Protective role of aqueous medicinal herbal extracts against oxidative stress on Glucose-6-phosphate dehydrogenase activity and red blood cells fragility

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Abstract: We investigated the protective effect of the aqueous extracts of Salvia officinallas, Thymus vulgaris and trigonella Foem–graecum against oxidative stress on Glucose-6-phosphate dehydrogenase (G6PD) activity and red blood cells (RBC) hemolysis. The NO donor compounds nitrosocysteine and nitrosoarginine caused strong inhibition on G6PD activities. Those oxidative compounds promoted RBC hemolysis in parallel to their inhibition extents on G6PD activities. The complete protection of both G6PD activity and red blood cells hemolysis against oxidative stress induced by NO donor compounds was achieved by preincubation with the above mentioned aqueous extracts. Those extracts were able to protect hemolysis in parallel to their activation extents on G6PD activities. Compared to nitrosoarginine, nitrosocysteine showed more effect on both G6PD activity and RBC hemolysis. Thus, more aqueous extracts concentration is needed for protection against nitrosocysteine.


Keyword: Salvia officinallas; Thymus vulgaris; trigonella Foem–graecum extracts; Glucose-6-phosphate dehydrogenase; Nitrosocysteine; Nitrosoarginine; aqueous extract.

1. Introduction:

Glucose-6-phosphate dehydrogenase is the first enzyme in the pentose phosphate pathway, with main physiological function to produce NADPH and pentose sugars (Sodeinde, 1992; Mehta, 1994; Prchal and Gregg, 2005). G6PD is not the sole NADPH producing enzyme in nucleated cell, but in red blood cell only G6PD enzyme can generate NADPH (Scriver, 1995). The NADPH plays important role in erythrocytes, by preserving the integrity of red blood cell membrane sulphhydryl groups and detoxifies harmful hydrogen peroxide and oxygen radicals. Also it is used in the regeneration of reduced glutathione which prevents hemoglobin denaturation (Ciftci et al., 2004; Abboud and Awaida, 2010).

Hemolysis is the destruction or removal of red blood cells from the circulation before the completion of their 120 days normal life span (Beutler, 1959; Bowman et al., 2004). Decrease in G6PD enzyme activity leads to decrease in NADPH and reduced Glutathione. It is believed that a lack of reduced glutathione availability causes early hemolysis in the spleen (Tian et al., 1999). G6PD deficient erythrocytes are sensitive to oxidative stress (Gerli et al., 1982).

A deficiency in G6PD activity was connected with increases accumulation of cellular reactive oxygen species (ROS) while an overexpression of G6PD activity decreases the accumulation of these species in response to oxidative stress (Leopold et al., 2003; Fico et al., 2004). Sodium nitroprusside, spermidine and nitroso derivatives are nitro compounds that act as oxidants (Kuhn and Arthur, 1997; Gobble et al., 1997; Xian et al., 2000; Abboud and Awaida, 2010). Nitric oxide (NO) and its derivatives directly inactivate Glutathione peroxidase enzyme, resulting in an increase of intracellular peroxides that are responsible for cellular damage (Asahi et al., 1997).

Herbs and spices have been widely used as food flavors for natural antioxidants. Spices and aromatic herbs are considered to be essential in diets or medical cures for delaying aging and biological tissue deterioration (Frankel, 1996). Salvia officinallas is an old medicinal plant, which is rich source of different chemical constituents including terpenoids, polyphenol as well as essential oils (Wang et al., 2000; Bozin et al., 2007). Salvia officinallas extract is used to stabilize fat and fat-containing food against oxidation (Schwarz and Ternes, 1992). The main antioxidant compounds in the sage are carnosic acid and carnosol rosmarinic acid (Cuvelier et al., 1996).

Thymol and carvacrol were found to be the major components of Thymus vulgaris essential oil. However, the thymol showed more effectiveness than carvacrol in preventing oxidation during the autoxidaion of lipids (Yanishlieva, 1999; El-
Nekeety et al., 2011). Aqueous extract of thyme showed a degree of reductive and radical scavenging capacity (Dorman et al, 2003). Fenugreek (trigonella Foem–graecum) being rich in phytochemical components have encouraged its use as medicinal plant (Zaman et al 1998; Dixit et al, 2005). Simultaneous administration of aqueous extract of fenugreek seeds with ethanol for 60 days in rats prevented the enzymatic leakage and rise in lipid peroxidation and enhanced antioxidant potential thus offering protection against ethanol toxicity (Thirunavukkarasu et al., 2003). This work aims to investigate protective role of aqueous medicinal herbal extracts against oxidative stress on both Glucose-6-phosphate dehydrogenase activity and RBC fragility

2. Materials and methods

The protocol for this study was approved by the Ethics Committee of the science faculty of American University of Madaba (AUM) and all subjects gave their informed consent.

Preparation of human erythrocytes

Fresh heparinized blood samples from healthy subjects or patients were centrifuged under cooled conditions for 5 min at 1000 rpm. After removing plasma, the washed buffy coat pellet was resuspended in phosphate buffered saline (PBS) at pH 7.4 (Masahiko et al., 2002).

Analysis of nitric Oxide (NO) donor compounds (Nitroso amino acids) induced hemolysis

The erythrocyte suspension was incubated with different concentration of the oxidizing agent for 60 min at 37 °C. The sample was then centrifuged at 1000 rpm for 10 min and the absorbance of the supernatant at 415 nm was measured spectrophotometrically. The extent of hemolysis was expressed as a percentage value relative to complete hemolysis of similar sample (blank) in deionized water. A control experiment was conducted under similar conditions without adding oxidizing agent.

Preparation of enzyme extract and determination of enzyme activity

Fresh unhemolyzed blood sample was collected and kept no longer than 7 days at 2–8 °C before being processed for G6PD extraction. A blood sample of 200 μL was washed 3 times with 2 mL aliquots of saline solution. The washed pellet was re-suspended in solution of digitonin (0.02%) and sodium azide (0.1% w/v) then allowed standing for 15 min at 2–8 °C. After centrifugation the supernatant was used as an enzyme preparation (enzyme extract) within 2 h of the extraction procedure. Activity of the enzyme G6PD was measured kinetically. An extracted fresh enzyme of 15 μL was added to a cuvette with 900 μL of 50 mM Tris buffer at pH 7.6 containing 1mM MgCl2 and a substrate glucose 6-phosphate at 500 μm concentration. The reaction was initiated by adding NADP+ (250 μm) and the rate of increase in absorbency at 340 nm due to the conversion of NADP+ to NADPH was determined for a period of 2 min (Tian et al., 1994).

Preparation of nitric Oxide (NO) donor compounds (Nitroso amino acids 10 mM):

The Nitroso amino acids (10 mM) were prepared freshly according to Hogg’s method (Hogg, 1999).

Preparation of aqueous medicinal herbal extracts:

Leaves from Salvia officinalis, Thymus vulgaris and seeds from Fenugreek (trigonella Foem – graecum) were harvested and dried in the dark at 35°C, before being stored at -20°C in paper bags until analysis. The aqueous extracts were prepared by boiling the dried plant material with 12 times the volume of water (wt/vol) for 1 h. After filtration and concentration of the supernatant under water suctioning, the sample was dried in oven at 50 °C (Dragland et al., 2003).

Antioxidants protection against the nitric Oxide (NO) donor compounds (Nitroso amino acids) caused inhibition of G6PD activity:

Aqueous extract with different concentrations (0.01%, 0.02%, 0.03%, 0.08%, 0.1%) were preincubated for 5 minutes with 15μl of G6PD enzyme before adding nitric Oxide (NO) donor compounds. Each measurement was repeated three times and the mean values were calculated.

Statistical analysis

Descriptive statistical analysis was performed with SPSS.

3. Results

Effects of nitric oxide (NO) donor compounds on G6PD activity:

The effects of NO donor compounds nitrosoarginine and nitrosocysteine on G6PD activity were investigated. Each oxidant was added to the enzyme assay at concentration range of 0.01-2 mM. Before testing nitroso derivatives, the effects of their corresponding amino acids (arginine and cysteine) on enzyme activity were examined. Neither cysteine nor arginine produced effects on G6PD activity (Figures 1). In contrast, the nitric oxide (NO) donor compounds nitrosoarginine and nitrosocysteine showed inhibition effects on G6PD activity (Figures 2). The calculation of I50 values (concentration of the compound that gives 50% inhibition in enzyme activity) showed 950 μm for nitrosoarginine and 300 μm for nitrosocysteine.
Figure 1: Effect of amino acids cysteine and arginine on G6PD enzyme activity. Different concentrations of amino acids cysteine and arginine were added directly to the assay G6PD enzyme. Using control experiments without amino acids (100% activity).

Figure 2: Effect of nitric oxide (NO) donors on G6PD activity. Different concentrations of nitrosoarginine and nitrosocysteine were added directly to the assay G6PD enzyme. Using control experiments without nitric oxide (NO) donors compounds (100% activity).

Effect of preincubation time on the reversibility of G6PD inhibition by nitric oxide (NO) donors compounds:

Nitric oxide (NO) donor compounds showed inhibition actions on G6PD activities. The objective was to investigate the reversibility of G6PD inhibition by these compounds after long preincubation time. Figure 3 shows that the G6PD inhibition by these compounds was not altered after long preincubation time. At the end of this period, the G6PD activity remained at a level of less than 60% from the control of enzyme activity without adding nitric oxide (NO) donor compounds.

Figure 3: Effect of preincubation time on reversibility of G6PD inhibition by 950 µm nitrosoarginine and 300 µm nitrosocysteine. Using control experiments without nitric oxide (NO) donors compounds (100% activity).

Effects of nitric oxide (NO) donor compounds on the fragility of red blood cells

The effects of arginine, cysteine, nitrosoarginine and nitrosocysteine compounds on the fragility of red blood cells were investigated. Figure 4 indicates that the compounds arginine and cysteine did not have hemolysis effect on the fragility of red blood cells. In contrast, The NO donor compounds nitrosocysteine and nitrosoarginine induced concentration dependent red blood cells hemolysis (Figure 5). Control experiments in the absence of NO donor compounds indicated no hemolysis of RBC from either normal or G6PD deficient patients during the period of incubation. The calculation of I₅₀ values (concentration of the compound that induces 50% hemolysis of RBC) showed 1100 µm for nitrosoarginine and 750 µm for nitrosocysteine.

Figure 4: Effect of amino acids on the hemolysis of red blood cells. 10% RBC suspension was incubated for 60 min with different concentration of cysteine and arginine.
Figure 5: Hemolysis of RBC by (NO) donors: 10% RBC suspension was incubated for 60 min with different concentration of nitrosoarginine and nitrosocysteine. A control sample subjected to similar conditions but without (NO) donors showed insignificant hemolysis during the same period of incubation.

Effect of aqueous medicinal herbal extracts on G6PD activity completely inhibited by nitric oxide (NO) donors:

Figures 6 and 7 show the effect of preincubated G6PD enzyme with different aqueous herbal extract concentrations from Salvia officinallis, Thymus vulgaris and Trigonella Foem–graecum before testing the enzyme with the 1.75 mM of nitrosoarginine and 0.75 mM of nitrosocysteine, respectively. All above aqueous herbal extracts managed to produce 100% protection of the enzyme from the inhibition by nitrosoarginine and nitrosocysteine and sometimes led to activation of G6PD activity compared with the control. It should be noted that when the NO donors were preincubated first with the enzyme, no protection could be detected with aqueous herbal extracts.

Protection of RBC hemolysis

The protection of RBC hemolysis was examined using different concentrations of aqueous herbal extract from Salvia officinallis, Thymus vulgaris and Trigonella Foem–graecum. Various concentrations of aqueous herbal extracts had neutral effects on RBC hemolysis (Figure 8). Furthermore, each aqueous herbal extract was preincubated with RBC for 5 min before the hemolysis was initiated by NO donors. Figure 9 and 10 show these aqueous herbal extracts were able to protect the RBC from hemolysis by nitrosoarginine and nitrosocysteine, respectively.
4. Discussion:

As an antioxidant enzyme, G6PD is present in all human cells but it is particularly essential to red blood cells because these cells have no alternative source for producing NADPH (Luzzatto, 1967; Beutler, 1994). The reduced glutathione (GSH) and NADPH are dependent on the availability of G6PD activity, however both are considered to have contribution in the resistance of RBC against oxidative damage (Gerli et al., 1982; Abboud and Awaida., 2010). A decrease in G6PD enzyme activity leads to decrease in NADPH and reduced Glutathione. It is believed that a lack of reduced glutathione availability causes early hemolysis in the spleen (Tian et al., 1999; Leopold and Loscalzo., 2005). It has been documented that G6PD structure possesses two critical sulphydryl groups that may play important roles in the enzyme catalytic function (Desforges et al., 1960). It is possible that one of these enzymatic sulfhydryl groups is attacked by oxidant compounds. Enzyme inhibitions by NO donor compounds commonly imply the oxidation of specific tyrosine residues (Yamakura et al., 1998) or cysteine residual groups (Spooren et al., 1998; Gow and Ischiropoulos., 2001) and both residues have been established in the crystal structure of human G6PD (Shannon et al., 2000). Also, the toxicity of these NO donor compounds on erythrocytes have been documented when peroxynitrite formed in vivo via the reaction of nitric oxide and the superoxide anion, was found to cause high rate of hemolysis on bovine erythrocytes (Wrobel et al., 2003). Several enzymes such as 5-nucleotidase (Siegfried et al., 1996) and tryptophan hydroxylase (Kuhn and Geddes, 1999) are inactivated through S-nitrosylation interaction between oxidant and reactive enzymes sulfhydryde group. Usually these oxidants are NO generator that initiates the S-nitrosylation step followed by oxidation of sulfhydryde groups to Disulfide Bridge. This might be applied for the action of nitrosoarginine, nitросocysteine oxidants on G6PD activity.

The irreversibility of G6PD inhibition by NO donor compounds was not altered after long preincubation time, the conversion of enzymatic thiols to disulfides may explain the irreversible inhibition observed by all these inhibitors. Zhou et al (2000) found that dopamine β-hydroxylase enzyme was irreversibly inhibited by diethylamine..

An imbalance between cellular pro-oxidant and antioxidant levels leads to the oxidative stress resulting in tissue damage. The antioxidant enzyme interacts directly with reactive oxygen species (ROS) to convert them to non-radical products. We had previously demonstrated that the overproduction of these radicals by cigarette smoking has an inhibitory effect on the enzymes responsible for removal of ROS such as CAT, G6PD and GPx (Al-Awaida et al., 2013).

Preincubated G6PD enzyme with aqueous herbal extract were able to protect the enzyme activity against inhibition and overcome red blood hemolysis effects due to richness of these extracts with antioxidants compound. Abboud and Awaida, 2010 showed the NO donor compounds promoted RBC hemolysis in parallel to their inhibition extents on G6PD activities. A decrease in G6PD enzyme activity was connected with increases accumulation of cellular reactive oxygen species (ROS) while an overexpression of G6PD activity decreases the accumulation of these species in response to oxidative stress (Leopold et al., 2003; Fico et al.,
Nitrosocysteine showed more effect than nitrosoarginine on both G6PD activity and red blood cells hemolysis. Thus, more aqueous extracts concentration is needed for protection against nitrosocysteine. This result indicate the nitrosocysteine release more nitric oxide. Salvia officinalis, Thymus vulgaris and trigonella Foem–graecum have been widely used as a source of natural antioxidants. Salvia officinalis is an old medicinal plant, which is rich source of different chemical constituents including terpenoids, polyphenol as well as essential oils (Wang et al., 2000; Bozin et al., 2007). Salvia officinalis extract is used to stabilize fat and fat-containing food against oxidation (Schwarz and Ternes, 1992). The main antioxidant compounds in the sage are carnosic acid and carnosol rosmarinic acid (Cuvelier et al 1996). Thymol and carvacrol were the major components of Thymus vulgaris essential oil. However, the thymol was more effective than carvacrol in preventing oxidation during the autoxidaion of lipids (Yanishlieva et al., 1999; El-Nakeety et al., 2011). Aqueous extract of thyme showed a degree of reductive and radical scavenging capacity (Dorman et al, 2003). Fenugreek (trigonella Foem–graecum) being rich in phytochemical components have encouraged its use as medicinal plant (Zaman et al., 1998; Dixit et al., 2005).

Simultaneous administration of aqueous extract of fenugreek seeds with ethanol for 60 days in rats prevented ethanol toxicity (Thirunavukkarasu et al., 2003).

5- Conclusion

The NO donor compounds (nitrosocysteine and nitrosoarginine) caused strong inhibition on G6PD enzyme activities, these NO donor compounds promoted RBC hemolysis in parallel to their inhibition extents on G6PD enzyme activities. The protection of both G6PD enzyme activity and red blood cell hemolysis against oxidative stress were achieved by preincubation with aqueous extracts from Salvia officinalis, Thymus vulgaris and trigonella Foem–graecum. Those extracts were able to protect hemolysis in parallel to their activation extents on G6PD activities. The nitrosocysteine showed more effect than nitrosoarginine on both G6PD inhibition and red blood cells hemolysis. Thus, more aqueous extracts concentration is needed for protection against nitrosocysteine.

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