Ascorbic acid counteracts the prooxidant effect of alloxan in erythrocytes in vitro

Poonam Yadav*, S. Sarkar**, Nisha Rathore, Susan John, Manisha Kale and D. Bhatnagar

Department of Biochemistry, D.A. University, Khandwa Road, Indore 452 001, India
*Department of Biochemistry, **Centre for Reproductive Biology and Molecular Endocrinology, Indian Institute of Science, Bangalore 560 012, India

Alloxan-induced experimental diabetes has been reported due to the production of toxic O² and OH radicals and H₂O₂. Alloxan induced lipid peroxidation (LPO) in rat erythrocytes in the absence of ascorbic acid (AA). Superoxide dismutase (SOD) activity decreased while catalase (CAT) activity increased in erythrocytes with alloxan treatment without AA. Alloxan treatment in presence of AA showed no significant changes in LPO, SOD and CAT activities in erythrocytes, indicating neutralization of alloxan induced free radical production. Treatment with glucose in presence of AA showed no significant changes in LPO and SOD and CAT activities in erythrocytes. Erythrocytes incubated with alloxan and glucose without AA showed increased LPO and decreased activity of SOD and CAT. However, LPO decreased and enzyme activities were comparable to control when treatment with alloxan and glucose was followed in presence of AA.

Alloxan is known to cause cytotoxicity to β-cells of pancreas by producing highly reactive free radical species. The action of alloxan is inhibited by superoxide dismutase (SOD) and the enzyme has been shown to have therapeutic value in alloxan-induced diabetes. These and other studies showed the involvement of free radical species such as O₂ and OH radicals in the action of alloxan. In the present study, rat erythrocytes exposed to alloxan have been used as an in vitro model to show the action of alloxan. As the cyclic reaction involving alloxan and its reduced product dialic acid spontaneously produce O₂ and OH radicals and H₂O₂ (refs 2, 3), it was intended to show whether alloxan causes changes in antioxidant enzymes in the erythrocytes. Further, whether addition of free radical scavengers such as ascorbic acid (AA) inhibits the action of alloxan. Ascorbic acid was used to investigate whether it protects erythrocytes from the prooxidant effect of alloxan. Glucose was added in physiological amounts to erythrocytes to compensate for any glucose loss during incubation and to observe the effects of alloxan in presence of glucose.

In the present study, the action of alloxan in vitro and the protective action of AA on erythrocyte lipid peroxidation (LPO) and antioxidant enzymes such as superoxide dismutase (SOD) and catalase (CAT) were determined. The report indicates that alloxan induced LPO and changes the activities of SOD and CAT, and the action is mitigated by AA in the rat erythrocyte system.

Male Wistar rats weighing 150-180 g were housed in polypropylene cages under standard conditions with free access to drinking water and basal diet. The animals
were sacrificed by decapitation and blood was collected in 2% citrated vials by heart puncture. Blood was centrifuged and erythrocytes were washed twice in 0.1 M phosphate buffered saline (PBS, 1:9, v/v) pH 7.4 and adjusted to 5% packed cell volume (PCV). The 5% PCV was incubated in 0.1 M PBS pH 7.4 along with glucose (5 mM), alloxan (100 mM) or both with (5 mM) or without AA and incubated at 37°C for 1 h in a well-oxygenated water bath. Alloxan, glucose and AA were prepared fresh in 0.1 M PBS pH 7.4 and added immediately to the mixture. In our earlier studies, the amount of alloxan used showed significantly increased LPO in erythrocytes incubated with alloxan. The erythrocytes were washed thrice in PBS and were used for the assays.

LPO was estimated as malonidialdehyde (MDA) formed by thiobarbituric acid (TBA) reaction. Two ml of 5% PCV was exposed to 10 mM H₂O₂ and 2 mM sodium azide in a total volume of 4 ml 0.1 M phosphate buffer pH 7.4. The mixture was incubated at 37°C for 1 h followed by the addition of 2 ml trichloroacetic acid (28%). The cell suspension was centrifuged at 1000 g for 5 min. Four ml of the supernatant was transferred to a boiling tube to which 1 ml TBA (1%) was added and the contents were boiled for 15 min, cooled immediately and the absorbance at 532 nm was recorded in a spectrophotometer. Blank sample was prepared without H₂O₂.

SOD and CAT were analysed in erythrocyte lysate prepared by the method of McCord and Fridovich. Each enzyme assay was performed in duplicate in two-fold concentration range. The SOD activity was determined by the method of Marklund and Marklund by the ability of the enzyme to inhibit the autooxidation of pyrogallol. CAT was assayed by the measurement of the decomposition of H₂O₂ (ref. 9). Haemoglobin was estimated as described by Dacie and Lewis. Protein content in the haemolysate was determined by the method of Lowry et al.

The statistical analysis was done using Student's t-test and the probability level of less than 5% was considered significant.

Erythrocytes were exposed to glucose (5 mM), alloxan (100 mM) or both with (5 mM) or without the presence of AA (Table 1). Glucose in physiological amounts (5 mM) showed no significant change in LPO with or without AA compared to the respective control. Alloxan increased LPO in erythrocytes compared to its control in absence of AA. However, in presence of AA, alloxan treatment showed no significant change in LPO compared to control. Alloxan and glucose without AA stimulated an increase in LPO. However, such an increase in LPO was not observed in alloxan and glucose-treated erythrocytes in presence of AA. On the contrary, the presence of AA decreased LPO in erythrocytes induced by alloxan and glucose as compared to its control.

Erythrocytes in presence of glucose without AA showed increase in SOD and CAT activities (Table 2), however, glucose in presence of AA showed no significant increase in these enzymes as compared to respective controls. Alloxan treatment without AA decreased SOD activity while the CAT activity increased in erythrocytes as compared to respective controls. In presence of AA, alloxan showed no significant change in SOD and CAT activity as compared to control. Alloxan and glucose treatment without AA inhibited SOD and CAT activity in erythrocytes. However, such an inhibition in SOD and CAT activities by alloxan and glucose treatment was overcome in presence of AA.

Alloxan induced LPO and altered the reactive oxygen scavenger enzymes such as SOD and CAT, and the effect of alloxan was mitigated with AA in rat erythrocytes. The plasma AA at increased concentrations has been reported to be an effective scavenger of free radicals.

| Table 1. Effect of various treatments on LPO in erythrocytes |
|------------------|------------------|------------------|
| Treatment        | −AA              | +AA              |
| Control          | 277 ± 08         | 230 ± 10         |
| Glucose          | 295 ± 16         | 249 ± 09         |
| Alloxan          | 354 ± 11         | 206 ± 15         |
| Alloxan + glucose| 400 ± 16         | 185 ± 22         |
|                  | P < 0.001        | P < 0.05         |

LPO was expressed as nmols of MDA formed/hg Hb. Values are mean ± SD of 6 animals. Erythrocytes were incubated in 0.1 M PBS pH 7.4 at 37°C for 1 h with glucose (5 mM), alloxan (100 mM) or both at the concentration shown, with (5 mM) or without ascorbic acid (AA). The values in treated groups were compared with respective controls. The value of LPO in erythrocytes without incubation (0 h) was 252 ± 10 nmols of MDA formed/hg Hb.

Table 2. Effect of various treatments on the antioxidant enzymes in erythrocytes

<table>
<thead>
<tr>
<th>Treatment</th>
<th>SOD</th>
<th>CAT</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>−AA</td>
<td>+AA</td>
</tr>
<tr>
<td>Control</td>
<td>269 ± 11</td>
<td>275 ± 15</td>
</tr>
<tr>
<td>Glucose</td>
<td>349 ± 19</td>
<td>299 ± 09</td>
</tr>
<tr>
<td>Alloxan</td>
<td>200 ± 10</td>
<td>257 ± 15</td>
</tr>
<tr>
<td>Alloxan + glucose</td>
<td>133 ± 12</td>
<td>298 ± 11</td>
</tr>
<tr>
<td></td>
<td>P &lt; 0.001</td>
<td>P &lt; 0.001</td>
</tr>
</tbody>
</table>

SOD and CAT activities are expressed as units/mg protein and nmols of H₂O₂ decomposed/min/mg protein, respectively. Values are mean ± SD of 6 animals. Erythrocytes were incubated in 0.1 M PBS pH 7.4 at 37°C for 1 h with glucose, alloxan or both with or without ascorbic acid (AA) at the concentrations shown in Table 1. The values were compared with respective controls. The value of SOD and CAT in erythrocytes without incubation (0 h) was 267 ± 5 units/mg protein and 267 ± 5 nmols of H₂O₂ decomposed/min/mg protein, respectively.
radicals in aqueous phase, more effective than any other endogenous antioxidant.\textsuperscript{12} Alloxan induced LPO in rat erythrocytes, however, such an effect was not observed in presence of AA. Glucose in physiological amounts showed no significant change in erythrocyte LPO with or without AA. However, high amounts of glucose have been shown to produce toxic oxygen species in presence of transition metals.\textsuperscript{13}

Alloxan with or without glucose increased oxidative reaction and generation of oxygen-free radicals. These effects of alloxan were observed in absence of AA. In presence of AA no significant increase in erythrocyte LPO was observed in alloxan-treated erythrocytes with or without glucose, suggesting the role of$O_2^-$ anion in the induction of alloxan-induced LPO in erythrocytes. AA acts as scavenger of the free oxygen species, thus lowering LPO in erythrocytes treated with AA.

The increase in SOD and CAT activity due to glucose in absence of AA may be an attempt by erythrocytes to counteract any oxidative change. However, in presence of AA, no significant change was observed in erythrocyte SOD and CAT activities or in LPO, which indicate that free radical species are effectively neutralized by AA.

Alloxan due to the production of free radical species such as$O_2^-$ inhibited SOD activity which may be one of the reasons for increased LPO in erythrocytes. The increase in CAT activity may be due to increased production of$H_2O_2$ in presence of alloxan. As the changes in SOD and CAT activity in alloxan-treated erythrocytes recover towards normal in presence of AA, it is suggested that these enzymes are modified in presence of alloxan and the action is mitigated by AA. Reactive oxygen species (ROS) not only induce LPO but also modify enzymes. The involvement of ROS in the modification of protein kinase C has been suggested to be an effective on/off signal mechanism to influence cellular events.\textsuperscript{14} ROS has been shown to induce signalling including activation of protein kinase, induce protein phosphorylation and also act as second messenger for the expression of genes involved in the immune response.\textsuperscript{15} It is probable that ROS may modify reactive oxygen scavenger enzymes and in presence of AA may show dual activation-inactivation of these enzymes.

It is concluded that alloxan due to the production of free radical species increased LPO and altered antioxidant enzymes in erythrocytes. The treatment with glucose in physiological amounts showed no effect on erythrocyte LPO but increased SOD and CAT in glucose-treated erythrocytes in absence of AA. However, no significant changes were observed in LPO, SOD and CAT activities in erythrocytes treated with glucose in presence of AA. Alloxan and glucose treatment in absence of AA increased LPO and decreased SOD and CAT activities in erythrocytes. None of these changes was observed in presence of AA. Thus, it may be one of the processes, how the cells respond to and mitigate the ill effects of reactive oxygen intermediates engendered in biological systems by diverse processes.


Received 7 February 1996; revised accepted 1 August 1996

---

Plant extracts: A non-chemical approach to control Fusarium diseases of mulberry

V. P. Gupta, Govindiah and R. K. Datta

Central Sericultural Research and Training Institute, Srirampura, Mysore 570 008, India

Leaf extracts of Azadirachta indica, Calotropis gigantea, Eucalyptus sp., Parthenium hysterophorus and Pongamia pinnata were evaluated for their antifungal activity against Fusarium pallidoroseum and F. moniliforme var. intermedium causing leaf blights, and F. oxysporum causing leaf spot diseases in mulberry (Morus alba L.). Leaf extract (1:5) of P. pinnata was highly fungitoxic to F. pallidoroseum and F. moniliforme var. intermedium inhibiting their mycelial growth in plates by 78.2% and 84.3%, respectively; whereas C. gigantea and A. indica were most effective.

CURRENT SCIENCE, VOL. 71, NO. 5, 10 SEPTEMBER 1996