



Effect of Thylakoid Supplementation on Activities of Glucose Metabolizing Enzymes in Rats

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Abstract

Background and aims: Thylakoids are the photosynthetic sites present in the green plant cell which acts as appetite suppressant resulting in loss of body weight and enhancing satiety in animals and humans. The aim of present study was to investigate the effect of thylakoids on body weight gain and enzymatic activity of certain enzymes of glucose metabolism.

Methods: Thylakoids from spinach leaves were isolated and freeze dried. Supplementation of spinach thylakoids to *Sprague dawley* male rats (n=6) at an oral dose of 0.5 g/kg body weight for 4 days was carried out. Food intake, changes in body weight of treated and control rats were monitored and enzymatic activities in liver and muscle tissues were estimated at the end of the experiments.

Results: The gain in body weight was less in treated rats in comparison with control (control 12.1 g and treated 9.6 g, $p < 0.05$). There was a significant increase ($p < 0.001$) in specific activities of glucose 6-phosphate dehydrogenase (control vs. treated, liver: 6 times; muscles: 11.1 times), lactate dehydrogenase (control vs. treated, liver: 5.9 times; muscles: 6.8 times), succinate dehydrogenase (control vs. treated, liver: 1.5 times; muscle: 2.5 times) and malate dehydrogenase (control vs. treated, liver: 1.4 times; muscle: 5 times).

Conclusion: Dietary intake of spinach thylakoids increase the activity of glucose metabolizing enzymes, which indicates increased utilization of substrates for energy production in addition to regulate body weight gain in rats. This may be responsible for observed beneficial effects of thylakoid supplementation.

Keywords: Obesity; Appetite; Spinach; Enzymatic activity; Glucose metabolism

Introduction

The increasing problem of overweight and obesity is posing a major health concern in the countries that are in transition from under-nourished to over-nourish. The fundamental cause for this is calorie imbalance between energy intake and energy expended. Sedentary lifestyle and intake of energy dense foods makes people vulnerable to metabolic disorders like obesity, cardiovascular diseases (CVDs), type-2 diabetes, etc. [1,2]. Various pharmaceutical drugs like orlistat, sibutramine and natural plant products like coffee, nuts, vegetable juice, etc influence the functioning of enzymes involved in glucose metabolism in order to decrease the uptake of glucose from the food and help in decreasing hunger and appetite [3,4]. A chemical drug, orlistat functions by hindering the intestinal lipase to act on lipids [3]. Similarly, the succulent plant, *Hoodia gordonii* is also known for appetite suppressant action in rats and humans [5,6].

As a natural product, spinach thylakoids have been suggested as an appetite suppressant and satiety promoting food component [7,8]. Thylakoids are the disc shaped structures present in the chloroplast of plant cell where various photosynthetic pathways occur. Several studies have shown that supplementation with thylakoids results in reduced food intake and weight loss in both animals and humans [7-9]. The reduction in food intake by thylakoid supplementation is possibly due to increased secretion of satiety signal cholecystokinin (CCK), leptin and decreased levels of ghrelin, the hunger hormone [9]. Supplementation with thylakoid also promote appetite control by suppression of hedonic hunger, elevation of plasma glucagon-like peptide 1 (GLP-1) and glucose concentration [10-13]. Thylakoid membrane proteins cause hindrance to the digestion of lipids by prolonging lipolysis due to binding with dietary fat and fat digestive enzymes co-lipase and lipase [7]. The

absorptive function of the gut is impaired as shown by reduction in glucose and delayed free fatty acid absorption [14]. In addition, the thylakoids act as probiotics by modulating gut microbiota which in turn helps in delaying lipid metabolism [8]. Appetite suppressant action of thylakoids is reported with both high fat as well as high carbohydrate diets [9-15]. The thylakoid feeding results in decrease in the body weight and improve insulin sensitivity in healthy humans [9].

Various enzymes concerned with glucose metabolism are known to play important role in the intermediary metabolism. Appetite suppression can be considered to force body in utilizing the available substrate for energy production. It is reported that intermittent fasting results in increased enzymatic activities of carbohydrate metabolism [16]. It is hypothesized that supplementation of thylakoids to rats might result in specific metabolic adaptations which affect the uptake of glucose via activating glycogenolysis to maintain negative energy balance [16]. Therefore, effect of spinach thylakoids was evaluated on selected enzymes of glucose metabolism, aspartate amino transferase (AST), alanine amino transferase (ALT) along with body weight and food intake monitoring.

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Materials and Methods

Preparation of thylakoids

Thylakoids were isolated from spinach leaves following method described by Kohnke et al. [9]. In brief, fresh spinach leaves (240 g) were ground and filtered with a muslin cloth, the pH was adjusted to 4.7 and allowed to precipitate at 4°C. Supernatant was discarded and sediments were washed with water and final volume was adjusted to pH 7.0. Finally, thylakoid powder was prepared by freeze-drying.

Estimation of β -carotene and vitamin E in thylakoids

The estimation of β -carotene in the powdered thylakoid extract was performed colorimetrically [17]. In brief, 1 g thylakoid extract was mixed with 1 ml ethanol and extracted with 10 ml of petroleum ether. After extraction, the ether layer was taken and optical density was measured at 450 nm against petroleum ether as reagent blank using Biorad Smart Spec 3000 spectrophotometer. The vitamin E estimation of the extract was done by using bathophenanthroline and ferric chloride [18].

Estimation of phenolic content and total reducing power of thylakoids

Total phenolic content was determined using Folin and Ciocalteu's reagent and expressed as gallic acid equivalents (GAE) [19]. Total reducing power of the thylakoid extract was estimated and represented as ascorbic acid equivalents [20]. In brief, 0.5 ml extract is mixed with 2.5 ml of 0.1 M phosphate buffer (pH 6.6) along with 2.5 ml 1% potassium ferricyanide. After 30 min of incubation at 50°C, 2.5 ml 10% TCA was added and centrifuged at 1000 rpm for 10 min. Finally, 2.5 ml of diluted supernatant along with 0.5 ml of ferric chloride (0.1%) were mixed and incubated for 10 min and the optical density was measured at 700 nm.

Supplementation with thylakoids

Animal experimentation was approved by the Ethics Committee of the institute. The study was conducted on the male rats of *Sprague dawley* strain (180-200 g, N=12) bred at the experimental animal facility of the institute. The rats were grouped in two sets of 6, one control and other treated. The animals were fed on standard food pellets consisting of following components: crude protein 21%, ether extractable fat 5%, crude fiber 4%, ash 8%, calcium 1%, phosphorous 0.6%, nitrogen free extract 53% enriched with stable vitamin A, D₃, E, K, B₁, B₂, B₆, B₅, B₁₂, C, choline chloride, folic acid, trace elements. The diet provided 3600 kcal/kg of metabolic energy.

Rats were kept in the separate cages for food intake and body weight monitoring. In the morning between 0900 h-1000 h, the food pellets were removed and weighed. The food pellets were restored after weighing to the cages between 1500 h-1600 h and the consumption were recorded.

Suspension of thylakoids was prepared with 1% gum acacia for feeding rats and the control rats were given normal drinking water as an oral dose. The suspension of thylakoids at dose of 0.5 g/kg of body weight was given orally for 4 days; this was almost equivalent to 5 g dose used for humans. The rats were weighed every day during the course of the study. After 4 days, the rats were kept on fasting before the day of sacrifice. The rats were anesthetized and sacrificed for collection of blood, liver and leg muscles. Tissue homogenates (10% w/v) were prepared using polytron homogenizer and centrifuged at 1000xg 10 min at 4°C. Supernatants were stored at -80°C in aliquots.

Plasma was recovered from blood by centrifugation at 1000xg 10 min and stored at -80°C until assayed for different variables. The variables studied included liver and muscle glycogen, enzymes for carbohydrate metabolism and enzymes for liver damage.

Estimation of liver glycogen

A small portion of rat liver was weighed and processed for glycogen estimation [21]. In brief, tissues with 0.5 ml 30% potassium hydroxide were dissolved by placing tubes in boiling water for 5 min. After dissolving, 250 μ l of saturated sodium sulphate and 3 ml ethanol was added and kept overnight. The next day tubes were centrifuged and the pellet were dissolved in 1 ml hot water and again precipitated with 3 ml of ethanol. The tubes were incubated for 120 min at 4°C. Following the incubation, tubes were centrifuged and pellet was dissolved in 2 ml of hot water. An aliquot of 50 μ l was mixed with 1 ml of water to which 100 μ l of 80% phenol was added. Brown color was developed by quick addition of 5 ml concentrated sulphuric acid and OD was read at 490 nm against blank. The amount of glycogen was calculated using standard curve of glycogen.

Estimation of specific activity of enzymes

The specific activities of enzymes of carbohydrate metabolism viz lactate dehydrogenase (LDH, EC 1.1.1.27), succinate dehydrogenase (SDH, EC 1.3.99.1), malate dehydrogenase (MDH, EC 1.1.1.37), glucose-6-phosphate dehydrogenase (G6PDH, EC 1.1.1.49) were estimated using standard methods [22,23]. Activities of aspartate aminotransferase (AST, EC 2.6.1.1) and alanine aminotransferase (ALT, EC 2.6.1.2) were also estimated [24].

Protein content of tissue homogenates was determined by colorimetrically using method of Lowry et al. and used for calculation of specific activities of enzymes [25].

Statistical analysis

The values of observations are expressed as mean and SD. Unpaired Student's t-test was used for comparison between the groups. Changes in the variables were considered significant at p value p<0.05.

Results

The concentration of bioactive compounds having antioxidant activity (β -Carotene, vitamin E, total phenolic compounds) and reducing power are depicted in Table 1.

There was significant difference in body weight gain between the two groups in 4 days of study (12.1 ± 6.9 g body weight gain in the case of control and 9.6 ± 2.9 g in treated rats ($p<0.05$). The thylakoid supplementation showed no significant effect on average food intake of rats. The average food intake was 19.1 ± 1.3 g in case of control and 18 ± 2.4 g in case of treated rats at a dose of 0.5 g/kg body weight.

The glycogen level in liver of treated rats was decreased by 28.6 % in comparison with untreated control rats and there was 9.3% increase in muscle glycogen content of treated group in comparison to control group (Table 2).

The supplementation of thylakoids for 4 days at dose 0.5 g/kg significantly affected the activity of enzymes involved in glucose metabolism in the liver and leg muscles (Tables 3 and 4). There was significant increase ($p<0.001$) in specific activities of G6PDH (in case of liver 6 times and muscles 11.1 times), LDH (in case of liver 5.9 times and muscles 6.8 times) and SDH (in case of liver 1.5 times and muscle 2.5 times) and malate dehydrogenase (control vs. treated, in case of

Bioactive Compound	Concentration (mg/g)
β-Carotene	0.282
Vitamin E	0.193
Total Phenolic Content**	5.386
Total Reducing Power	2.916

Values are expressed in mg/g and mean of the determinants. *α-tocopherol equivalents, **Gallic acid equivalents

Table 1: Content of bioactive compounds in thylakoid.

Tissues	Control Rats (n=6) (mg/g wet tissue)	Treated Rats (n=6) (mg/g wet tissue)
	Mean	Mean
Liver	2.48 ± 0.8	1.77 NS ± 1.3
Muscles	0.86 ± 0.2	0.94 NS ± 0.1

Values are mean ± SD. Student's t-test: NS: not significant in comparison with control group.

Table 2: Effect of thylakoids on glycogen content in liver and leg muscles tissues.

Enzyme	Control (n=6) Mean	Treated (n=6) Mean
Glucose-6-Phosphate Dehydrogenase (nmol NADP+/min/mg protein)	0.3 ± 0.6	1.8 ^{**} ± 0.3
Malate Dehydrogenase (nmol NADH/min/mg protein)	9.3 ± 20.8	13.8 NS ± 23.9
Lactate Dehydrogenase (nmol pyruvate formed/min/mg protein)	18.5 ± 47.1	108.3 ^{***} ± 14.3
Succinate Dehydrogenase (nmol Potassium Ferricyanide reduced/min/mg protein)	0.57 ± 0.08	0.84 ^{**} ± 0.04
Aspartate Aminotransferase (μmol pyruvate/min/mg protein)	1.449 ± 0.2	1.286 NS ± 0.1
Alanine Aminotransferase (nmol pyruvate/min/mg protein)	50.4 ± 32.9	97.5 [*] ± 16.8

Values are mean ± SD. Student's t-test: Mean values were significantly different from those of the control group: *p<0.01, **p<0.001, NS: Not Significant in comparison with control group.

Table 3: Effect of thylakoids on specific activities of enzymes of glucose metabolism in liver.

muscles 5 times) in treated animals when compared with controls. MDH activity was also increased in liver (1.4 times) of treated rats but it was statistically not significant. Increase in specific activities of ALT (control vs. treated, in case of liver 1.93 times and muscle 2.4 times) was observed while AST was increased in muscle (1.33 times) and decreased in liver but it was statistically insignificant.

Discussion

In the present study, the body weight and food intake was observed for 4 days. Treated group of rats showed lesser weight gain as compared to control group which suggests that spinach thylakoids have acted as an appetite suppressant. We observed no change in food intake in treated group which may be due to use of low dose of thylakoids which is 0.5 g/kg body weight. Inhibition of food intake in rats with a diet containing thylakoids equivalent to 40 mg and 132 mg chlorophyll is reported [7,8,26]. In case of humans a diet containing 25 to 50 g of thylakoids has been used to decrease body weight and food intake [14]. Also, a dose of 0.5 g/kg of thylakoids were supplemented orally to pigs gave significant results showing increased cholecystokinin (CCK) and decreased blood glucose concentration [27]. A dose equivalent to 2-4 g/kg body weight will be required to produce similar effects in rat.

The plant thylakoids were hypothesized to act as a good antioxidant,

Enzyme	Control (n=6) Mean	Treated (n=6) Mean
Glucose-6-Phosphate Dehydrogenase (nmol NADP+/min/mg protein)	0.17 ± 0.3	1.9 ^{***} ± 0.8
Malate Dehydrogenase (nmol NADH/min/mg protein)	4.3 ± 4.1	21.7 [*] ± 19.2
Lactate Dehydrogenase (nmol pyruvate formed/min/mg protein)	35.5 ± 92.3	244.1 ^{***} ± 47.1
Succinate Dehydrogenase (nmol Potassium Ferricyanide reduced/min/mg protein)	0.36 ± 0.24	0.90 ^{***} ± 0.19
Aspartate Aminotransferase (μmol pyruvate/min/mg protein)	2.032 ± 0.3	2.703 ^{**} ± 0.3
Alanine Aminotransferase (nmol pyruvate/min/mg protein)	9.1 ± 5.5	22.3 [*] ± 8.8

Values are mean ± SD. Student's t-test: *p<0.05, **p<0.01, ***p<0.001, in comparison with control

Table 4: Effect of thylakoids on specific activities of enzymes of glucose metabolism in leg muscle.

when isolated and supplemented, as naturally it protects the plant from photo oxidation and deterioration of leaves [11]. We observed the isolated thylakoids as rich source of antioxidants as shown by analysis of related bioactive compounds (Table 1). Fresh vegetables are rich resource of β-carotene, vitamin E, total phenolics and reducing compounds. Spinach contains high amount of β-carotene and vitamin E. Reported values for β-carotene considers more than 8000 ug/100 g in spinach [28,29]. Similarly, lipid-soluble antioxidant Vitamin E had been reported to be 2.33 mg/100 g in raw spinach. However, there is no report on β-carotene and vitamin E in isolated spinach thylakoids and we found β-carotene and vitamin E in high amount i.e (282 ug/g and 19.3 mg/100 g spinach extract respectively) in comparison to the reported values. Total phenolic content can be expressed in equivalents of gallic acid. Total Spinach has 7167 ± 73 mg/100 g of total phenolics expressed as ferulic acid equivalents and 208.8 mg GAE/100 g of total phenolics expressed as gallic acid equivalents [30,31]. Apparently, total phenolic contents can be in high amounts in isolated thylakoids which is evident from the data we obtained i.e. 538.6 mg GAE/100 g. We also found spinach as good reducing agent as data shows total reducing power of isolated thylakoids was 291.6 mg/100 g in terms of L-ascorbic acid equivalents.

In general, under negative energy balance, body undergoes glycogenolysis for energy production. The data shows 28.6% decrease in glycogen level in liver in comparison to control rats specifying the use of stored glycogen to meet its energy needs thereby maintaining a negative energy balance which may be associated with the decreased gain in body weight. A small increase in muscle glycogen of treated rats can be considered as stable muscle glycogen which may be due to switching of body to more utilization of fatty acid for energy than the glycogen, also termed as glycogen sparing effect.

Spinach thylakoids supplementation causes modulation of activities of enzymes involved in glucose utilization and formation in liver and muscle. Glucose-6-Phosphate Dehydrogenase is the first and rate-limiting enzyme of the Hexose Monophosphate Shunt. Activity of G6PDH was increased in the treated animals, indicating its active participation in gluconeogenesis and suggesting that fat metabolism is activated instead of glucose oxidation. This may promote the formation of fatty acids and pyruvate which release acetyl-CoA, which enters the Krebs cycle and subsequently increase the activity of SDH and MDH in muscles and liver. The increased level of SDH and MDH suggests the energy delivery from the Krebs cycle to muscle and liver. The LDH is increased in both liver and muscle of treated rats, demonstrating the conversion of pyruvate, which is final product of glycolysis, into lactate

via an active de novo gluconeogenesis. This may be required by the body to maintain its blood glucose levels, which is partially maintained by glycogen depletion in the liver. The gluconeogenesis requires energy, which comes from fatty acid oxidation and is thereby responsible for weight reduction with low carbohydrate diets [32]. It has also been reported earlier that enzymes involved in carbohydrate metabolism (SDH, MDH and LDH) were elevated in intestine and liver of rats when kept fasting signifying its role in glycogenolysis and gluconeogenesis [16]

A possible reason for weight loss or decreased weight gain in rats can be as depicted in Figure 1. After supplementation of thylakoids, a direct path to hypothalamus can be considered where the signal of satiety is being programmed, due to elevated activity of pancreatic lipase/co-lipase in stomach, resulting in increased secretion of satiety hormones like cholecystokinin (CCK) and leptin and reduced glucose uptake and hunger hormone, ghrelin [7,9,12,14,15]. This may directly retard the absorption of carbohydrates and fat present in the diet which may leads to decreased gain in body weight. Alternatively, the other path can be indirect way of loss of weight in which spinach thylakoids indirectly activates the enzyme cascade of Krebs' cycle, Cori's cycle and gluconeogenesis which may increase the utilization of substrate as reported in case of fasting [16].

Aspartate aminotransferase and alanine aminotransferase are the main enzymes studied to detect the toxicological effect (like liver damage) when measured in serum. An increased level of ALT and AST signifies the on-going process of gluconeogenesis in muscle and liver. In liver, there is a decrease in AST and a small increase in ALT supporting a non-toxic effect.

The inhibition of glucose and lipid uptake and decreased insulin

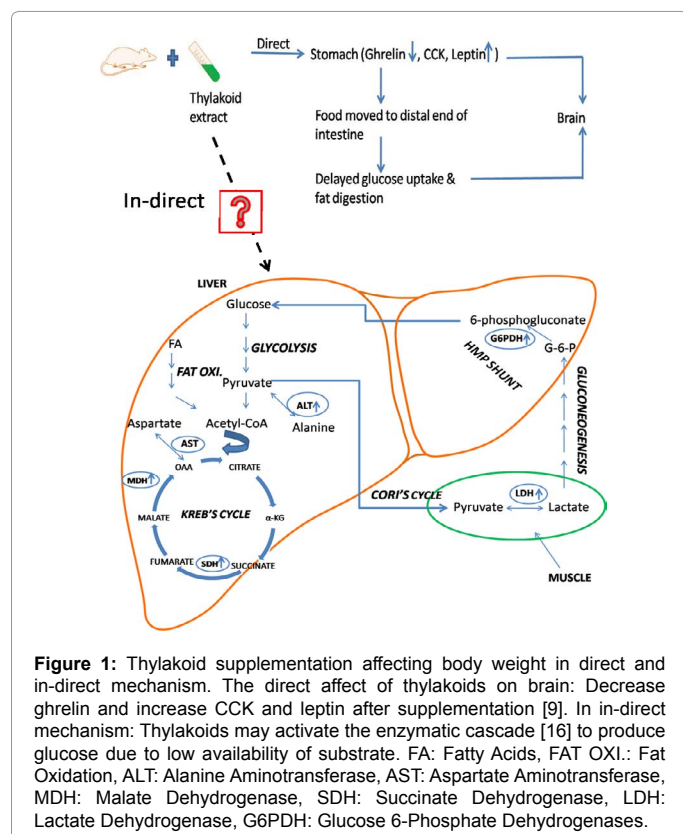
levels with weight reduction are already reported [8,14,27,33]. The present finding of an increase in the activity of enzymes of glucose metabolism by thylakoid supplementation in rats offers a possibility of use of thylakoids to improve conditions of impaired glucose metabolism. However, further studies on intermediary metabolism with thylakoid supplementation are required.

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