Different functional responsibility of the small intestine to high-fat/high-energy diet determined the expression of obesity-prone and obesity-resistant phenotypes in rats

Zuzana Šefčíková, Tomáš Hájek, Ľudovít Lenhardt¹, Ľubomír Raček and Štefan Možš

Institute of Animal Physiology, Slovak Academy of Sciences, Košice, Slovak Republic
040 01 and ¹Department of Pathology, University of Veterinary Medicine, Košice, Slovak Republic 041 81

Running head: Intestinal function in diet-induced obesity and resistance

Address for correspondence:
Dr. Štefan Možš
Institute of Animal Physiology, Slovak Academy of Sciences, Šoltésovej 4,
040 01 Košice
Slovak Republic
Fax: +421 55 7287842
E-mail: mozes@saske.sk
Summary

The objective of the present experiment was to assess the involvement of small intestine in expression of susceptibility or resistance to the high fat/high energy diet. The investigation was carried out in adult male Sprague-Dawley rats fed 1) standard laboratory diet (3.2 kcal/g, 9.5% fat) or high fat (HF) diet (4.04 kcal/g, 30% fat) for 4 weeks and 2) in HF rats that were retrospectively designated on the bases of their higher or lower weight gain as sensitive (DIO) or resistant (DR) to obesity. Our results revealed in HF group significant increase in energy intake, food efficiency, weight gain and Lee’s index of obesity. Moreover in comparison with controls a significantly increased duodenal and jejunal alkaline phosphatase (AP) and \( \alpha \)-glucosidase activity as well as hypertrophy of jejunal mucosa (increased protein/DNA ratio) was observed in HF fed rats. In contrast, intestinal functionality was inversely related to energy intake and development of adiposity in DIO vs. DR phenotypes. The DR rats had significantly greater AP and \( \alpha \)-glucosidase activity and more pronounced suppression of energy intake than obese DIO rats. It indicates that the increase of enzyme activities and the lowered effectiveness of nutrient absorption might be a significant factor preventing the expression of obesity proneness. This information contributes to a better understanding of a complex interaction between HF diet feeding and small intestinal adaptability, which determines the energy homeostasis and predict the ability to resist or develop obesity in these phenotypes.

Key words

Diet-induced obesity, intestinal functionality, feed efficiency, energy homeostasis
Introduction

The modern lifestyle associated with the overconsumption of palatable high-fat diet and the sedentary behaviour is considered as main causes of the worldwide increase of obesity (Stubbs et al. 2004). Although the prominent role of dietary fat in this phenomenon is suggested and the relationship between the development of obesity and the efficiency of energy utilization have yet to be clarified, in fact, high dietary fat consumption does not lead always to obesity and even a significant number of high fat consumers could be either of normal weight or underweight (Macdiarmid et al. 1996). It could be hypothesized that the different effectiveness of nutrient absorption in the small intestine can also be reflected in the susceptibility or resistance to a high fat diet. This is consistent with more effective absorption of the meal in the small intestine of obese subjects (Wright et al. 1983, Wisen and Johansson 1992) and with the efficacy of dietary or pharmacologically induced fat malabsorption to promote weight loss in obese humans (Hollander et al. 1998).

There is considerable animal data suggesting that, like humans, physiological processes of digestion and nutrients absorption may also be a significant contributing factor in the development of obesity. The relevance of this is supported by lower satiating effect of fats in the small intestine of obesity-prone rats than that in obesity-resistant rats (Greenberg et al. 1999) and by studies using the rodent models of outbred Sprague-Dawley rats fed high fat/high energy diet. These data revealed the existence two phenotypes of animals i.e. rats in which developed diet-induced obesity (DIO) associated with higher weight gain and enhanced food intake and diet resistant rats (DR) with lowered weight gain, retroperitoneal fat pad weight and energy intake (Levin and Keesey 1998). Moreover, in DIO rats the high-fat diet induced a significant increase in circulating levels of insulin, leptin and adiponectin as
compared to DR rats (Levin and Keesey 1998, Ricci and Levin 2003, Perez-Echarri et al. 2005). With regard to regulation of energy homeostasis, it was found that the DIO rats displayed a significant increase in food efficiency (the ratio of weight gained to calories consumed) than HF diet feed DR animals (Chandler et al. 2005).

The mammalian small intestine contains several enzymes in the enterocyte brush border probably playing an important role in the digestion of the individual food components and their activity varied also with the dietary substrate levels. It has been proved that in rats and mice intestinal peptidase, disaccharidase and alkaline phosphatase (AP) activities were simultaneously stimulated with increased dietary protein, carbohydrate and fat intake (Ferraris et al. 1992, Tanaka et al. 1998, Kaur et al. 1996, Mahmood et al. 2003) and several forms of obesity in laboratory rodents were also accompanied with substantially elevated activity of intestinal disaccharidases and AP activity (Ramaswamy and Flint 1980, Flores et al. 1990, Adachi et al. 2003, Mozeš et al. 2004). Moreover, some studies revealed that long-lasting exposure of mice and rats to a high fat diet led to obesity associated with mucosal hypertrophy i.e. increase in the mucosal protein/DNA ratio (Estornell et al. 1995) and with increased activity of AP in the small intestine (Mozeš et al. 2006). At present time, however, the relationships between increased intestinal enzyme activities and DIO obesity is not fully understood particularly from aspect of differences that develop in DIO and DR phenotypes. It is therefore conceivable that investigation of biochemical and functional characteristics of the small intestine may help us to understand the feeding and growth perturbations that predict susceptibility or resistance to obesity after long-term high fat/energy diet feeding.

Accordingly, the mucosal DNA, protein as well as activity of brush border bound AP, α-glucosidase and aminopeptidase in the duodenum and jejunum were examined in DIO and DR rats (designated on the base of higher or lower weight gain) receiving HF diet and compared with rats feed a standard laboratory diet.
Materials and Methods

Subjects

Adult outbred Sprague-Dawley male rats (Charles River Laboratories, Prague) were individually housed in Plexiglass cages in a temperature-controlled environment of 22±2°C with a relative humidity of 60±15 % and a12 L:12 D regime (light on 06.00 - 18.00 h). The animals had free access to a standard laboratory diet (Laboratory diet M1, Prague; 3.2 kcal/g, with 26.3% energy as protein, 9.5% as fat and 64.2% as carbohydrate) and tap water.

After 2 weeks of adaptation period rats were divided into two dietary groups: 1) control group (n=16) in which the rats were continued on a standard pellet diet for 4 weeks, 2) high energy group (n=16) in which the rats were switched to receiving HF diet (Research Diet 53316 Test Diet, USA; 4.04 kcal/g, with 14.55% energy as protein, 30% as fat and 55.5% as carbohydrate) for 4 weeks. During this time period the animals of both dietary groups were kept on the above mentioned laboratory condition with ad libitum of food and water intake and consumption of their respective diets were monitored weekly. After 4 weeks on HF diet, the 9 rats with the higher weight gain were designated as DIO and the 7 rats with lowest weight gain were designated as DR. Between 08.00 and 10.00 control and HF rats were killed by decapitation with removal of epididymal plus retroperitoneal adipose depots and small intestinal segments. The Lee’s index of obesity was calculated from data \sqrt[3]{body weight (g)/nasoeanal length (cm)}. All animal work was in compliance with the Animal Ethics Committee of the Institute of Animal Physiology SAS, Košice.

Enzyme assays and chemical analysis

For enzyme assay small (0.5 cm) segments were immediately removed, the lumen rinsed in distilled water, and frozen in hexane (-70 °C). Segments of the frozen tissue
(proximal duodenum and middle part of the jejunum) were cut (8 μm) in the cryostat at -25 °C and the tissue slices were transferred to glass slides and air-dried.

The analysis of alkaline phosphatase activity was performed using a modified simultaneous azo-coupling method (Lojda et al. 1979). The incubation medium contained 2.0 mM naphthol AS-BI phosphate (Sigma, Deisenhofen, Germany), 0.8 mM Hexazotized New fuchsine (Serva, Heidelberg, Germany), 20 mM N,N-dimethylformamide (solvent of naphthol AS-BI phosphate) and 0.05 M veronal acetate buffer. The sections were incubated at 37 °C for 10 min at pH 8.9 (Mozeš et al. 1998). The analysis of aminopeptidase M activity was performed using the simultaneous azo-coupling method (Nachlas et al. 1960) with L-leucyl-4-methoxy-2-naphthylamide as a substrate, Fast Blue B, N, N-dimethylformamide and 0.1 M citric acid-phosphate buffer. The sections were incubated at 37 °C for 6 min at pH 7.0. Demonstration of α-glucosidase (maltase–glucoamylase complex EC 3.2.1.20) activity was performed using a simultaneous azo-coupling method (Lojda et al. 1979) with 2-naphthyl-α-D-glucopyranoside as substrate, Hexazotized New fuchsine (Serva, Heidelberg, Germany), N,N-dimethylformamide and 0.1 M citric acid-phosphate buffer. The sections were incubated at 37 °C for 20 min at pH 6.5.

The histochemically stained slides were visualized at low magnification (obj. x 4) using Olympus microscope (BX 51) and digital compact camera (Olympus DP 50) connected with a host computer. Sections were illuminated with white light from a 12V halogen lamp (100 W) after filtering with a 520nm monochromatic filter.

Image analysis was performed by the Ellipse program (ViDiTo, Slovakia) where the gray level of each pixel was given by a value in the 0–255 range. The correspondence between these gray level values and the known integrated absorbance values of the same section points was determined by the calibration. To define the standard density values of enzyme activities at the wavelength of 520nm (Frederiks et al. 1987) required for calibration, Vickers M85a
microdensitometer was used. A special semi-interactive algorithm was used to find relevant pixels along the villus length whose density was measured (Mozeš et al. 2006). The quantification of the enzyme activity (pixel intensities) was carried out along the villus length in a whole section of at least four duodenal and jejunal slides and the mean values recorded were referred to one animal.

For chemical analysis, the mucosa from the defrosted tissue segments of duodenum and jejunum was scraped off with a glass microscope slide and weighed. The mucosal samples were homogenized in ice cold PBS. The protein content was analysed by the method of Lowry et al. (1951) using bovine serum albumin as the standard. DNA was evaluated by the fluorometric method (Karsten and Wollenberger 1977) modified by Koppel et al. (1981). Calf thymus DNA was used as the standard. Values are presented in μg/mg mucosa of duodenum and jejunum.

Statistical analysis

Statistical analyses were carried out using the statistical package Statistica 6.1 (StatSoft CR, Prague, Czech Republic). Data were expressed as mean ± SE and the statistical significance was accepted at the P< 0.05 level. Statistical evaluation of the somatic and small intestinal responses to standard diet vs. HF diet receiving groups was carried out by one-way analysis of variance (ANOVA) and the post-hoc Fisher’s LSD test was used to compare the differences between the groups. Data from the assessment of the energy intake responses from 1st to 4th week period in standard diet vs. HF diet rats was analyzed by two-way ANOVA with post-hoc LSD test. Pearson’s R was used to assess relationships between Lee´s index of obesity, fat pad weight and body weight gained on the HF diet.
Results

Table 1 shows, that exposure of rats to high fat (HF) diet led to significant increase in body weight, weight gain, body fat and food intake parameters. As compared to the standard diet receiving controls in HF diet fed rats 80% higher weight gain and 40% higher final epididymal plus retroperitoneal fat pads weight and significantly enhanced Lee’s index of obesity has been observed. Moreover these dietary obese rats consumed during 4 weeks about 20% more energy and displayed 30% higher food efficiency than the lean controls. During the same period the DIO rats attained about 70% higher weight gain and their energy intake and food efficiency were also significantly higher than in DR-HF rats. Although, the DIO rats displayed significantly increased Lee’s index of obesity as compared to DR animals, the differences in body fat pad weights between these phenotypes did not fully correspond with expression of somatic and feeding variations between these groups. Accordingly, there was very good agreement between Lee’s index of obesity and weight gain (r=0.6892) whereas magnitude of correlation between body fat % and weight gain showed lowered values (r=0.4202).

Comparison of energy intake between standard diet receiving control rats and rats on HF diet suggested that the energy intake on 1\textsuperscript{st} week was significantly higher in both DIO and DR groups (about 50% and 40%), whereas in the next periods a different adaptive changes to this nutritional challenge has been observed. While after 1\textsuperscript{st} week the energy intake in DR rats substantially decreased on the level of controls, in DIO rats despite gradual decrease of energy intake on 2\textsuperscript{nd}, 3\textsuperscript{rd} and 4\textsuperscript{th} week their values remained significantly higher than in DR rats. Moreover, by two-way ANOVA a significant time (F=41.3, P< 0.0001), dietary (F=104.9, P< 0.0001), and time x diet interaction (F=12.3, P< 0.0001) was found (Fig.1).

Changes in intestinal activities of alkaline phosphatase (AP), \(\alpha\)-glucosidase and aminopeptidase in the standard diet and the HF diet fed rats are shown in Table 2.
Comparison between these groups indicate that in rats receiving HF diet the AP activity was significantly higher in duodenum and in jejunum and $\alpha$-glucosidase activity in jejunum than in control rats. In contrast, no significant effect of HF diet on intestinal aminopeptidase activity has been found. Moreover, the obese DIO and lean DR rats displayed a different susceptibility to this nutritional challenge and in DR group HF diet elicited greater adaptive changes. As compared to standard diet receiving controls in DR rats AP activity significantly raised by 22% - 23% in both intestinal segments and $\alpha$-glucosidase activity in jejunum by 16%, whereas the values of AP activity in DIO rats increased only by 7% and 12% in duodenum and jejunum, respectively, and $\alpha$-glucosidase activity in jejunum by 5%.

Protein and DNA content in duodenum overall did not differ between control and HF groups (Table 2). In contrast, 4 weeks consumptions of HF diet significantly decreased the jejunal DNA content (about 20%; in DIO rats 17% and DR rats 26%) but not altered their protein content. Due to these changes the protein/DNA ratio was significantly higher in the HF diet fed rats (40%; in DIO rats 34% and in DR rats 46%) as compared with control rats.

**Discussion**

The results from our experiment are in agreement with earlier research suggested on development of two different phenotypes of rats after 4 week HF diet exposition (Levin and Keesey 1998, Chandler *et al.* 2005). The metabolically efficient so called DIO-rats gained significantly more weight and due to higher energy intake became obese, while DR rats despite higher overall energy intake failed to reveal any significant increase in weight gain, food efficiency and Lee’s index of obesity. Moreover, our results revealed a different pattern of the feeding response to HF diet in DIO and DR rats. This was apparent in later periods where DIO rats consumed more HF food than DR rats in contrast with 1st week when in both
groups a substantially elevated food intake has been found. From this point of view, our results suggested that the early hyperphagia is the primary reason that facilitates the expression of obese DIO and lean DR phenotypes and those other factors might be important in mediation of susceptibility or resistance to a HF diet intake of at that time.

Our results suggested that the consumption of HF diet besides enhancement of obesity has profound effects on the intestinal function of rats. As compared to standard diet fed control group this nutritional intervention significantly increased AP and $\alpha$-glucosidase activities in duodenum and jejunum and increased jejunal protein/DNA ratio in HF group. This coincides with previous reports about a similar relationship between increased body fat and small intestinal changes in HF diet fed rats (Estornell et al. 1995, Mozeš et al. 2006). However, with regard to the presented data, it is not clear whether the effectiveness of nutrient absorption, the sustained increase of enzyme activity or both may be of importance for the maintenance of elevated body fat weight once dietary obesity had been established. Moreover some studies have shown that similar mechanisms of an increased enzyme ”set point” may be involved in the modulation of body fat in several forms of obesity in which body weight and body fat changes cannot be explained by hyperphagia only. Indeed, it has been found that genetically obese mice and MSG obese rats exhibited a significantly higher intestinal disaccharidase and AP activity (Flores et al. 1990, Mozeš et al. 2000) that surprisingly precedes the development of excessive body weight.

Our present results provide first experimental evidence to support an important role of the small intestine in induction of susceptibility and resistance to diet induced obesity. These results indicates that the small intestinal plasticity can be perpetuated by nutritional conditions when caloric density of the diet is raised, nevertheless the intestinal changes were differently involved in weight gain and food intake regulation in DIO and DR phenotypes. In fact, rats identified later as DR had significantly greater AP and $\alpha$-glucosidase activities and more
pronounced suppression of energy intake than DIO rats after exposition to HF diet. In mature laboratory animals the brush-border-bound AP and several disacharidases are in general, considered as enzymes involved fat and glucose absorption; their activity increased after eating fat and carbohydrates (Malathi et al. 1973, Tanaka et al. 1998, Mahmood et al. 2003).

Considering the finding that enzymatic changes were proportional to the size of previous overfeeding in these different phenotypes, it is possible that the excess of overnutrition was sufficient in DR rats to induce maximal elevation of enzyme values and adjustment their activities at the higher level, but this was not the case in DIO rats. An indication of this is the fact that the higher activity of enzymes in DR group were fixed for later life i.e. at the time when their previously enhanced energy intake lowered to the almost similar level as in lean control rats.

In light of this knowledge it is feasible to assume that small intestine of DIO rats appears to be less responsive than DR rats to dietary challenge. The obesity-prone DIO rats increased their weight gain and adiposity without substantial changing of enzyme activities, while DR rats reduced their weight gain and body fat accretion and these changes were reflected in simultaneously enhanced AP and \( \alpha \)-glucosidase activities. One of potentially interesting aspects of our present results is that the energy efficiency and size of body fat tissue observed in these groups is inversely related to the small intestine function. From this point of view it is more probable that a higher effectiveness of nutrient absorption and lower enzyme activities may be of importance for the maintenance of obesity in DIO rats, while in DR rats increased enzyme ”set point” can be a major determinant of their ability to maintain both energy homeostasis and fat pad content at lowered level. Such a possibility seems to be supported by evidence suggesting on inverse relation between intestinal AP and the rate of fat absorption. Consistently, in AP deficient KO mice after long-time exposure to HF diet, a
faster body weight gain, elevation of serum triglyceride levels and accelerated transport of fat
droplets in comparison with wild-type controls has been observed (Narisawa et al. 2003).

Regarding the enzymatic and biochemical small intestinal changes observed in HF rats it remains unclear how the range of these responses are generated. There exist the data suggesting that besides dietary influences the genetic background may play a significant role in development of DIO and DR phenotypes. The higher body weight and obesity (despite feeding a low fat diet) has been observed in selectively bred (3-5 generations) DIO as compared to DR rats (Levin and Dunn-Meynell 2002, Ricci and Levin 2003) and when fed HF diet DIO rats became more obese than DR rats (Ricci and Levin 2003). Since human obesity is not homogenous regarding its genetic and nutritional background, the used model of out-bred Sprague-Dawley rat is helpful for further exploration the involvement of nutritional imprint in development the diet-induced obesity. From this point of view the present findings extend our knowledge about the interaction between small intestinal function, food intake control and the energetic metabolism and allow better understanding the intestinal mechanisms that may limit or prevent the expression of susceptibility or resistance to the high-energy diet in DIO vs. DR rats.

Acknowledgments
This work was supported by the grant 2/5141/25 and 2/6131/26 of the Slovak Academy of Sciences and by EU project QLK 1-2000-00515 / QLRT-2002-02793.

References


STUBBS RJ, HUGHES DA, JOHNSTONE AM, HORGAN GW, KING N, BLUNDELL JE:


Table 1. Body weight, weight gain, food intake, body fat and Lee’s index in adult male Sprague-Dawley rats after 4 weeks exposure to control diet (control group) and HF diet (experimental groups)

<table>
<thead>
<tr>
<th></th>
<th>Body weight (g)</th>
<th>Weight gain (g)</th>
<th>Food intake kcal/day</th>
<th>Food efficiency</th>
<th>Body fat %</th>
<th>Lee’s index</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control diet</td>
<td>547.8±5.2</td>
<td>44.7±3.1</td>
<td>92.2±1.2</td>
<td>2.03±0.13</td>
<td>2.8±0.2</td>
<td>0.311±0.001</td>
</tr>
<tr>
<td>n=16</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HF diet</td>
<td>576.5±9.6</td>
<td>82.4±6.4</td>
<td>110.4±2.4</td>
<td>2.63±0.17</td>
<td>4.0±0.3</td>
<td>0.317±0.001</td>
</tr>
<tr>
<td>n=16</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DIO</td>
<td>593.0±11.9</td>
<td>100.5±5.6</td>
<td>117.0±2.0</td>
<td>2.98±0.19</td>
<td>4.2±0.3</td>
<td>0.320±0.002</td>
</tr>
<tr>
<td>n=9</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DR</td>
<td>555.3±12.2</td>
<td>59.0±4.4</td>
<td>102.0±2.1</td>
<td>2.19±0.20</td>
<td>3.7±0.6</td>
<td>0.314±0.002</td>
</tr>
<tr>
<td>n=7</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Values are means ± SE. Body fat (%) represents epididymal plus retroperitoneal fat pads. Food efficiency = weight gain/calories consumed during 4 weeks. Lee’s index = \( \sqrt{\text{body weight (g)/nasoanal length (cm)}} \). Values with different superscripts in each groups are significantly different at P<0.05 by LSD multiple comparison test after ANOVA. \( \dagger \) significant differences between DIO vs. DR groups.
Table 2. Duodenal and jejunal parameters of enzyme activities in adult male Sprague-Dawley rats after 4 weeks exposure to control diet (control group) and HF diet (experimental groups)

<table>
<thead>
<tr>
<th></th>
<th>DUODENUM</th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Alkaline phosphatase</td>
<td>α-glucosidase</td>
<td>Aminopeptidase</td>
<td>DNA</td>
<td>Protein</td>
</tr>
<tr>
<td>Control diet n=16</td>
<td></td>
<td>14.4±0.3^a</td>
<td>9.3±0.3^a</td>
<td>12.9±0.9^a</td>
<td>3.7±0.1^a</td>
<td>159.9±3.4^a</td>
</tr>
<tr>
<td>Control diet n=16</td>
<td></td>
<td>16.3±0.6^b</td>
<td>9.7±0.3^a</td>
<td>11.3±0.5^a</td>
<td>3.9±0.2^a</td>
<td>154.0±3.9^a</td>
</tr>
<tr>
<td>DIO n=9</td>
<td></td>
<td>15.4±0.7^a †</td>
<td>9.7±0.4^a</td>
<td>11.5±0.3^a</td>
<td>4.1±0.2^a</td>
<td>152.4±6.3^a</td>
</tr>
<tr>
<td>DR n=7</td>
<td></td>
<td>17.5±0.9^b</td>
<td>9.8±0.6^a</td>
<td>11.0±1.0^a</td>
<td>3.6±0.7^a</td>
<td>156.1±4.2^a</td>
</tr>
</tbody>
</table>

|                | JEJUNUM |                                                                 |                                                                 |                                                                 |                                                                 |                                                                 |
| Control diet n=16 |          | 13.8±0.4^a | 14.6±0.4^a | 19.7±0.9^a | 4.2±0.1^a | 140.8±3.9^a | 33.8±1.2^a |
| Control diet n=16 |          | 16.1±0.5^b | 16.1±0.4^b | 20.3±0.6^a | 3.3±0.2^b | 147.5±4.4^a | 47.1±3.3^b |
| DIO n=9         |          | 15.3±0.5^b † | 15.4±0.3^a † | 20.1±0.9^a | 3.5±0.3^b | 150.9±7.2^a | 45.2±4.2^b |
| DR n=7          |          | 17.0±0.7^b | 16.9±0.7^b | 20.4±0.8^a | 3.1±0.2^b | 143.6±4.8^a | 49.3±5.4^b |

Values are means ± SE. Enzyme activities are given as a density values (pixel intensities) in duodenal and jejunal entrecytes at wavelength of 520 nm. Values with different superscripts in each groups are significantly different at P<0.05 by LSD multiple comparison test after ANOVA. † significant differences between DIO vs. DR groups.
Figure legend

Fig. 1. Energy intake (kcal/day) of adult male Sprague-Dawley control group, DIO and DR rats fed either a standard diet or HF diet (experimental groups) for 4 weeks. Values are means ± SE. Bars with differing superscripts differ from each other at P < 0.05 by post hoc test. Two-way ANOVA (time, dietary, time x diet interaction) showed significant intergroup differences at (P<0.0001).