic stage of the oxidation of hemoglobin. The ability of hesperidin to scavenge superoxide may be responsible since superoxide is implicated in promoting the autocatalytic stage of oxidation of hemoglobin by nitrite.

Hesperidin is a potent scavenger of reactive oxygen species like superoxide anion and hydroxyl radical[1]. Its antioxidant property is further shown by its capacity to inhibit lipid peroxidation in rat brain mitochondria[2]. The oxidation of hemoglobin to methemoglobin by nitrite has been widely studied[3-5]. The formation of methemoglobin occurs in two stages. The first is a slow catalytic stage, and the second is a rapid autocatalytic stage[3,4]. Superoxide has been implicated in the autocatalytic stage of oxidation[5], which is inhibited by superoxide dismutase[3]. Since hesperidin is a potent scavenger of superoxide, the present work was undertaken to ascertain its ability to inhibit nitrite-induced oxidation of hemoglobin to methemoglobin.

Curcumin and hesperidin were obtained from Sigma Chemical Co., St. Louis, MO, USA, and sodium nitrite of analytical grade was procured from S. D. Fine Chemicals, Mumbai. Human blood, collected into acid-citrate-dextrose, was from the blood bank of Kasturba Hospital, Manipal.

Blood samples were centrifuged (2500xg, 20 min) to remove plasma and the buffy coat of white cells. Erythrocytes thus obtained were washed three times with phosphate-buffered saline and were hemolysed by suspending them in 20 volumes of 20 mM phosphate buffer, pH 7.4. The hemolysate was then centrifuged at 25 000xg for 60 min to remove the membrane, and then diluted with phosphate buffer (pH 7.4) to yield a final concentration of oxyhemoglobin suitable for spectrophotometric analysis. The reaction was initiated by the addition of sodium nitrite (final concentration, 0.6 mM) to the solution of hemolysate and the formation of methemoglobin was measured by monitoring absorbance at 631 nm[6] using a Shimadzu Graphicord UV 240 Spectrophotometer. Hesperidin was added before or at various time intervals after the addition of nitrite. Control experiments were conducted without the test compound and all experiments were in triplicate. Results were compared to that of standard drug, curcumin.

The washed erythrocyte suspension was incubated with curcumin (standard) or hesperidin for 30 min followed by addition of sodium nitrite (final concentration, 1.8 mM) for further 120 min. The suspension was centrifuged at 2500xg for 20 min to remove excess test compounds and nitrite. The cells were washed thrice with phosphate-buffered saline and lysed with 20 mM phosphate buffer, pH 7.4. The hemolysate was then centrifuged at 25 000xg for 60 min to remove the membrane, and the clear supernatant was removed and absorbance at 631 nm was measured[6]. Control experiments were conducted without the test compound and all experiments were in triplicate. Results were compared to that of standard drug, curcumin. Statistical evaluation of the data was done by one way ANOVA (Graph PAD Instar Software). A value of $p<0.05$ was considered to be significant.

Nitrite causes a rapid oxidation of hemoglobin to methemoglobin. In the presence of hesperidin, the oxidation process was delayed in a dose-dependent manner (Table 1). The time required to convert 50% of the available hemoglo-

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TABLE 1: INHIBITION OF NITRITE-INDUCED METHEMOGLOBIN FORMATION BY HESPERIDIN IN HEMOLYSATE AND ERYTHROCYTES.

Sample	Time to form 50% methemoglobin in hemolysate (min ± S.E.)	% Inhibition of oxidation in erythrocytes (± S.E.)
Control	18.1±1.8	—
Curcumin		
0.02 mM	116.5±5.8[a]	13.5±4.8
0.4 mM	—	48.4±10.8
Hesperidin		
0.02 mM	18.5±2.0[b]	nil
0.2 mM	28.6±1.5[b]	nil
0.4 mM	45.2±1.3[a,b,c]	9.2±0.3

All the values are mean±standard error (S.E.), n=3; Results were analysed by one-way ANOVA. (a) $p<0.05$ compared to control, (b) $p<0.05$ compared to curcumin (0.02 mM) treated group, (c) $p<0.05$ compared to hesperidin (0.02 mM) treated group.

bin to methemoglobin was 18.1 min in control, where as with 0.4 mM hesperidin the time increased to 45.2 min. Different treatment groups were compared by multiple comparison procedure (one-way ANOVA). Although hesperidin showed significant inhibition of hemoglobin oxidation compared to control, the activity was significantly ($p<0.05$) less than that of the standard, curcumin, which showed higher activity at much lower concentrations.

Fig. 1 describes the effect of hesperidin on the time-course of nitrite-induced oxidation of hemoglobin. With out hesperidin, the time-course of oxidation shows a characteristic pattern of slow initial transformation followed by a rapid autocatalytic process. When hesperidin was added along with nitrite, i.e. at 0 min, the formation of methemoglobin was inhibited to a great extent. Addition of hesperidin 5 min after nitrite did not result in significant protection. However, when hesperidin was added at the end of autocatalytic stage, i.e. at 10 min, no protection was observed, it actually accelerated the formation of methemoglobin. Hesperidin was able to inhibit the formation of methemoglobin in intact erythrocytes, but to a very low extent as compared to curcumin. There was no protection when hesperidin was added after nitrite.

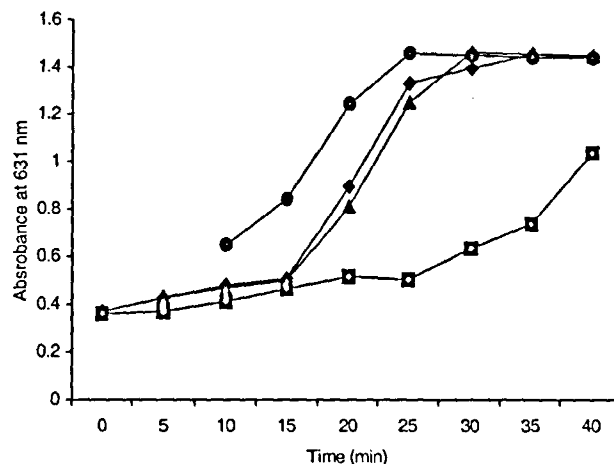

Fig. 1: Effect of hesperidin on time course of methemoglobin formation.

Hemolysate containing hemoglobin (150 μM) was treated with nitrite (0.6 mM). Hesperidin (0.4 mM) was added at 0 min (◘), 5 min (▲), and at 10 min (●). Control without Hesperidin (◆).

The present study has shown that hesperidin can protect hemoglobin from oxidation by sodium nitrite both in hemolysate and intact erythrocytes. However, it did not reverse the effect of nitrite if added at a later stage. It is well established that oxidation of hemoglobin takes place in two stages. There is a slow initial stage followed by a rapid autocatalytic stage, which carries the reaction to completion[3]. Hesperidin was able to prevent the onset of autocatalytic stage. Since superoxide is implicated in the autocatalytic stage[3], and the fact that hesperidin is a potent scavenger of superoxide[1], suggests that the protective action be by scavenging superoxide generated during the oxidation. The inability of hesperidin to protect intact erythrocytes as efficiently as curcumin suggests that they may not cross the membrane of erythrocytes as effectively as curcumin. Many antioxidants like ascorbic acid, uric acid, 3-ribosyl uric acid and glutathione protect hemoglobin from oxidation by nitrite[7]. These antioxidants also inhibit the onset of the autocatalytic stage of nitrite if added at a later stage. Thus the effect of hesperidin may be similar to these antioxidants in protecting hemoglobin from nitrite ions.

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