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Effect of Dietary Administration of *Lathyrus sativus* Pulse on Intestinal Biochemical Parameters in Normal and Scorbutic Guinea Pigs

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Objective In order to investigate that ascorbic acid deficiency is responcible for lathyrus toxicity, the effect of dietary feeding of lathyrus pulse in normal and scorbutic guinea pigs for 3 months, on intestinal biochemical parameters was undertaken. **Methods** The intestinal brush border membrane (BBM) marker and xenobiotic metabolising enzymes (XME) were assayed. **Results** Exposure to 80% lathyrus alone and in scorbutic conditions showed significant inhibition of alkaline phosphatase (28%-30%), sucrase (19%) and γ-glutamyl transpeptidase (GGT) (15%-27%) enzymes, while Ca^{2+}-Mg^{2+}-ATPase was significantly inhibited (38%) in scorbutic plus lathyrus treated group. The phase I XME (AHH) remained unchanged while the phase II enzyme glutathione-S-tranferase (GST) was significantly decreased (20%-22%) in lathyrus and scorbutic plus lathyrus treated groups. Quinone reductase (QR) activity was found to be significantly decreased in lathyrus exposed group (20%). The intestinal biomarker contents including hexose (25%-34%) and phospholipids (20%-40%) were significantly reduced in lathyrus and scorbutic plus lathyrus exposed animals, while sialic acid showed a significant decrease (28%) in scorbutic plus lathyrus treated group. However, cholesterol levels were signifcantly enhanced (15%-28%) in lathyrus and scorbutic plus lathyrus treated animals. **Conclusion** The results indicate that oral feeding of lathyrus pulse to guinea pigs can alter BBM parameters as well as XME, which may result in the intestinal toxicity. Further, ascorbic acid deficiency could be one of the pre-disposing factors of lathyrus toxicity.

Key words: Ascorbic acid; BBM; *Lathyrus sativus*; Small intestine; XME

INTRODUCTION

The small intestine provides a major route for exposure to xenobiotics via ingestion of food and liquid. The primary function of gastrointestinal tract is ultimate digestion of food to its basic constituents (amino acids, sugars, lipids, purines, pyrimidines) and its absorption into the blood stream[1]. The cells responsible for the absorption and transport are the epithelial cells, lining the villus-crypt surface of the intestinal lumen[1]. The intestine microvillus membrane plays an important role in determining the rate and specificity of intestinal transport

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Abbreviations: AHH, Aryl hydrocarbon hydroxylase; AsA, Ascorbic acid; BBM, Brush border membrane; β-L-ODAP, β-N-oxalyl-L-α, β-diaminopropionic acid; GGT, Gamma glutamyl transpeptidase; GST, Glutathione-S-transferase; QR, Quinone reductase; XME, Xenobiotic metabolising enzyme

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processes[2]. It is rich in enzymes that hydrolyse the selected nutrients to absorbable solutes and also contains several carrier systems including phosphatases and sucrases for uphill transport[3]. The bioconstituents like sialic acid, hexose, cholesterol and phospholipids are the important structural components of intestinal membrane, the concentration of which affects various physical parameters of the membrane.

Enzymatic biotransformation in intestinal mucosal cells has the potential to detoxify xenobiotics by directly facilitating their excretion[4-6]. Studies have indicated that activity of xenobiotic metabolising enzymes (XME) of small intestine could be modulated thereby modifying metabolism of xenobiotics and hence affecting their bioavailability[4,5].

Lathyrism, the neurodegenerative disease, is attributed to excessive consumption of *Lathyrus sativus* pulse which contains an unusual, free, excitatory amino acid β-L-ODAP, considered to be the chief toxic principle of lathyrus plant. Several experimental studies have been conducted using ODAP in pure form or as alcoholic extract of lathyrus seed through intraperitoneal injections or intrathecal routes or by direct focal injection in hippocampus region of brain to localise the site of action[7-11]. Furthermore, parenteral administration of ODAP has also been shown to cause retinal damage which is not the major target organ[12].

Dietary surveys carried out in lathyrism endemic states of India have shown the diet was deficient in vitamin A and C[13]. Experimental studies indicate the increased occurrence of lathyrism in guinea pigs made subclinically deficient in ascorbic acid and fed with lathyrus seeds[14]. However, toxicological implications of oral feeding of either lathyrus alone or lathyrus in scorbutic conditions on the exposure site viz. intestinal mucosal cells remains obscure. Hence an attempt was made in the present investigation to study the effect of dietary supplementation of lathyrus in scorbutic guinea pigs and in the control group on intestinal brush border membrane parameters and xenobiotic metabolising enzymes which may have some bearing on neurotoxicity of lathyrus.

MATERIALS AND METHODS

Chemicals

Bovine serum albumin (BSA), 1-chloro-2,4-dinitrobenzene (CDNB), dichlorophenol indophenol (DCPIP), flavin adenine dinucleotide (FAD), glucose-6-phosphate, L-γ-glutamyl-p-nitroanilide, glutathione reduced (GSH), glycyl-glycine, nicotinamide adenine dinucleotide phosphate (NADP), nicotinamide adenine dinucleotide reduced (NADH), nicotinamide adenine dinucleotide phosphate reduced (NADPH), and 2-amino-2-(hydroxymethyl)-propane-1,3-diol (Tris) were obtained from Sigma Chemical Co., St. Louis, Moo, USA. Benzo(a) pyrene was procured from Aldrich Chemicals Co, Milwaukee, WI, USA. Acetic acid and ammonium molybdate were purchased from Merck, Mumbai, India. Nicotinamide was a product from BDH, Poole, England. Ferric chloride, D-galactose, anthrone reagent and dihydrogen phosphate (KH_2PO_4) were products of BDH, Mumbai, India. Glucose oxidase, N-acetyl nuraminic acid and maleic acid were procured from Sisco Research Laboratories, Mumbai, India. All other chemicals used were of analytical grade and obtained from commercial sources.

Lathyrus pulse was procured from state of Bihar and was found to contain 0.6 g ODAP/100 g pulse. Bengal gram was used as a substitute for the control group and was purchased from the local market of Lucknow, India.

Animal Treatment

The cooked diet containing 80% lathyrus was supplemented with 20% wheat. The corresponding control cooked diet was prepared by mixing 80% bengal gram with 20% wheat. Vitamin mixture was added in the cooked diet according to the composition given by Oser and Oser[15]. The scorbutic diet was prepared by mixing all vitamins in the cooked diet except ascorbic acid. A sustenance dose of 2 mg AsA/Kg bwt was added in the scorbutic diet for survival of animals.

Adult male albino guinea pigs (300±10 g) were procured from ITRC animal breeding colony and were maintained on water *ad libitum*. The animals had free access to cooked diet prepared daily for the experiments. The animals were divided in 4 groups of 12 each and were exposed daily for 90 d as follows:

Group I: Animals were fed with cooked diet containing 80% bengal gram and 20% wheat daily for a period of 90 d. This group is referred to as control group.

Group II: Animals were fed with cooked diet containing 80% lathyrus and 20% wheat daily for a period of 90 d. This group is referred to as lathyrus alone group.

Group III: Animals were fed with scorbutic cooked diet containing 80% bengal gram and 20% wheat daily for a period of 90 d. This group is referred to as scorbutic group.

Group IV: Animals were fed with scorbutic cooked diet containing 80% lathyrus and 20% wheat daily for a period of 90 d. This group is referred to as scorbutic plus lathyrus group.

The animals were sacrificed by cervical dislocation with minimal suffering following rules laid down by Animal Welfare Committee of ITRC.

Preparation of Intestinal Brush Border Membrane

The brush border membrane was essentially prepared according to the method of Forstner *et al.*[2]. Small intestine was removed and placed in ice-cold normal saline (0.9% NaCl). It was gently washed 2-3 times with cold normal saline to remove the remaining food material. After averting the intestine the epithelial layer was scrapped off conveniently with the help of a glass slide. The mucosal cell scrapping was weighed, placed in 75 volume of 5.0 mmol/L EDTA adjusted to pH 7.4 with NaOH (EDTA buffer) and homogenized in Ultra Turax Polytron. The homogenate was centrifuged at 450×g for 10 min at 4℃, the sediment was washed three times by suspension and centrifugation in 5 volume of EDTA buffer. The washed sediment of "crude" brush border fraction was then suspended in 2 volume of 90 mmol/L NaCl-0.8 mmol/L EDTA buffer, mixed throughly and kept until a well defined sediment had developed. This step usually required 20-30 min. The supernatant and sediment were then poured in succession thruogh a pad of glass wool to remove aggregated praticles. After the glass-wool pad had been washed with a further 20 mL of 5 mmol/L EDTA buffer, brush borders on the total washing were sedimented by centrifugation at 450 ×g for 10 min. and washed once with 2.5 volume of 2.5 mmol/L EDTA. The final pellet was suspended in a convenient volume of 2.5 mmol/L EDTA buffer and used for estimation of biochemical parameters.

Assay of Intestinal Brush Border Enzymes

The enzyme activity of sucrase was determined essentially by the method of Dahlqvist[16] using glucose as standard. The enzyme activity was expressed as umoles glucose formed per minute per mg protein. The method of Weiser[17] was followed for measurement of alkaline

phophatase using *p*-nitrophenol as standard. One unit of enzyme activity was taken as μmoles of *p*-nitrophenol formed per minute per mg protein. The Ca^{2+}-Mg^{2+}-ATPase activity was determined according to Hidalgo *et al.*[18] by measuring the liberation of inorganic phosphate (Pi)[19]. The γ-glutamyl transpeptidase activity was estimated by the method of De Young *et al.*[20] using L-γ-glutamyl-*p*-nitroaniline as substrate. The enzyme activity was calculated by the molar extinction coefficient of 10020 $(mol/L)^{-1} \cdot cm^{-1}$. AHH activity was assayed according to the method of Dehnen *et al.*[21]. AHH activity was expressed as pmoles 3-hydroxybenzo(a)pyrene formed per minute per mg protein. Glutathione-s- transferase (GST) activity was assayed by the method of Habig *et al.*[22] using 1-chloro-2,4-dinitrobenzene (CDNB) as substrate. The enzyme activity was calculated by the using millimolar extinction coefficient value of 9.6 $(mmol/L)^{-1} \cdot cm^{-1}$ and expressed as nmoles CDNB conjugates formed per minute per mg protein. Quinone reductase (QR) activity was assayed according to the method of Ernster[23] (1967) using 1,2-dichlorophenol indophenol (DCPIP) as substrate. The calculations were made using millimolar extinction coefficient value of 2.1 $(mmol/L)^{-1} \cdot cm^{-1}$ and expressed as nmoles DCPIP disappeared formed per minute per mg protein.

Assay of Intestinal Brush Border Membrane Constituents

Total hexose was estimated by anthrone reagent according to the method of Roe[24] using D (+) galactose as standard. The estimation of sialic acid content was carried out according to the method of Warren[25] using N-acetyl nuraminic acid as standard. Phospholipid content was essentially estimated by the method of Folch *et al.*[26] and the inorganic phosphate was estimated by the method of Wagner *et al.*[27] using potassium dihydrogen phosphate (KH_2PO_4) as standard. Cholesterol levels were estimated according to Zlatkis *et al.*[28]. The protein content was measured by the method of Lowry *et al.*[29] using bovine serum albumin (BSA) as standard.

Statistical Analysis

The analysis of variance (ANOVA) with rank ordering was employed to calculate significance of differences between the treatment groups[30]. *P* Values of less than 0.05 were considered to be significant.

RESULTS

Oral feeding response of lathyrus in the control and scorbutic guinea pigs on some intestinal biomarker enzymes is illustrated in Table 1. There was a significant ($P<0.05$) inhibition in alkaline phosphatase activity in lathyrus (28%), scorbutic (19%) and scorbutic plus lathyrus (30%) groups, when compared with the control group. Furthermore, the scorbutic plus lathyrus group also showed significant ($P<0.05$) inhibition when compared with the scorbutic group. There was no change in intestinal Ca^{2+}-Mg^{2+}-ATPase activity in animals of the scorbutic or lathyrus groups as compared with the control. Interestingly, the activity was found to be significantly ($P<0.05$) inhibited (32%-38%) in the scorbutic plus lathyrus group, when compared with scorbutic, lathyrus and control groups. Lathyrus treatment in control and scorbutic guinea pigs showed significant ($P<0.05$) decrease of 19% in sucrase activity when compared with the control and scorbutic treated group. The GGT activity was significantly inhibited in scorbutic (33%) and lathyrus (27%) treated groups, when compared with the control. However, scorbutic plus lathyrus group showed a significant ($P<0.05$) enhancement of 26% in GGT activity when compared with the scorbutic group alone.

TABLE 1

Effect of Lathyrus in Control and Scorbutic Guinea Pigs on Intestinal Brush Border Membrane Enzymes

Parameter	Control	Scorbutic	Lathyrus	Scorbutic Plus Lathyrus
Alkaline[d] Phosphatase	57.34±0.99	46.65±1.42[a]	41.59±0.75[a]	40.03±0.53[a,b]
Ca^{++}-Mg^{++}-ATPase[d]	0.39±0.008	0.37±0.006	0.35±0.22	0.24±0.021[a,b,c]
Sucrase[d]	0.05±0.003	0.05±0.005	0.03±0.002[a]	0.03±0.003[a,b]
GGT[e]	27.9±2.1	18.7±2.2[a]	20.5±2.0[a]	23.6±1.1[b]

Note. Data represents $\bar{x}\pm s$ of 5 animals; [a] $P<0.05$ when compared to control; [b] $P<0.05$ when compared to scorbutic; [c] $P<0.05$ when compared to scorbutic lathyrus; [d] µmoles/mg protein; [e] nmoles/min/mg protein

Table 2 depicts the oral feeding response of lathyrus in control and scorbutic guinea pigs on intestinal xenobiotic metabolizing enzymes. There was no significant change in the AHH activity in any of the treated groups. A significant ($P<0.05$) inhibition of 20%-22% was observed in GST activity in scorbutic, lathyrus and scorbutic plus lathyrus groups when compared with the control. The QR activity was found to be significantly ($P<0.05$) decreased in lathyrus group (20%) when compared with the control. However, scorbutic animals showed a significant enhancement (46%) of intestinal QR activity when compared with the control. The QR activity in scorbutic plus lathyrus group showed a significant ($P<0.05$) increase in activity (36%-37%) when compared with the control or lathyrus group.

TABLE 2

Effect of Lathyrus in Control and Scorbutic Guinea Pigs on Intestinal Xenobiotic Metabolizing Enzymes

Parameter	Control	Scorbutic	Lathyrus	Scorbutic Plus Lathyrus
AHH[d]	12.1±1.9	11.5±0.7	10.9±0.6	12.3±0.8
GST[e]	3560±120	2830±240[a]	2770±280[a]	2860±210[a]
QR[e]	390±22	569±107[a]	312±22[a]	530±141[a,c]

Note. Data represents $\bar{x}\pm s$ of 5 animals; [a] $P<0.05$ when compared to control; [b] $P<0.05$ when compared to scorbutic; [c] $P<0.05$ when compared to lathyrus; [d] pmoles/min/mg protein; [e] nmoles/min/mg protein

Dietary feeding response of lathyrus in control and scorbutic guinea pigs to intestinal brush border membrane constituents is shown in Table 3. The total hexose content was significantly ($P<0.05$) reduced in lathyrus group (25%) while no change was evident in scorbutic group as compared with the control. Furthermore, the total hexose level in the scorbutic plus lathyrus group witnessed a significant ($P<0.05$) decrease (33%-34%) when compared with the control and scorbutic groups. The sialic acid content was significantly ($P<0.05$) decreased in scorbutic (18%) group, while treatment of lathyrus alone showed no change, when compared with the control. Further, scorbutic plus lathyrus group also showed significant ($P<0.05$) depletion of sialic acid content (21%-24%) when compared to control or lathyrus group. The intestinal brush border membrane cholesterol level was found to be significantly increased ($P<0.05$) in lathyrus exposed group when compared with the control. Scorbutic plus lathyrus group also showed significant ($P<0.05$) increase in cholesterol level when compared to scorbutic, lathyrus and control groups. Conversely, the phospholipid content was significantly ($P<0.05$) decreased in scorbutic (31%) and lathyrus groups (31%)

when compared with the control. The scorbutic plus lathyrus group depicted a further decrease (20%-43%) in phospholipids when compared with the lathyrus, scorbutic and control groups (Table 3).

TABLE 3

Effect of Lathyrus in Control and Scorbutic Guinea Pigs on Intestinal Brush Border Constituents

Parameter	Control	Scorbutic	Lathyrus	Scorbutic Plus Lathyrus
Total Hexose[d]	110.84 ±0.95	109.7 ±4.54	83.39 ±1.61[a]	73.33 ±4.72[a,b]
Sialic acid[d]	17.51 ±0.7	14.30 ±0.93[a]	16.66 ±0.71	13.27 ±1.14[a,c]
Cholesterol[d]	37.91 ±0.38	38.60 ±1.64	43.42 ±1.48[a]	48.49 ±0.74[a,b,c]
Phospholipids[d]	29.13 ±1.48	20.63 ±1.64[a]	21.14 ±0.68[a]	16.48 ±0.45[a,b,c]

Note: Data represents $\bar{x}\pm s$ of 5 animals; [a] $P<0.05$ when compared to control; [b] $P<0.05$ when compared to scorbutic; [c] $P<0.05$ when compared to lathyrus; [d] µg/mg protein

DISCUSSION

The small intestine forms the initial site of exposure to lathyrus and could be one of the major target tissues in addition to brain and liver. The brush border membrane enzymes viz. alkaline phosphatase, Ca^{2+}-Mg^{2+}-ATPase, sucrase and GGT are found to be highest in the differentiated cells of the villus region and helpful in digestion, absorption and transport of nutrients[31]. The inhibition of alkaline phosphatase, Ca^{2+}-Mg^{2+}-ATPase and GGT in the lathyrus treated animals as observed in the present investigation could lead to the impairment of the uptake and transport of micronutrients which in turn may be responsible for lathyrus toxicity. Disaccharidases including sucrase, associated with the hydrophobic core of BBM are localised on luminal surface of the membrane[31]. The decline in sucrase in lathyrus exposed animals further signifies the alteration in digestive function.

The protein bound sialic acid and hexose form a set of another important component of intestinal cell lining required for its normal functioning[32,33]. It has been observed to establish cross linkage between glycoprotein molecules and to aid in the promotion of molecular integrity[32]. The mucus glycoprotein is determined in terms of hexose content[33]. Reduced levels of hexose and sialic acid content in lathyrus exposed animals as observed in the present investigation suggests an alteration in the integrity of BBM. The sugars, including sialic acid have been shown to scavenge the hydroxyl radicals ($OH^{\bullet}$) formed in the lumen thus protecting it from the oxidative stress[34]. During the process hydroxyl radical destroys the sugar to give various products, thus loosing its visco-elasticity. The decrease of sialic acid content in the scorbutic group and a further decline in the scorbutic plus lathyrus treated group may be due to loss of elasticity of the BBM as a result of increased oxidative stress in the scorbutic group.

Phospholipids and cholesterol are important structural components of the biological membranes. The concentration of cholesterol and phospholipids in the membrane affects various physical parameters of membrane including the membrane fluidity[35]. The exposure of lathyrus and scorbutic plus lathyrus treatments to animals resulted in an increase of cholesterol and decrease of phospholipid contents, leading to an increase in the C/P ratio. The increased C/P ratio may result in an enhanced fluidity of BBM[36].

XME have been found to be localised on the villus-crypt surface of the intestinal epithelium, predominantly active in the upper and mid villus cells[5,6]. The phase II reactions

that occur parallel to oxidative reaction (Phase I) are more dominant in intestinal mucosa than in hepatic and extra-hepatic tissues[37]. In the present study no significant change could be observed in phase I (AHH) enzyme of intestine in any of the treated groups. A significant decline in phase II viz. GST was observed in the lathyrus treated group, suggesting the overall decreased detoxification potential. The results emphasize intestinal exposure site of lathyrus to alter detoxification potential which may be of relevance in retaining ODAP in the body or target tissue to exert toxicity following continuous exposure to lathyrus. Furthermore, the increased QR activity in the intestine of scorbutic animals may be a defense phenomenon against oxidative stress, which was not altered by co-exposure to lathyrus and requires further investigation to unravel the mechanism of action.

The overall results suggest that oral feeding of lathyrus pulse to guinea pigs can alter the BBM parameters as well as XME, which may result in the intestinal toxicity. Furthermore, AsA deficiency could be one of the pre-disposing factors of lathyrus toxicity.

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