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Assessment of Lipid Peroxidation and Activities of Antioxidant Enzymes in Phosphide- Powder Residue Exposed Rats

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Abstract

Cowpea, *Vigna unguiculata (L.) Walpers* is an important edible legume crop, is a good source of vegetable protein for millions of people. The estimation of global post-harvest losses caused by insect damage is enormous; therefore to prevent such wastage the use of synthetic chemical pesticides to protect stored grains is widespread. In some cases pesticide contamination of fumigated grains has been documented. The aim of this study to establish if oxidative stress is induced in animals exposed to such treated/contaminated cowpea. Eighteen female Wistar rats divided equally into three groups were used for the study. The rats in the first group were fed untreated cowpea and served as control while the rats in the second and third groups received phosphide-powder residue contaminated and uncontaminated cowpea respectively. Blood was drawn by retro-orbital bleeding. Analysis of blood to determine levels of malondialdehyde and glutathione as well as the activities of serum catalase, glutathione peroxidase, glutathione reductase, glutathione S transferase and superoxide dismutase in these rats revealed that while the level of reduced glutathione and activities of antioxidant enzymes were significantly reduced ($p<0.05$), the levels of malondialdehyde and oxidized glutathione were significantly increased ($p<0.05$) in rats fed with contaminated cowpea compared with control. These indices were not significantly changed ($p>0.05$) in rats fed with uncontaminated cowpea compared with control. The results of this study support the hypothesis that exposure of an animal to phosphide powder residue is capable of inducing oxidative stress.

Keywords: Phosphide residue; Cowpea; Antioxidant; Rat

Introduction

Cowpea, *Vigna unguiculata (L.) Walpers* is an important edible legume crop, mainly cultivated in dry lands. It is a good source of vegetable protein for millions of people. Cowpea is widely cultivated all over the world because of its high protein content; its adaptability to different types of soil and intercropping systems is well known. In addition to these, its resistance to drought, and its ability to improve soil fertility and prevent erosion makes it an important economic crop in many parts of the developing world especially Africa. Although it is cultivated all over the world, with more than 5.4 million tons of dried cowpeas produced worldwide, Africa alone accounts for approximately 5.2 million with Nigeria, the largest producer and consumer, being responsible for 61% and 58% of Africa and world production respectively.

The estimation of global post-harvest losses caused by insect damage, microbial deterioration and other factors is of the order of 10 ± 2.5% [1]. While approximately 12.5 million tonnes of these edible legumes are produced every year in India, as much as 18.6% of this is damaged by bruchids during storage. The bruchid beetle Callosobruchus maculatus (F.) has been associated with dried legumes for thousands of years [1]. To prevent such wastage the use of synthetic chemical pesticides to protect stored grains is widespread [2,3].

These chemicals being foreign may provoke free radical generation and which may consequently altered the activities of the enzymes of the anti-oxidant system in exposed animals. This may not be far-fetched since cowpea-consumption related poisoning has been linked with many clinical conditions and even death in Nigeria. The aim of this study is to determine the levels of malondialdehyde and glutathione as well as the activities of serum catalase, glutathione peroxidase, glutathione reductase, glutathione S transferase and superoxide dismutase in female Wistar rats exposed to phosphide powder residue contaminated and uncontaminated cowpea.

Methods

Animals

Female Wistar rats, 240-270 g in weight, purchased from the animal house of the Department of Veterinary Physiology, University of Ibadan were used for the study. They were allowed free access to standard laboratory diet and drinking water without any form of restriction. This study was carried out in conformity with national and international laws and Guidelines for Care and Use of Laboratory Animals in Biomedical Research; especially as promulgated and adopted by United States Institutes of Health (1985).

Treatments and samples

Cowpea was fumigated, using Protex (aluminum phosphide-57% inert ingredients-43%) manufactured by United Phosphorus Ltd, India. Using 2 tablets of phosphide perm^3 of space, cowpea was fumigated at an average temperature of 29°C over a period of 48 hours. At the end of the fumigation process, the grains were separated from the fumigant, and the treated cowpea was divided into two, and one part was deliberately contaminated with phosphide powder residue, i.e. residue of a quarter tablet of Protex was used to contaminate one kilogram of cowpea [4].

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The 3 different types of cowpeas for the three groups were given to the rats for a period over 8 hours. Twenty-four hours after the commencement of the study, from each rat, blood was drawn through retro-orbital bleeding and introduced into an anticoagulant free bottle. Serum was separated by centrifugation at 3000 g and stored in a refrigerator at -20°C.

Assay of level of MDA and activities of antioxidant enzymes: Twenty-four hours after the commencement of the study, blood was collected from each rat through retro-orbital bleeding which was left to clot and centrifuged for 10 minutes at 3000 g using a table centrifuge. The serum samples obtained were used for the estimation of indices of oxidative stress. These estimations were carried out using Hitachi 902 Automated machines (Roche Diagnostic®, Germany). The glutathione reductase (GR) activity was measured using the method of Zhou and Freed [5] while that of glutathione S transferase (GST) was by the method of Habig et al. [6]. Reduced and oxidized glutathione were determined using the methods of Prins and Loos [7] and Owen Joshua and Butterfield respectively. On the other hand, serum activities of superoxide dismutase, glutathione peroxidase, catalase and MDA were quantified by the methods [8-12].

Statistical analysis

The experimental results are expressed as mean ± standard error of the mean (SEM) for n=6. Differences between the control and the three treatment groups were determined by Student's t-test followed by one-way analysis of variance (ANOVA). A ($p<0.05$) was taken as an indication of a statistically significant difference.

Results

Table 1 shows the levels of malondialdehyde, reduced glutathione (GSH), oxidized glutathione (GSH) and reduced/oxidized glutathione ratio (GSH: GSSG). Rats fed phosphide residue contaminated cowpea had significant increase and decrease ($p<0.05$) in the levels of oxidized and reduced glutathione respectively. Moreover, in Table 1 while GSH:GSSG was significantly reduced; malondialdehyde was significantly increased in the same category of rats. On the other hand, rats fed treated but uncontaminated cowpea did not record any significant change ($p>0.05$) in the levels of GSH, GSSG, GSH:GSSG and malondialdehyde.

Serum activities of antioxidant enzymes of rats fed phosphide residue contaminated and uncontaminated cowpea are shown in table 2. While there were significant decrease ($p<0.05$) in the activities of superoxide dismutase, glutathione peroxidase, catalase, glutathione reductase and glutathione S transferase in rats fed phosphide residue contaminated cowpea; rats fed uncontaminated cowpea did not exhibit such changes, rather non-significant differences ($p>0.05$) were recorded for all the antioxidant enzymes.

Discussion

Even in the absence of any insult, generation of free radical occurs in the living system, since they have been identified as byproducts of a subset of enzymes that engage in electron transfer. Although the oxidative effects of phosphide powder residue has not been previously determined, according to Nath et al. [13] strong evidence exists to suggest that phosphine disrupts energy metabolism, particularly mitochondrial function. To understand the role of the mitochondria in phosphine-induced toxicity and its relationship with free radical generation, it will be essential to describe some of the features of mitochondrial energy metabolism.

The peculiar nature of membrane structure of the mitochondrion has been described as being critical to the energy metabolism of the cell. The mitochondrion itself, a double membrane bound organelle is characterized by a permeable outer membrane as well as a highly impermeable mitochondrial inner membrane (MIM), which ensures that the movement of molecules across the MIM between the intermembrane space and the central mitochondrial matrix is controlled, while a more relaxed exchange between the intermembrane space and the cytoplasm is maintained. This is important because the transfer of molecules across the MIM is dependent on the potential energy stored in a proton gradient across the inner membrane. In

	GSH (mol/ml)	GSSG (mol/ml)	GSH/GSSG Ratio	MDA (nmol/ml)
Controls	1.99 ± 0.21	0.10 ± 0.005	19.10	15.53 ± 3.7
Contaminated	1.40 ± 0.14 (0.002)*	0.30 ± 0.043 (0.021)*	4.66 (0.007)*	21.00 ± 8.8 (0.036)*
Uncontaminated	2.02 ± 0.11 (0.651)	0.11 ± 0.006 (0.439)	18.82 (0.544)	14.79 ± 2.5 (0.695)
F-value	30.99	26.95	110.83	106.86
P-value	0.006ǂ	0.006ǂ	0.005ǂ	ǂ0.005

Results are expressed as mean ± standard error of mean. *p<0.05 is significant when compared with control using Student's t test. ǂp<0.05 is significant using ANOVA. Abbreviations: GSH-reduced glutathione; GSSG-oxidized glutathione; GSH/GSSG- reduced/oxidized glutathione ratio; MDA- malondialdehyde.

Table 1: Levels of malondialdehyde, reduced glutathione, oxidized glutathione and reduced/oxidized glutathione ratio.

	SOD (U/mg protein)	CAT (µmol H_2O_2 consumed/(min·mg protein))	Gln-px (µmol GSH consumed/ (min·mg protein)	Gln reduc (U/mg protein)	GST (U/mg protein)
Controls	12.66 ± 0.21	2.26 ± 0.24	10.11 ± 0.58	60.09 ± 4.54	0.71 ± 0.090
Contaminated	12.03 ± 0.31 (0.013)*	1.79 ± 0.05 (0.005)*	9.12 ± 0.37 (0.019)*	41.28 ± 4.06 (0.010)*	0.25 ± 0.085 (0.008)*
Uncontaminated	12.59 ± 0.37 (0.518)	2.15 ± 0.08 (0.418)	9.57 ± 0.19 (0.332)	58.69 ± 6.72 (0.282)	0.74 ± 0.067 (0.427)
F-value	94.523	34.722	110.950	89.53	104.69
P-value	0.022ǂ	0.010ǂ	0.009ǂ	0.009ǂ	0.003ǂ

Results are expressed as mean ± standard error of mean. *p is significant when compared with control using Student't' test. ǂp is significant using ANOVA. Abbreviations: SOD-superoxide dismutase; CAT- catalase; Gln-Per- glutathione peroxidase; Gln Red- glutathione reductase; GST- glutathione S transferase.

Table 2: Serum activities of antioxidant enzymes of rats fed phosphide residue contaminated and uncontaminated cowpea.

addition, key metabolic reactions, especially the phosphorylation of ADP to ATP have also been linked to this process.

The results of our study revealed presence of free radical activity because of the significant increase in the level of malondialdehyde (MDA). Increase in MDA levels is usually associated with loss of membrane integrity, as well as disintegration of polyunsaturated fatty acids in the membrane bilayer, which exerts unfavorable effects on the susceptible organ structure and function. This then raises the possibility that this potential i.e. mitochondrial membrane potential will not be sustained by the electron transport chain (ETC), which is embedded within the MIM in phosphide residue exposed rats. Moreover, the three protein complexes, namely protein complexes, I, III, and IV that normal should sequentially extract the energy of these electrons (i.e. electrons derived from glycolysis, TCA and beta oxidation), and transport protons from the matrix to the intermembrane space, thereby creating a difference in electrochemical potential between the mitochondrial matrix and the mitochondrial intermembrane space will not be able to function effectively.

Free radical are also known to have deleterious effects on several critical molecular and cellular components such as proteins, DNA, and membrane lipids and although the primary targets of these species are cell-membrane polyunsaturated fatty acids, which, in turn, result in disruption in cell structure and function; the fact that DNA is altered by increase in free radical generation raises the possibility that mitochondria DNA might be affected and therefore suggest the possibility of the involvement of free radical in derailment of the oxidative phosphorylation in phosphide residue exposed rats.

The NADH that is produced through a number of different sources e.g. during glycolysis and other metabolic processes in the cytosol will be unable to penetrate the matrix and therefore cannot be used by Complex I directly. Normal functioning of these components and sequence of energy metabolism (oxidative phosphorylation) are important but in phosphide-induced toxicity Nath et al. [13] have indicated that glycerophosphate dehydrogenase may have a very significant role to play. Nakakita et al. [14] as well as Chefurka et al. [15] on the other hand, have revealed that complex IV, cytochrome c oxidase, is the primary site of in vitro interaction between phosphide and the electron transfer chain. Whereas, Kashi and Chefurka [15] who examined the oxidation state of the two cytochromes within complex IV, identified that phosphide treatment caused cytochrome a, but not cytochrome a_3 to become highly reduced. Irrespective of the site of action of phosphide, the disruption of this oxidative pathway results in free radical generation.

It is interesting to note that some of the molecules responsible for the generation of free radicals which eventually results in cellular oxidative stress e.g. reactive oxygen species (ROS), predominantly superoxide (O_2^{-}), hydrogen peroxide (H_2O_2), and reactive nitrogen species (RNS), predominantly nitric oxide ($^{\cdot}NO$) and peroxynitrite ($OONO^{-}$), are themselves byproducts of a subset of enzymes that engage in electron transfer. Both ROS/RNS are potentially highly destructive to biological macromolecules, which may eventually result in cell death. Normally, the mitochondrial ETC is the primary source of ROS/RNS is the mitochondrial ETC [16], their production even occurs when electrons flow freely through the ETC, especially if there is inappropriate transfer of electrons to molecular oxygen at complexes I and III. But then when an agent (e.g. phosphide) impedes this free flow of electrons, production of large quantities of the superoxide can occur [17]. The result of this study of significant decreases in the activities of superoxide dismutase, catalase, glutathione peroxidase, glutathione reductase, glutathione S transferase as well as the significant increase in the level of MDA suggests that phosphide residue especially if it is unspent may act through this mechanism. This observation is in agreement with earlier ones, phosphide, the gas derivative of phosphide has been reported to inhibit cytochrome c oxidase, in actual fact depress the activity of glycerophosphate dehydrogenase [18] leading to increase generation of hydrogen peroxide [19].

Miwa et al. [20] have also corroborated this, in which inhibition of the ETC in Drosophila melanogaster resulted in significant superoxide generation from glycerophosphate dehydrogenase. Although it was phosphide residue contaminated cowpeas that was fed to these rats and yet we recorded significant changes in the activities of these antioxidant enzymes, direct fumigation of experimental animals such as insect and mouse has been noted to also inhibit the activities of peroxidase and catalase but moderately induce superoxide dismutase [19,21].

Furthermore, Quistad et al. [22] have revealed that phosphine also reacts chemically with hydrogen peroxide to generate an even more reactive oxygen species, the hydroxyl radical [23] such that oxidative damage to macromolecules has been described in not only insects but also in nematodes [24] and mammalian cell lines [25] as well as rats [26]. That phosphine and by extension phosphide residue is oxidative in nature is evident by the submission of [24] who identified glutathione as the strongest protective antioxidant and hydrogen peroxide as the most significant ROS. This is more so as these workers observed that glutathione not only protected against oxidative damage, but enhanced cell survival as well. Moreover treatment of rats with glutathione depleting chemicals enhanced the toxic effects of phosphine [26].

Significantly low level of glutathione was recorded in phosphide powder exposed rats, the precise involvement of glutathione in phosphide toxicity has not been fully defined but enigmatic; this is despite the fact that antioxidants, most notably melatonin, prevents most of the oxidative damage induced in a range of tissues in rat [25] and this it did by protectively maintaining the levels of glutathione [27]. The primary toxic effect of phosphide is the oxidative pathway, but glutathione involvement in derailment of energy metabolism is not a common occurrence. This raises the possibility that phosphine may results in generation of other reactive species through the cytochrome P450 cytochrome pathway or other routes.

Conclusion

This study confirms that exposure of rats to phosphide residue contaminated cowpea is capable of inducing oxidative stress as the levels or activities of antioxidant (glutathione, superoxide dismutase, catalase, glutathione peroxidase, glutathione reductase) were significantly reduced and products of oxidation namely; malondialdehyde and oxidized glutathione were significantly increased.

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