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The Adaptation of Small Intestine Nitregic Myenteric Neurons on Rats (*Rattus norvegicus*) to High Fat Diet

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ABSTRACT

High fat diet can result in the loss of nitregic neurons in the myenteric plexus. The study aimed at finding out the effect of high fat diet on the adaptation of nitregic nerve of rat intestine. It used 15 male rats (*Rattus norvegicus*) of a month of age with mean body weight of 53.73 gr. The rats were adapted for 7 days to individual cages with ad libitum feeding. After random adaptation, all of the rats were assigned to 3 groups of five rats, namely K-7, K-10, and K-13 groups. Feed and drinking water were given ad libitum. The treatment of the high fat diet lasted for 7 weeks. After the treatment, all of the rats were fasted for 12 hours and then killed. Subsequently, small intestine segments (duodenum, jejunum, and ileum) were taken. The intestinal segments were prepared by using NADPH-d histopathological technique to determine the morphometric changes of nitregic myenteric neurons. During the treatment the rats were weighed every week and at the end of the study orbitalis vein blood measurement was carried out to see its glucose, cholesterol and cholecystokinin (CCK) plasma levels. The data of body weight, glucose, cholesterol, CCK levels, the total number of the nippergenic myenteric segments of the small intestine were statistically analyzed using Anova. The results of the study showed that the treatment of 7% to 13% fat diets for 7 weeks did not indicate any weight gain and any increase in cholecystokinin level, and any decrease in glucose level. However, it indicated significant increase in cholesterol level. The treatment of 10% and 13% fat diets increased the total number of neurons in the jejunum and the ileum. Thus, it was concluded that the treatments of the high-fat diet of the rats (K-7, K-10, and K-13) for 7 weeks had significant effect on the adaptation of the neurons of the jejunum and the ileum.

Keywords: Fat diet, Myenteric, Nitregic nerve, Rats, Small intestine

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Introduction

Consumption of dietary fat is among the most important environmental factors of obesity. The motility of gastrointestinal tract (GIT) is directly controlled by enteric inhibitory neurons and motor excitations that innervate smooth muscle layers. The nitregic myenteric neuron is an inhibitory motor neuron that serves relaxation and accommodating functions and controls the opening of sphincter. Therefore, it plays an important role in regulating normal GIT motility (Cunningham, 2002; Herdt, 2002; Brookes, 1993). Dysfunction of nitregic neurons can cause various GIT diseases (Oliveira and Goncalves, 2010; Bartho and Lefebvre, 1995).

Nitric oxide (Nitric oxide, NO) is an important relaxant neurotransmitter in GIT smooth muscle, which is synthesized from L-arginine by nitric oxide synthase (Nitric oxide, NOS). Neurons containing NO have been classified as Non-

Adrenergic Non-Cholinergic Neurons (NANC), which are important in regulating normal GIT motility (Lefebvre, 1995; Williams and Parsons, 1995).

The main organ that performs digestive activity and absorption of nutrients is small intestine. The small intestine performs peristaltic action. The peristaltic action moves the cimus along the gut and causes the shift of the cimus on the surface of the intestinal mucosa so that the digestion tract can digest and absorb nutrients (Thomas, 2003; Cunningham, 2002). The system that regulates bowel movements is the intestinal nervous system consisting of intrinsic sensory myenteric neurons, some types of myenteric interneurons and motor neurons myenteric stimuli and barriers (Grider, 2003; Thomas, 2003). The main cause of the increase and the decrease in the peristaltic motion is the density of the myenteric neurons. If the density of the myenteric neuron decreases, the innervation decreases and it

alters the local sensitivity of the control and hence causes peristaltic motion to decrease. On the contrary, if the density of the myenteric neuron increases, the innervation increases so that the peristaltic wave increases (Aube *et al.*, 2006). The change in the population of intestinal myenteric neurons is an adaptation response to increased workload (Natali *et al.*, 2003). The study aimed at finding out the effect of the high fat diet on the neuron apoptosis in the small intestinal segment.

Materials and Methods

All of the methods in conducting the study have been approved by LPPT Research Ethics Committee on the approval paper Number 00061/04/LPPT/V/ 2017. The study was conducted in the Experiment Animal Farming Unit (EAFU) of Universitas Gadjah Mada, including the maintenance, the weight measurement, and the blood taking of the experiment animals. The measurement of blood glucose level, CCK level, the preparation and the NADPH-d staining on the small intestinal segment were conducted in the Physiology Laboratory of the Faculty of Veterinary of Universitas Gadjah Mada. The measurement of blood cholesterol level was conducted in LPPT.

Instruments

The instruments used in the study are individual rat cages and equipments, digital glucometer (Accu-chek® Advantage, Roche Diagnostic, Germany), light microscope (type 102, Nikon, Japan); scissors, tweezers for surgery, 25 ml beaker, 10 µl, 100 µl, 1,000 µl Eppendorf tubes (Gilson, France), Eppendorf micropipette tips (Gilson, France), incubator (Mettler, Germany), object glass, glass cover, electrical digital scales (Germany), vortex, refrigerator (Panasonic, 825 AF), light microscope, stereo microscope for NADPH-d staining technique.

Animals

The experimental animals used were 15 white rats (*Rattus norvegicus*) of a month of age with mean weight of 53.73 grams obtained from the Experiment Animal Farming Unit (EAFU) of Universitas Gadjah Mada, 7%, 10% and 13% fat diets for normal rats (produced by PT Japfa Comfeed Indonesia, Sidoarjo, consisting of 53% com-pars Pars with 12% water content, 11% protein, 4% fat, 7% fiber, 8% grains, 1.1% Ca, 0.9% phosphorus, antibiotics, 53% coccidiostat and 23.5% wheat flour and 23.5% water) with additional pork fat, 10% formalin solution, 30% PBS, nitro blue tetrazolium powder, β NADPH-d crystal, 0.5% Triton solution X-100, gelatin, Tris-HCL buffer containing 0.01% 3,3'-diaminobenzidine, and aquades for NADPH-d staining, Rat CCK (Cholecystokinin), Elisa kit (Cat No. ER0372), Finetest, China for measuring rat cholecystokinin levels.

Treatment

Fifteen rats (*Rattus norvegicus*) were adapted for 7 days to individual cages with ad libitum feeding. After random adaptation, all of the rats were assigned to 3 groups of 5 rats, namely K-7, K-10, and K-13. Before the feeding treatment, they were fasted for 12 hours. Feed and drinking water were given ad libitum. The treatment of the high fat diet lasted for 7 weeks. After the treatment, they were fasted for 12 hours and Subsequently, small intestine segments (duodenum, jejunum, and ileum) were taken. The intestinal segments were prepared by using NADPH-d histopathological technique to determine the morphometric changes of nitregic myenteric neurons. During the treatment the rats were weighed every week and at the end of the study orbitalis vein blood measurement was carried out to see its glucose, cholesterol and cholecystokinin (CCK) plasma levels.

Determining blood glucose level

Blood samples were drawn through the tail tip of the rats that have been cleaned using 70% alcohol and then the tail tip was punctured with a small needle (1 cc syringe). Once the blood has come out, a digital glucometer strip was used to touch it. Blood glucose levels expressed in mg/dL will be read on the glucometer screen.

Determining choleistokknin level

The CCK measurement was carried out using enzyme-linked immunosorbent assay (ELISA) Rat CCK (Cholecystokinin) Elisa kit, Finetest, China following the ELISA kit procedure (No. Catalog ER0372). Samples were placed in the well and Biotin-detection antibody was added. The enzyme-substrate reaction is terminated by adding TMB solution. Color will soon turn yellow. Immediate absorbance of OD is read on 450nm with elisa reader.

The examination of small intestine myenteric neurons with NADPH-d staining technique (Lee and Nam, 2006; Ramos-Vara, 2005; Neunlist *et al.*, 2003)

Once the small intestinal segments have been washed using PBS, they were fixed using 4% formalin solution for an hour. And then, the muscles and the mucosa were separated under a stereomicroscope. Subsequently, the small intestinal segmental muscle was incubated in a mixed solution of NBT and β NADPH-d in 0.05 M buff Tris-HCL buffer (pH 7.4) at 37°C. After that, the muscle was covered using gelatin and glass cover. The total number total of the myenteric neurons/1 cm² was objectively calculated under a microscope at 40 x magnifications. And then, optilabs were connected to a computer system for image analysis. There were totally 40 myenteric neurons of each rat.

Statistical analysis

The data of body weight, glucose, cholesterol, CCK levels, the total number of the

nitregic myenteric neurons of the small intestine segment were statistically analyzed using direct pattern Anova. If the resulting data were indicative of significant difference, Tukey test was then carried out.

Results and Discussion

Small intestinal nitregic neurons

The results of the observation of the form of the neuron in the duodenum, the jejunum and the ileum show that they are the same (i.e., oval nucleus form). The neuronal form in the duodenum of the K-7 group is shown in Figure 1. The mean numbers of the duodenal nitregic neurons, the jejunum and the ileum of the rats in the K-7, K-10 and K-13 groups treated using high fat diets for 7 weeks are summarized in Table 1.

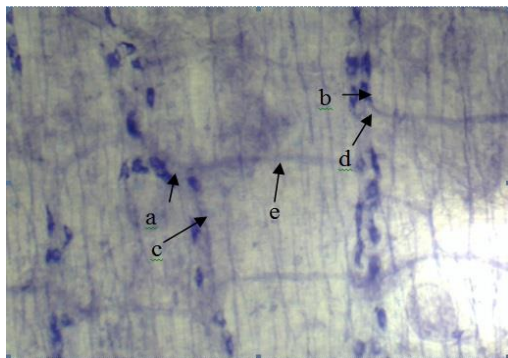


Figure 1. Form of duodenal neuron group of K-7 rats. Description: a. Neurons, b. nucleus, c. dendrites, d. axons, e. nerve fibers. NADPH-d staining, light microscope, 20x10 magnification.

The number of duodenal nitregic neurons

The mean numbers of the nitregic duodenum/1 cm² neurons of the rats in the K-7, K-10 and K-13 groups (Table 1) groups showed that the number of K-10 and K-13 neurons increased significantly (K-7) (Figure 2). However, the K-7, the K-10 and the K-13 groups were statistically not significantly different (p>0.09). It meant that the high-fat diet does not significantly affect the number of duodenal neurons of the K-7, K-10 and K-13 groups (Figure 2).

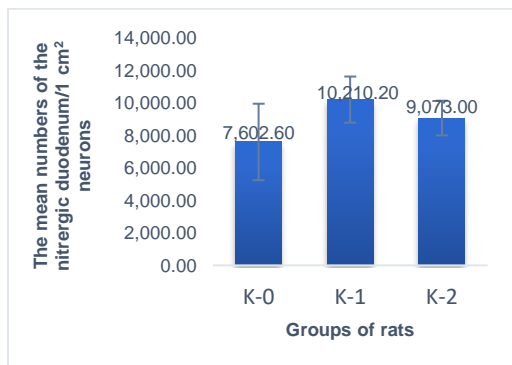


Figure 2. Changes in the number of duodenal nitregic neurons of the K-7, K-10 and K-13 group of high-fat diets.

The number of jejunal nitregic neurons

The mean total numbers of the jejunum/1 cm² nitregic neurons of the rats in the K-7, K-10 and K-13 groups (Table 1) statistically showed that they were significantly different (p<0.000). The results of the Tukey's test showed that the K-7 group and the K-10 group were not significantly different (p>0.173), while the K-7 group and the K-13 group were significantly different (p<0.001) and the K-10 group and the K-13 group were very significantly different (p<0.000). It meant that the high-fat diet affects the number of the jejunal neurons in the K-10 group. The number of the jejunal neurons of the K-10 decreases, while that of the K-13 increases and is higher than that of the K-7 (Figure 3).

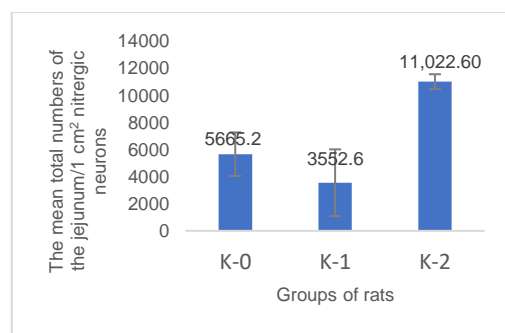


Figure 3. Changes in the number of jejunal nitregic neurons of the K-7, K-10 and K-13 high-fat diets.

The number of nitregic ileal neurons

The mean total numbers of the nitregic ileum/1cm² neurons of the rats in the K-7, K-10 and K-13 groups (Table 1) statistically showed that they were significantly different (p<0.000). The results of the Tukey's test showed that the K-7 group and the K-10 group were significantly different (p<0.000), while the K-7 group and the K-13 group were not significantly different (p>0.893), and the K-10 group and the K-13 group very significantly different (p<0.000). The number of the ileal neurons of the K-10 group increased significantly and was higher than that of the K-7 and K-13 groups. It meant that the high-fat diets increased the number of the neurons in the high ileum of the rats in the K-10 group and was higher than that of the K-7 and K-10 groups (Figure 4).

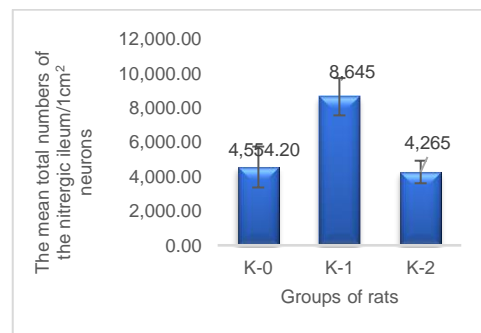


Figure 4. Changes in the number of nitregic neurons of rat ileum of the K-7, K-10, and K-13 diets high in fat.

Table 1. Average number of nitergic intestine of small intestine / 1 cm² group of K-7, K-10 and K-13 high fat diet for 7 weeks

| Rat Group | Number of nitergic neurons of the small intestine/1 cm ² | | |
|-----------|---|------------------|------------------|
| | Duodenum | Jejunum | Ileum |
| K-7 | 7,602.60±2,354.71 | 5,665.2±1,610.06 | 4,554.2±1,190.33 |
| K-10 | 10,210.20±1,421.45 | 3,552.6±2,468.14 | 8,645.0±1,085.71 |
| K-13 | 9,073.00±1,068.34 | 11,022.6±547.06 | 4,265.0±656.52 |

K-7: group of rats with diet containing 7% fat, K-10: group of rats with diet containing 10% fat, K-13: group of rats with diet containing 13% fat.

Although a general neuron population is maintained, the high-fat diet affects nitergic subpopulations, which causes the increase in the density of nNOS-IR neurons. This increase may indicate the change in chemical code and in turn it causes the change in intestinal motility because the myoregic neurons of the small intestine of the rats that contain nNOS are mostly motor neurons inhibitors (90%) that conserve the muscle layer and also the interneuron descendents (10%) involved in local motility reflex. It means that slower intestinal transition increases nutrient retention and absorption and it amplifies the morphometric results obtained from the intestinal villi of the duodenum and the jejunum. The rats with a high-fat diet treatment show longer gastrointestinal transition time (Soares *et al.*, 2015) and hence it confirms the hypothesis of the study.

The general population of the myenteric neurons that is reflected in immunohistochemistry to detect myosin-V proteins, neuron cell markers, does not change in the duodenum or the jejunum with high-fat diet treatment. The highest neuronal density in the ileum observed in the treatment group is more likely to be a consequence of the decrease in wall thickness and circumference of the segment, which results in higher neuronal concentration per area as reported in other experimental models. The loss of the neurons takes place in the CF1 rat colon with the fat diet treatment of 60% kcal for 11 weeks (Soares *et al.*, 2015). The high fat diet increases the density of the myosin-V-IR neurons in ileal and in the nNOS-IR neurons in all segments of the small intestine. There is not any significant difference in the density of the general neuronal population (myosin-V-IR) observed between the control and the treatment groups ($p>0.05$). Concerning with nitergic subpopulations, HFD increases the density of the nNOS-IR neurons in the treatment group (23%, $p<0.05$). The neuronal density of the myosin-V-IR population in the jejunum of the control and the treatment groups did not change ($p>0.05$). However, the increase in nNOS-IR of the subgroup (24%, $p<0.01$) was observed in the treatment group as compared to the control group. The high fat diets significantly increased the neuron density of the myosin-V-IR population (16%, $p<0.01$) and the nNOS-IR subpopulation (28%, $p<0.01$) in the OB group ileum (Soares *et al.*, 2015). In C57BL/6 mice, the neuron loss was also observed in the duodenum with the high fat diet treatment of 72% kcal for 8 weeks and in the ileum and the colon with the high fat diet treatment of 45% kcal after 6 months. The study shows that the loss of the neurons may vary with different

lipid levels in food, different intestinal segments, rat strains, and feeding periods. It is not known in the study whether the number of the neurons is maintained without any neurodegeneration because it is impossible to assess the area of the ileum (by measuring its length) as a result of the absence of macroscopic constraints on the segment. Using the same experimental model, we previously observed a significant decrease in the length and the circumference of the large intestine, and we used correction factors to calculate regional reduction indicative of neuron loss (Soares *et al.*, 2015). Interestingly, changes are observed in length and circumference and hence it corroborates the morphometric results of the small intestine wall. The villus height and thickness of the intestinal wall increased in the duodenum and jejunum and decreased in the ileum of the treatment group. The changes indicate adaptations to the digestibility and the consistency of the high-fat diet or the increased nutrient retention in the duodenum and the proximal jejunum as a result of the decreased motility in the segment. Prior study has shown that nutrient uptake mostly occurs in the proximal bowel, and few nutrients reach the ileum. Furthermore, high-fat diet reduces duodenal motility. The decrease in the motility in the study may be the result of the increased density of the nitergic neurons (inhibitors). Consequently, the high fat diet may have affected bowel absorption capacity, which results in weight gain and visceral adiposity. Different changes in the ileum indicate the involvement of other factors, such as microbiota that is typically found in the ileum and affects the morphology of villi and crypts. Despite the changes in villous morphometry, mucosal changes do not occur because of the changes in cell proliferation rate (Soares *et al.*, 2015). According to Soares *et al.* (2015), the treatment of high fat diet containing 59% kcal fat for 8 weeks has induced changes in myenteric plexus (myenteric neuroplastic) and adaptation to mucous walls and cells along the intestinal segment. The loss of neurons in the colon of CF1 rats with the treatment of the diet 60% kcal fat for 11 weeks decreases in the area of the cell body of neurons and varicosities. It showed the decrease in the metabolic activity that may be associated with cell injury. In this study, the diets that contain fat 7%, 10% and 13% did not affect the number of the neurons in the duodenum, while the diet containing 13% fat increases the number of neurons.

The changes in body weight of the rats in the K-7, K-10 and K-13 groups showed that the body weight of the K-10 and K-13 groups were

lower than K-7 (control) (Figure 5). However, the differences in body weight of the rats in the K-7, K-10 and K-13 groups were not significant ($p>0.057$). It means that the treatment of the high-fat diets in the K-7, K-10 and K-13 groups does not affect the difference in weight gain.

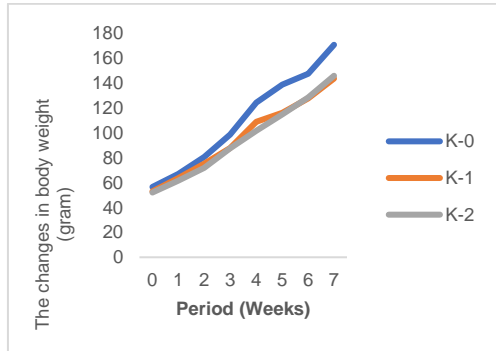


Figure 5. Changes in body weight of K-7, K-10 and K-13 diets high fat diet for 7 weeks.

The rats treated with high-fat diet (59% fat) for 7 weeks showed a 14% increase in body weight and a 144% increase in visceral fat weight, which results in moderate obesity. The obesity results from the increased resilience of the hypothalamus and the anorexigenic hormone, leptin and insulin that cause progressive loss of balance between dietary intake and thermogenesis (Morales *et al.*, 2009). It means that the concentration of the fat does not triggered the loss of balance between the food intake and the thermogenesis.

Blood glucose level

The mean and standard deviation of the blood glucose, cholesterol and cholecystokinin levels of the rats in the K-7, K-10 and K-13 groups with the treatment of the high fat diets were summarized in Table 2. The blood glucose levels of the rats in the K-7, K-10 and K-13 groups (Table 2) showed statistically significant differences ($p<0.021$). The results of the Tukey test showed that the rats in the K-7 group were significantly different from those in the K-10 group ($p<0.017$), while the rats in the K-7 group were not significantly different from those in the K-13 group ($p>0.145$), and the rats in the K-10 group were not significantly different from those in the K-13 group ($p>0.145$) (Figure 6). It means that the treatment of the high fat diet lowers the blood glucose levels of the rats in the K-10 and K-13 groups as compared to those in the K-7 group (Figure 6).

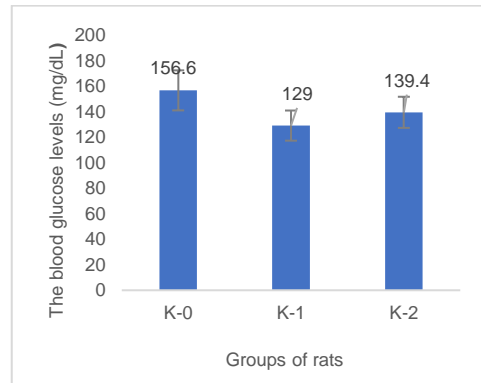


Figure 6. Changes in blood glucose levels of the K-7, K-10 and K-13 diets high-fat diet group at week 7.

Cholesterol level (mg/dL)

The mean cholesterol levels of the rats in the K-7, K-10, and K-13 groups (Table 3) showed statistically significant difference ($p<0.001$). The results of the Tukey tested showed that the rats in the K-7 group were significantly different from those in the K-10 group ($p<0.021$), while the rats in the K-7 group differed significantly from those in the K-13 group ($p<0.000$), and the rats in the K-10 group differed significantly from those in the K-13 group ($p<0.024$) (Figure 7). It means that the treatment of the high fat diet increases the blood cholesterol levels of the rats in the K-10 and K-13 groups as compared to the rats in the K-7 group. The higher is the fat level, the higher the cholesterol level will be (Figure 7).

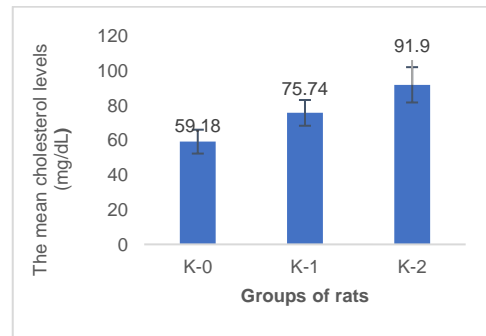


Figure 7. Changes in blood cholesterol levels of the K-7, K-10 and K-13 high-fat diets.

Cholecystokinin level (pg/mL)

The mean plasma CCK levels of the rats in the K-7, K-10, and K-13 groups (Table 2) statistically do not show any significant difference ($p>0.41$). The low fat diet does not have any effect

Table 2. Mean and standard deviation of blood glucose levels, cholesterol levels and cholecystokinin levels of K-7, K-10 and K-13 diets high in fat

| Group rat | Blood gluc lev (mg/dL) | Chol. lev (mg/dL) | Cholesist. lev (pg/dL) |
|-----------|------------------------|-------------------|------------------------|
| K-7 | 156.6±15.68 | 59.18±6.88 | 258.47±66.86 |
| K-10 | 129.0±11.81 | 75.74±7.41 | 239.38±59.72 |
| K-13 | 139.4±12.2 | 91.90±10.17 | 243.28±65.48 |

K-7: group of rats with diet containing 7% fat, K-10: group of rats with diet containing 10% fat, K-13: group of rats with diet containing 13% fat.

Table 3. Average weight of rats chart of K-7, K-10 and K-13 high-fat diets for 7 weeks

| Group | No. Rat | Body weight (gram) week - | | | | | | | |
|-------|---------|---------------------------|-------|-------|-------|--------|--------|--------|--------|
| | | 0 | 1 | 2 | 3 | 4 | 5 | 6 | 7 |
| K-7 | X | 56.48 | 66.98 | 80.60 | 98.52 | 124.14 | 138.56 | 147.46 | 170.66 |
| | Sd | 3.13 | 6.34 | 7.99 | 10.56 | 13.54 | 15.06 | 13.79 | 20.91 |
| K-10 | X | 52.92 | 63.62 | 75.20 | 87.74 | 108.82 | 115.92 | 127.70 | 143.54 |
| | Sd | 4.03 | 6.24 | 12.38 | 19.71 | 17.70 | 14.68 | 14.68 | 18.06 |
| K-13 | X | 52.00 | 61.48 | 71.80 | 87.60 | 101.72 | 114.72 | 128.48 | 145.78 |
| | Sd | 4.48 | 5.52 | 5.40 | 5.51 | 11.65 | 10.11 | 10.83 | 12.84 |

K-7: group of rats with diet containing 7% fat, K-10: group of rats with diet containing 10% fat, K-13: group of rats with diet containing 13% fat.

on the elevated levels of cholecystokinin. It means that the treatment of the high fat diet does not affect the increase in the CCK levels of the rats in the K-7, K-10 and K-13 groups.

According to Cheung *et al.* (2009), it has been reported in some studies that if the CCK levels increase, the duodenum may decrease glucose production and increase the changes in insulin circulation. Furthermore, the CCK levels of the duodenum require CCK receptor intestinal activation and the liver-brain-nerve intestinal axis to decrease glucose production. The recent CCK duodenum failed to decrease the glucose production in the early high fat diet treatment that triggers insulin resistance. The role of the intestinal CCK is to decrease the production of glucose through neural tissue and the CCK intestine can cause hyperglycemia as a result of high fat diet intake.

Conclusions

The treatments of 7%, 10% and 13% dietary fats in this study did not affect the metabolic activity in the duodenum, but there was an increase in metabolic activity in the jejunum and the ileum that might cause cell injury indicative of the adaptation to the innervation of the jejunum and the ileum. The total number of the neurons in the duodenum, the plasma control CCK level (K-7%) and the treatment (K-10, K-13) were equal. However, the blood glucose levels of the rats in the K-10 and K-13 groups decreased. The means of the treatment of 7%, 10% and 13% dietary fats for 7 weeks have shown the role of innervations in the duodenum to decrease blood glucose level. It was concluded that the treatment of the high fat diets of the rats (K-7, K-10, K-13) for 7 weeks had shown high fat effect on adaptation of the neurons in the jejunum and ileum.

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