RBC: Tool for Oxidant Agents Screening Test

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Abstract: Oxidative stress occurs when the balance between oxidants and biochemical antioxidants is disrupted because of the excess of ROS. RBC is used to determine the extent of oxidative stress in vitro. Interaction of ROS and free radicals with hemoglobin can denature it. The denatured hemoglobin can form aggregates that bind to the cell membrane to form inclusions called Heinz bodies. The enhancement of oxidative stress causes an increase in the number of Heinz bodies. In this study, RBCs were incubated with different concentration of xenobiotics at 4°C for 18 hours. The concentration was induced in 50% of RBCs with Heinz bodies calculated. It is known as Heinz Induction Concentration 50% or HIC50%. HIC50% is a comparative scale for detecting the extent of oxidative stress. It is concluded that hemoglobin may be useful biomarker to monitor oxidative stress in the biological system. This assay is considered a useful tool for the preliminary assessment of oxidative stress.

Key words: Oxidative Stress; Screening Test; Hemoglobin;

INTRODUCTION

Oxidative stress results from excessive levels of reactive oxygen species (ROS). The pathologies that have been attributed to ROS-induced cell dysfunction include 1) cardiac stunning and arrhythmia; 2) skeletal muscle injury; 3) neurological conditions, e.g., neuronal damage in Parkinson’s disease; 4) neurotoxicity; 5) Alzheimer’s disease; 6) diabetes, apoptosis of T lymphocytes, and gastric mucosal injury; and 7) hypertension Kourie, (1998).

ROS are generated in two pathways: Endogenous sources and exogenous sources. Major sites of Endogenous generation of ROS in cells are mitochondria, microsomes, endoplasmic reticulum, and nuclei. Exogenous sources are xenobiotics, ionizing radiation, and ultraviolet light Carletti and Cantiello (2007).

The non-enzymatic and enzymatic antioxidants are provided in cells; the former include glutathione (GSH), vitamin E, ascorbic acid, β-carotene and taurine acid; enzymes involved in antioxidant defense include superoxide dismutase, selenium dependent glutathione peroxidase, selenium independent glutathione peroxidase, catalase, alkyl hydperoxide reductase, glutathione s-transferase (GST) and glutathione reductase. Non-enzymatic antioxidants are obtained from nature through nutrition, as vitamin C, vitamin E, and carotenoids Junqueira and Barros (2004).

The glutathione pathway is an important factor against the toxic ROS. Reduced glutathione (GSH), a tripeptide with a free sulfhydryl group, combats oxidative stress by reducing ROS to harmless forms. The glutathione is now in the oxidized form (GSSG) and it must be reduced to regenerate GSH. As the levels of GSSG increase, its metabolism by GSH reductase will decrease the levels of NADPH, a cofactor that is potentially involved in the reduction of glutathione. NADP-dependent GSH reductases are thought to be major enzyme systems that maintain GSH in its reduced form. NADPH is produced by the pentose phosphate pathway (PPP). In fact, the electron source for glutathione is NADPH from the PPP and a fundamental function of PPP is to maintain GSH in a reduced state.

The normal erythrocytes generate relatively high levels of ROS. The respiratory function of RBCs generates oxidative stress on a continuous basis. Nevertheless, they have a well-integrated network of antioxidant mechanisms to combat this oxidative stress Banerjee et al., (2004). They have several mechanisms to protect against oxidative injury especially glutathione and pentose phosphate pathway. But, if RBCs are exposed to oxidant xenobiotic, ROS production will be uncontrolled and antioxidant systems will not be sufficient to protect the cell against ROS. The oxidants xenobiotics are themselves either converted to, or stimulate the formation of ROS (Knight, 1995). In fact, many of the environmental, occupational and industrial chemicals are able to generate free radical species, primarily through their metabolism Panayiotidis, (2008).
Smooth endoplasmic reticulum contains enzymes detoxifying xenobiotics, among them cytochrome P-450 isozymes, especially CYP2E1. These enzymes are able to produce ROS (Bartose, 2008). These products denature and oxidize critical proteins including hemoglobin.

More than 90% of the erythrocyte’s protein content consists of hemoglobin Boelsterli and (2007). The oxidation of these groups can denature hemoglobin and decrease its solubility. The denatured hemoglobin can form aggregates that bind to the cell membrane to form inclusions called Heinz bodies, a hallmark of oxidative injury to erythrocytes. Accumulations of oxidant agents induce the oxidation of these groups. This causes an increase in the number of Heinz bodies respectively. Moreover, the iron of hemoglobin is oxidized. Heme proteins contain redox-active transition metal iron that makes it susceptible to causing oxidative damage (Vollard et al., 2005). Iron is oxidized to the ferric and methemoglobin is formed. The primary reaction that reduces methemoglobin back to hemoglobin is catalyzed by the reduced form of nicotinamide adenine dinucleotide (NADH)-cytochrome b5 reductase(b5R). Electrons are transferred from NADH (generated by glyceraldehydes 3-phosphate in the glycolytic pathway) to an enzyme, NADH cytochrome b5 reductase, and then to cytochrome b5. Cytochrome b5 transfers electrons directly to methemoglobin to reduce it to hemoglobin (Prchal and Gregg, 2005). In oxidative stress, both the free cysteins and heme iron can be oxidized. These factors produce oxidative changes like Heinz bodies formation.

In this study, RBC and hemoglobin are suggested for ranking compounds with oxidant properties.

MATERIALS AND METHODS

Chemicals:

The two food preservatives; calcium propionate (98% purity), ascorbic acid (99% purity), and lindane(98% purity) as positive control were chosen.

Experimental Design:

Antioxidant factors (enzymatic and enzymatic antioxidants) are different among intraspecies. These differences in antioxidant activity will cause in defense response among intraspecies. For instance, sheep with low-GSH erythrocytes were more susceptible to oxidative agent than were sheep with high-GSH erythrocytes (Wetterstroom and Brewer, 1984). Furthermore, the quantity of nutrition is different between animals consequently; the level of non-enzymatic antioxidants such as vitamin E, ascorbic acid, and β-carotene or level of glucose will be different. Vitamin E is a fat–soluble vitamin that exists in eight different forms. α-tocopherol, the most active form of vitamin E, is a powerful biological antioxidant which is considered to be the major membrane bound antioxidant employed by the cell. Its main antioxidant function is protection against lipid peroxidation (Nemmiche et al., 2007). RBCs with elevated glucose concentration showed an increase in NADPH levels (Jam, 1989). NADPH needs to reduce oxidized hemoglobin in glutathione pathways. Therefore, blood should be taken from one animal for deleting intraspecies variability, and cow or sheep are suitable animals' model, because a lot of blood can be taken from one animal.

Blood sample was collected from a healthy cow. Blood sample was centrifuged (2000xg, 5min) and the plasma and white blood cells were removed. Erythrocyte packets were prepared by washing the erythrocytes three times with isotonic saline. Any kind of factors which will cause damage to membranes were avoided.

1 ml of erythrocytes was exposed to different concentrations of calcium propionate, ascorbic acid and lindane (0.1, 1, 10mg/ml). All the dilutions should be prepared with isotonic saline. In the control group, isotonic saline was added. RBCs were incubated at different temperatures (room temperature and 4C) and times (4, 18, and 24h). Heinz bodies were stained by 100μL erythrocytes with 50μL brilliant cresyl blue (1% in isotonic saline).

We should attempt to establish relationships between the amount of xenobiotics and the number of Heinz bodies. The number of RBCs with Heinz bodies per 100 RBCs counted can be calculated. The concentration was induced in 50% of RBCs with Heinz bodies calculated by linear regression analysis. It is known as Heinz Induction Concentration 50% or HIC50%. HIC50% is a comparative scale for detecting the extent of oxidative stress between different of xenobiotics.

RESULTS AND DISCUSSION

In this study, the incubation at 4C for 18h was better than other experiments. The activity of chemicals was compared with the activity of lindane as positive control. Lindane is an organochlorine pesticide with its
oxidative properties well documented. Lindane enhanced lipid peroxidation level in the brain of mice 18 h after the administration of single subcutaneous dose Bist and Bhatt, (2008). In the other study, the induction of oxidative stress was observed in rat testis as early as 12 h following exposure to a single dose of lindane Saradha et al., (2008). Therefore, lindane is chosen as positive control. Heinz bodies were not detected in negative control (isotonic saline).

The other chemicals did not show activity as strong as the positive control (Table 1). Calcium propionate has been widely used in the food industry for many years as an important food additive. According to evaluations performed by the Joint FAO/W HO Expert Committee on Food Additives, there are no limitations for Acceptable Daily Intakes (ADIs). Tests related to the teratogenicity and reproductive toxicity for calcium propionate are negative (US EPA, 1991). In short-term feeding studies of mice and rats, they produced no adverse effects on growth, mortality, or hematological patterns and no histopathological effects Deshpande, (2002).

Vitamin C is not only a nutrient but also an important antioxidant in many foods. It is water soluble and can break down easily. Vitamin C is relatively nontoxic. It is a known antioxidant (Tripathi and Gaur, 2004; Omaye, 2004). It acts as a cofactor for NADP reductase required for glutathione metabolism Chavan et al., (2007). But, it is also mentioned, vitamin C in high levels and in the presence of metals such as iron and copper can cause oxidative damage Omaye, (2004).

Table 1: A comparison of HIC50% between xenobiotics at 4°C for 18h

<table>
<thead>
<tr>
<th>Sample</th>
<th>HIC50%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Positive control (lindane)</td>
<td>13 mg/ml</td>
</tr>
<tr>
<td>Vitamin C</td>
<td>213 mg/ml</td>
</tr>
<tr>
<td>Calcium propionate</td>
<td>467 mg/ml</td>
</tr>
<tr>
<td>Negative control (isotonic saline)</td>
<td>-</td>
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</table>

Conclusion:

Antioxidant systems such as GSH, peroxidase, glutathione superoxide dismutase, catalase are detected as biomarkers of oxidative stress. Sometimes, the oxidative damages are assayed by measurement of lipid, protein and DNA damages Zhang et al., (2008). Both of these assays need technical personal and time-consuming. Every day new chemicals are produced. The environmental contaminants are regularly released. Some of them generate high levels of ROS which lead to oxidative stress and only a low percent of them have been studied for their oxidative stress. The extent of oxidative stress generated by new chemical and contaminants should be assayed. For determining Acceptable Daily Intakes (ADIs), carcinogenicity, teratogenicity, reproductive toxicity, and mutagenicity are studied. But, there are not many studies on the oxidative stress. RBC is useful to assess oxidative stress in vitro and helps in ranking compounds with oxidant properties. This assay is simple, less time-consuming, economic, non-ethical problem, and validate for determining the extent of oxidative stress. The numbers of Heinz bodies reflect the extent of oxidation stress.

The P-450 enzymes are present in all tissue (Klaassen, 2001). But, liver has a greater capacity for oxidative metabolism. Therefore, like the Ames test that bacteria do not duplicate mammalian metabolism for activating chemicals, then rat or human liver homogenates are added to the incubation mixture Omaye(2004), in this test, adding liver homogenates(supernatant of the 9000×g centrifugation, S9) can be intended for further investigations. It seems that the sensitivity of this procedure in detecting ROS producers with adding mammalian liver homogenates might be increased.

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REFERENCES


