Effects of proanthocyanidin and methotrexate on glucose-6-phosphate dehydrogenase (G6PD) and glutathione reductase (GR) in an animal model

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Abstract

This study was designed to determine the effects of orally administered proanthocyanidins (PAs) and methotrexate (MTX) on glucose-6-phosphate dehydrogenase (G6PD) and glutathione reductase (GR) activities in serum and erythrocytes of Wistar rats. Seventy Wistar rats were divided into 3 groups: Group 1 included 30 rats administered 1.36 mg/kg of MTX and 30 rats of Group 2 were administered 0.87 mg/kg of PAs. Ten rats of Group 3 were maintained only on normal rat chow and water ad libitum and served as the control group. Five rats each from groups 1 and 2 were sacrificed weekly for 6 weeks while 5 rats from the control group were sacrificed on days 0 and 42, respectively. In all the groups, the serum and erythrocytes were analyzed spectrophotometrically for the activities of G6PD and GR and the results were compared with the control group. After 42 days of PAs administration insignificant differences (P>0.05) were observed in the activities of the two enzymes and there was also no significant difference (P>0.05) in the level of MDA when compared to the control group. However, the results showed significant differences (P<0.05) in the activities of both G6PD and GR even within 3 weeks of methotrexate administration. The MDA level was also significantly different from the control group following MTX administration for 21 and 42 days.

Key words: Oxidative stress, bioflavonoids, enzymes, supplement, drug administration.

Introduction

Proanthocyanidins are a group of polyphenolic bioflavonoids which are known to possess broad pharmacological activities and therapeutic potential 1. Proanthocyanidins are also called oligomeric procyanidins (OPCs) or procyanidolic oligomers (PCOs) and are known to protect against oxidant injuries during ischemia/reperfusion in rat heart 2. In a preliminary study, proanthocyanidins were reported to have anti-mutagenic activity and have been shown to improve aspects of vision in healthy people 3-6. Proanthocyanidins have been reported to protect against H2O2 induced oxidative damage in cultured microphages and neuroactive PC-12 cells and in rat primary glial cell cultures 6. Oligomeric proanthocyanidins significantly ameliorated free radical induced lipid peroxidation and DNA fragmentation in rat liver and brain tissues and provided better protection than vitamins C, E and β-carotene in a close relation 6,7.

Shao et al. 8 reported that proanthocyanidins attenuated both oxidant generation and cell death indicating its ability to confer protection against mitochondrial endogenous reactive oxygen species. Methotrexate, on the other hand, is a potent anticancer drug and found to be effective against a wide range of human cancers 8. It has been shown to inhibit dihydrofolate reductase (DHFR), a key enzyme required to maintain adequate intracellular levels of reduced folates 11. Furthermore, DHFR inhibition led to the accumulation of dihydrofolate polyglutamates within the cell, which can directly inhibit the folate dependent enzymes involved in the synthesis of thymidylate and purine nucleotides 10. However, the clinical uses of methotrexate have been limited due to its serious adverse effects which are likely to result in the generation of free radicals and lipid peroxidation 11. These adverse effects include anaemia, neutropaenia, increase risk of bruising and nausea 11. The higher doses of methotrexate often used in cancer chemotherapy can cause toxic effects to the rapidly dividing cells of bone marrow and gastrointestinal mucosa 11.

Several oxidants are produced during the course of metabolism in both blood cell and most other cells of the body 12. Glucose-6-phosphate dehydrogenase plays an important role in the catabolism of glucose through the pentose phosphate pathway (PPP) 13. Glucose-6-phosphate dehydrogenase supplies reducing equivalents to cell (notably erythrocytes) by maintaining the level of the co-enzyme NADPH. The NADPH in turn maintains the level of glutathione in these cells that helps protect the red blood cells against oxidative damage. In the states of oxidative stress, the rate of utilizing remaining glutathione significantly increases 14.

The objectives of this study was to evaluate the effects of orally administered PAs and MTX on glucose-6-phosphate dehydrogenase and glutathione reductase activities and the MDA levels in the serum and erythrocytes of Wistar rats.
Materials and Methods

Experimental animals: Seventy Wistar rats with a mean weight of 171.7g were obtained from the animal house of the Nigerian Institute of Medical Research (NIMR), Yaba, Lagos. The animals were housed in stainless laboratory cages and acclimatized for 7 days and maintained on standard rat chow and water ad libitum throughout the duration of the experiment. The animals were housed in a well-ventilated room under 12 h light: 12 h dark cycle. The rats were divided into 3 groups. Groups 1 and 2 consisted of 30 rats while Group 3 (the control group) consisted of 10 rats.

Drug administration: The animals in Group 1 were orally administered 1.36 mg/kg/bw of methotrexate daily for 6 consecutive weeks while the animals in Group 2 were orally administered 0.87 mg/kg/bw of proanthocyanidin for the same period. The control group received distilled water for the same duration.

Collection of blood samples and separation of serum: On weekly basis for 6 weeks, 5 rats from Groups 1 and 2 were sacrificed under chloroform anaesthesia and their blood was quickly collected, pooled and separated into serum and erythrocyte fractions by spinning at 1500 g for 6 min in a centrifuge. Rats in the control group were sacrificed on the first and last day of the experiment using the same processes for comparison. The sera were stored in a refrigerator immediately while the erythrocyte haemolysate was processed.

Preparation of haemolysate: The red blood cells (RBCs) earlier obtained were watched free of leucocytes in normal saline through centrifugation at 2500 g for 5 min and repeated three times. The rbc’s were then haemolysed with 5 volume ice cold distilled water and spun in a high speed centrifuge of 13,000 g for 15 min. The haemolysate was aspirated from the membrane ghost and stored at 4°C in a refrigerator.

G6PD activities in serum and erythrocyte: G6PD assays were done by using the method of Beutler 15. This assay measures the rate of reduction of NADP+ to NADPH when the haemolysate or serum is incubated with glucose-6-phosphate (G6P). The assay mixture consisted of 6 mM glucose 6-phosphate (0.1 ml), 2 mM NADP+ (0.1 ml), MgCl₂ (15 mM), Tris buffer pH 8.0 (0.68 ml) and either haemolysate or serum (0.02 ml). The assay for haemolysate and serum was monitored with a background wavelength of 550 nm and a real wavelength of 340 nm over a 5 min time course on the spectrophotometer at 25°C. A full-scale reading of 1.0 absorbance unit was maintained for the spectrophotometer (Beckmann 7400). G6PD activity at 37°C was calculated in haemolysates from the equation A = 1000A/Hb where A = number of enzyme units per ml and expressed as A = ΔAbs at 340/6.22 X VC/VA , where 6.22 is absorbance of 1 ml solution of NADPH). VA = volume of hemolysate or homogenate in the system. VC = volume of cuvette (1.0 ml). Hb = hemoglobin concentration in g/100 ml. G6PD activities (E) in serum were evaluated against total protein without the 1000 factor in the numerator, that is, E = A/total protein (mg/ml). The conversion factor from 25 to 37°C is given as G6PD activity at 25°C = 0.559 + 0.034 G6PD activity at 37°C.

Determination of GR activities: Activity was determined by following the oxidation of NADPH to NADP⁺ during the reduction of oxidized glutathione (GSSG) 16. The main reagent was prepared by combining 18 ml of KH₂PO₄ buffer (13.9 mmol/l, 0.76 mmol/l, EDTA; pH 7.4) and 2.00 ml of NADPH (2.5 mmol/l). The sample (20 µl), 220 µl of the main reagent, and 5 µl of FAD (315 µmol/l) plus 10 µl of KH₂PO₄ buffer were added to the cuvette, and the absorbance at 340 nm was monitored for 200 s (Step A). Then 30 µl of GSSG (22 mmol/l; Sigma) plus 10 µl of KH₂PO₄ buffer was added to start the reaction and the absorbance was followed for specified time. The final reaction volume was 315 µl. Using a molar absorptivity of NADPH at 6.22 × 103 L mol⁻¹ cm⁻¹ used the difference in absorbance per minute between steps B and A to calculate the enzyme activity. The unit is µmol of NADPH oxidized/min.

Statistical analysis: The data were analysed by using the two-tailed Student’s t-test and the level of significance was set at P<0.05.

Results and Discussion

The G6PD and GR were assayed from the serum and erythrocyte haemolysate of Wistar rats orally administered proanthocyanidins and methotrexate. The activities obtained were compared with that of control. The results are summarized in Tables 1-4.

There are many chemical compounds that are known to have adverse or beneficial effects on metabolic enzymes. The inhibition of some important enzymes that play a key role in metabolic pathway by these compounds may lead to physiological and/or pathologic changes. Glucose-6-phosphate dehydrogenase (G6PD), a vital enzyme in all cells, catalyzes the first reaction of the pentose phosphate pathway (PPP) allowing the conversion of glucose-6-phosphate to 6-phosphogluconolactone. In this reaction, nicotinamide adenine dinucleotide phosphate (NADP) is reduced to NADPH, which is also used in the protective process against physiologically high levels of reactive oxygen species and subsequently oxidative damage 17. In red blood cells, this is the only source of NADPH, making them more vulnerable than other cells to destruction by oxidative stress 18. NADPH is a key hydrogen donor for the reduction of oxidized glutathione (GSSG) to a tripeptide known as reduced glutathione (GSH). This tripeptide is used as a reducing agent by glutathione peroxidase, which is involved in the detoxification of hydrogen peroxide. In this process, GSH is converted to GSSG leading to a decrease in GSH levels. The regeneration of GSH occurs by the action of glutathione reductase, which catalyzes the reduction of GSSG to GSH in the presence of NADPH 19. Since there are no other sources of NADPH in red blood cells, G6PD is essential in protecting haemoglobin sulphhydril groups and preventing red blood cell membrane oxidation, therefore inhibition or destruction of G6PD and glutathione reductase adversely affects cellular integrity and cells’ ability to protect against reactive oxygen species 20.

It is important to note that proanthocyanidins (PAs) (one of the compounds administered to the rats in this study) are a common component of foods and beverages of plant origin 21. PAs, among the most abundant phenolic compounds in grape seeds, are present in substantial amounts in red wine when fermentation takes place in the presence of skin and seeds, thus PAs could at least in part account for the protective effect of red wine with respect to atherogenesis and cardiovascular disease. PAs have

attracted increasing attention in the fields of nutrition and preventive medicine due to their potential health effects. *In vitro*, PAs have strong antioxidant activity and scavenge reactive oxygen and nitrogen species, modulate immune function and platelet activation and produce vasorelaxation by inducing NO release from endothelium.

In this study, PAs was administered to Wistar rats and their effects on the G6PD and glutathione reductase activities in the serum and erythrocytes were studied. PAs did not demonstrate a significant effect on G6PD activity in both serum and erythrocytes following 21 and 42 days of administration when compared to the control (Tables 1 and 2). The effect of PAs on glutathione reductase (GR) in both serum and erythrocytes in this study is similar to that reported for G6PD (insignificantly different from the control group). The effect of PAs on the MDA level was also not significantly different from that of the control group. PAs is known to have antioxidant activity, however, this was not the case in the current study. This may partly be related to the concentration used in our study. Previous study by Sato et al. used 50 mg/l and they reported significant antioxidant activity compared to the control. In our study, a far less concentration of PAs was used, hence the expected antioxidant activity of PAs could not be noticed. The biological, pharmaceutical and medicinal properties of PAs have been reported.

**Table 1. Effects of proanthocyanidin on G6PD activities in rat serum and erythrocyte haemolysate.**

<table>
<thead>
<tr>
<th>Samples</th>
<th>21 days MEAN ± S.D</th>
<th>42 days MEAN ± S.D</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>SERUM</td>
<td>3.14 ± 0.33</td>
<td>2.99 ± 0.67</td>
<td>P&lt;0.05</td>
</tr>
<tr>
<td>ERYTHROCYTE</td>
<td>6.19 ± 0.76</td>
<td>6.39 ± 0.49</td>
<td>P&lt;0.05</td>
</tr>
</tbody>
</table>

The control of G6PD activities in serum and erythrocytes are 3.11 ± 0.41 and 6.43 ± 0.91, respectively. Mean was average of three readings from sets of experimental groupings. T was calculated and determined against the control values in the serum. P<0.05 is significantly while P>0.05 is insignificantly different from the control.

**Table 2. Effects of proanthocyanidin on glutathione reductase activities in rat serum and erythrocyte haemolysate.**

<table>
<thead>
<tr>
<th>Samples</th>
<th>21 days MEAN ± S.D</th>
<th>42 days MEAN ± S.D</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>SERUM</td>
<td>3.01 ± 0.32</td>
<td>3.10 ± 0.88</td>
<td>P&lt;0.05</td>
</tr>
<tr>
<td>ERYTHROCYTE</td>
<td>5.13 ± 0.41</td>
<td>5.19 ± 0.36</td>
<td>P&lt;0.05</td>
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</tbody>
</table>

The control of GR activities in serum and erythrocytes are 2.64 ± 0.33 and 5.18 ± 0.39, respectively. Mean was average of three readings from sets of experimental groupings. T was calculated and determined against the control values in the serum. P<0.05 is insignificantly while P>0.05 is insignificantly different from the control group. MDA level (control 2.15 ± 0.85) and for PAs group (2.31 ± 0.62) P<0.05.

**Table 3. Effects of methotrexate on G6PD activities in rat serum and erythrocyte haemolysate.**

<table>
<thead>
<tr>
<th>Samples</th>
<th>21 days MEAN ± S.D</th>
<th>42 days MEAN ± S.D</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>SERUM</td>
<td>4.61 ± 0.29</td>
<td>10.38 ± 1.05</td>
<td>P&lt;0.05</td>
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<tr>
<td>ERYTHROCYTE</td>
<td>9.87 ± 1.13</td>
<td>5.19 ± 0.36</td>
<td>P&lt;0.05</td>
</tr>
</tbody>
</table>

The control of G6PD activities in serum and erythrocytes are 3.11 ± 0.41 and 6.43 ± 0.91, respectively. Mean was average of three readings from sets of experimental groupings. T was calculated and determined against the control values in the serum. P<0.05 is insignificantly while P>0.05 is insignificantly different from the control.

**Table 4. Effects of methotrexate on GR activities in rat serum and erythrocyte haemolysate.**

<table>
<thead>
<tr>
<th>Samples</th>
<th>21 days MEAN ± S.D</th>
<th>42 days MEAN ± S.D</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>SERUM</td>
<td>3.91 ± 0.32</td>
<td>4.43 ± 0.34</td>
<td>P&lt;0.05</td>
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<tr>
<td>ERYTHROCYTE</td>
<td>8.11 ± 0.70</td>
<td>8.21 ± 0.88</td>
<td>P&lt;0.05</td>
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</table>

The control of glutathione reductase activities in serum and erythrocytes are 2.64 ± 0.33 and 5.18 ± 0.39, respectively. Mean was average of three readings from sets of experimental groupings. T was calculated and determined against the control values in the serum. P<0.05 is insignificantly while P>0.05 is insignificantly different from the control. MDA level (control 2.15 ± 0.85) and for MTX group (4.63 ± 0.63) P<0.05.

Future studies that would investigate proanthocyanidins-methotrexate combination effects are suggested to determine.
whether the simultaneous administration of the two xenobiotics could be beneficial in the case of cancer where MTX is sometimes administered.

Conclusions
This study shows that PAs did not demonstrate significant effects on G6PD and GR However, MTX administration after three weeks showed significant effects on the same enzymes as well as on the MDA. Future studies that would investigate PAs-MTX combination effects are suggested to determine whether the simultaneous administration of the two xenobiotics could be beneficial in the case of cancer where MTX is sometimes administered.

References