International Journal of Nutrition Sciences

Journal Home Page: ijns.sums.ac.ir

ORIGINAL ARTICLE

The Effect of Melanocortin 4 Receptor Agonist RM-493 on Cognitive Functions in Rats Fed with Western Diet

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ARTICLE INFO

Keywords:
Western diet
Cognitive functions
Brain
MC4R
RM-493

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Received: July 16, 2022
Revised: October 23, 2022
Accepted: October 30, 2022

ABSTRACT

Background: The central melanocortin system is among those that plays a key role in the homeostatic regulation of energy balance and eating disorders. This study investigated the effect of melanocortin 4 receptor (MC4R) agonist setmelanotide (RM-493) on changes in metabolic and cognitive functions.

Methods: Thirty two male Sprague-Dawley rats were divided into 4 groups including those fed with standard laboratory food and given phosphate buffered saline (PBS, ND group); fed with western-type diet and given PBS (WD group); fed with standard laboratory food and given RM-493 (RM-493 group); and fed with western-type diet and RM-493 (WD+RM-493 group). After injection with PBS and RM-493 injections for 5 days, they were followed by elevated plus maze test and a novel object recognition test.

Results: Nutrition with western-type diet resulted in an increase in serum cholesterol, high-density lipoprotein (HDL) and low-density lipoprotein (LDL) levels, respectively, and RM-493 treatment decreased these values. Proopiomelanocortin (POMC), MC4R and brain-derived neurotrophic factor (BDNF) expressions increased in groups fed with western-type diet and RM-493. Treatment with RM-493 in ND group increased the residence time in the open arm. In WD group, CA3 region of the hippocampus revealed edema in stratum lucidum layer and degeneration in the pyramidal neurons unlike the WD+RM-493 group.

Conclusion: POMC-mediated pathway was activated as a result of an increase in body fat caused by a western-type diet. RM-493 had alleviating effects on brain damages caused by a western-type diet and could improve cognitive functions.

Please cite this article as: Aslan B, Karakaya B, Sirvanci S, Yildirim A. The Effect of Melanocortin 4 Receptor Agonist RM-493 on Cognitive Functions in Rats Fed with Western Diet. Int J Nutr Sci. 2023;8(1):47-56. doi: 10.30476/IJNS.2023.97449.1212

Introduction

Brain plays an important role in the control of energy intake and expenditure. Studies in the mid-1900s identified the hypothalamus as the key brain area that controls food intake and regulates energy balance (1). Endocrine system plays a fundamental role in the regulation of fat, carbohydrate and protein metabolism. It is accepted that adipose tissue is under endocrine control and can act as an endocrine organ that secretes hormones (2). There is an evidence that the "western-type diet", which has a high fat and sugar ratio, accompanies learning and memory deficits (3). Leptin-Leptin receptor, alpha melanocyte stimulating hormone (α-MSH), melanocortin 4 receptor (MC4R) and brain derived neurotrophic factor (BDNF), tropomyosin receptor kinase B (TrkB) pathways function in the central control of energy balance in the body (4).

The central melanocortin system is among those that plays a key role in the homeostatic regulation of energy balance and eating disorders especially the involvement in the binge eating disorder and anorexia nervosa (5-8). Arcuate melanocortin neurons consist of two distinct neuronal populations including neurons expressing proopiomelanocortin (POMC)/cocaine amphetamine regulated transcript (CART) and neurons expressing neuropeptide Y/agouti-related peptide (NPY/AgRP). Some studies have proven that although neuronal activation of POMC reduces food intake and increases energy expenditure and thermogenesis, neuronal activation of NPY/AgRP increases food intake, decreases energy expenditure and thermogenesis. POMC neurons are vital with beneficial effects on leptin and serotonin metabolism (9, 10).

Serotonin is a monoamine involved in the regulation of food intake and body weight (11). There is also evidence that serotonin affects movement control and decision-making in the cognitive process (12). Like most neurons, serotonergic neurons also receive local gamma aminobutyric acid (GABA)ergic inhibitory inputs (13). While leptin hormone secreted from adipose tissue and acting on POMC inhibits the activity of NPY/AgRP neurons; it activates MC3R in the arcuate nucleus and MC4R in the paraventricular nucleus (14). Central MC4R activation accelerates satiety, decreases insulin secretion, increases insulin sensitivity and increases energy expenditure (15). Central MC4R activity may play an important role in coordinating behavioral and hormonal responses to psychological stress (16). These findings suggest that activation of the central melanocortin system involves endocrine and behavioral responses to stress (16, 17).

However, studies on this agonist have been limited to changing metabolic parameters in the body and protein expressions in brain signaling pathways. There is no study that has looked at the effects on possible diet-induced behavioral and memory changes with the melonocortin reseptör agonists. In light of all this information, animals were fed a high fat and high sugar western-type diet in the present study. Afterwards, it was aimed to investigate the effect of MC4R agonist RM-493 treatment on possible changes in metabolic and cognitive functions and the mediating mechanisms in male rats.

Materials and Methods

The study was approved by Marmara University Experimental Animal Research Ethics Committee**.** In the study, male Sprague-Dawley rats weighing 250-300 grams provided by Marmara University Experimental Animals Application and Research Center were used. The rats were kept in laboratory conditions of 22±2°C, 12 hours of light and 12 hours of dark with free access to water and food. Animals (n=32) were divided into 4 groups (each 8 rats) as described before (3); including those fed with standard laboratory food and given PBS (ND group); fed with western-type diet (MBD trade, Turkey) and given PBS (WD group); fed with standard laboratory food and given RM-493 (RM-493 group); and fed with western-type diet and given RM-493 (WD+RM-493 group). The dietary ingredients were shown in Table 1. At the end of 4 weeks, 100 mL/kg/day of PBS was injected subcutaneously for 5 days to two groups of animals, one fed standard diet and the other fed western-type diet. In the other two groups, RM-493 (MedChemExpress, USA) as a melanocortin 4 receptor agonist, was injected subcutaneously at 3.6 µmol/kg/day for 5 days as previously mentioned (18). Injections were given for 5 days between 9 and 10 am.

Elevated plus maze test was used to determine anxiety. Animals were placed in the center of the elevated plus maze test, which included open and closed areas with a height of 50 cm from the ground, a length of 50 cm and a width of 10 cm. A 5-minute video was shot with Sony (Cyber-Shot DSC –W530) brand camera. The numbers of times the animals entered the open and closed arms and the time they stayed in the open and closed arms were evaluated. The decrease of time the animals stayed in the open arm and the number of times they entered the open arm was interpreted as an increase in anxiety based on a previous report (19).

Novel object recognition test was followed for a previous study in order to evaluate the cognitive

Table 1: The dietary ingredients utilized for the animals.

Standard laboratory dietary content (3.4 kcal/g)	Western-type diet content (4.5 kcal/g)
18% fat	38% fat (saturated fat: Lard)
0% fructose	20% dextrose
58% carbohydrate	18% other carbohydrates
24% protein	24% protein

status of the rats at the end of the experiment. Good or bad memory was evaluated with reference by an index. This index was calculated as the ratio of the time to discover the novel object to the sum of the time to explore the novel and the old object. An increase in this ratio was interpreted as a better functioning of memory (20).

Blood was collected by cardiac puncture from animals under ketamine (100 mg/kg) and xylazine (10 mg/kg) anesthesia. After centrifugation in gel serum tubes, the serum was taken into eppendorfs and stored at -80°C. After blood collection, the hippocampus was removed from the brain. One hemisphere of the removed brain was stored in 10% buffered formaldehyde for histological examination, while the hippocampus removed from the other hemisphere was stored in 10% phosphate buffered saline (PBS) at -80°C.

In order to determine the effect of western-type diet on metabolic parameters in the body, serum cholesterol, triglyceride, high-density lipoprotein (HDL) and low-density lipoprotein (LDL) measurements were performed together. The serums prepared for this purpose were centrifuged again at 3000 rpm for 10 minutes. Then, 50 µL of samples were added to the eppendorfs. Next, the eppendorfs were inserted into the Siemens Dimension RxL Max (Newark, USA). In this device, appropriate reagent loading was done by the device for each parameter to be read automatically. Again, they were automatically mixed and processed, and the results were obtained.

To undertake Enzyme-Linked Immunosorbent Assay (ELISA), the collected blood was centrifuged at 3000 rpm for 10 minutes and stored at -80°C. Serum samples stored at -80°C were thawed. Leptin, serotonin and GABA levels in serum were detected by ELISA kits (Sunlong Biotech, China) as the protocol provided by the manufacturer. The ELISA plates were read at 450 nm in the ELISA reader and the results were recorded.

To conduct Western Blot, the hippocampus tissues lysed by cold RIPA buffer, and the samples were seperated using 10% Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The membranes were blocked at room temperature for 1 hour or overnight at 4°C using blocking buffer. Likewise, the membranes were incubated overnight at 4°C with the appropriate dilution of the primary antibodies; anti-POMC (abcam), anti-MC4R (GeneTex), and anti-BDNF (Novus). The membranes were washed 3 times with 1X Tris-Buffered Saline, 0.1% Tween® 20 Detergent (TBST) for 5 minutes each. The membranes were then incubated with conjugated secondary antibodies diluted in blocking buffer for 1 hour at room temperature. Washing was done 3 times with TBST for 5 minutes each. Excess reagent was removed and the membrane was covered with clear plastic gelatin. Images were then acquired using the Image-Lab imaging program.

For histological evaluation after the brain tissues from all groups were fixed in 10% formalin, they were dehydrated by passing through a series of rising alcohol. After dehydration, the tissues were cleared in xylene. The tissues, which were kept overnight in paraffin in a 60°C oven, were blocked in cassettes with an embedding device the next day. Hematoxylin and eosin stains were applied to the sections taken from paraffin blocks with a thickness of 5 µm for morphological evaluation. The stained sections were viewed and photographed with an Olympus BX51 photomicroscope (Tokyo, Japan) with an Olympus DP72 camera attachment.

For statistical analysis, data were expressed as mean±standard error. While comparing the evaluation parameters, GraphPad Prism 6 statistical program was used for all data. Data were compared with two-way ANOVA test and multiple comparisons with Tukey post hoc test. Values with $p<0.05$ were considered significant.

Results

Western-type diet group showed a significant increase compared to the normal diet group as a result of western-type diet ($p<0.0001$). While no change was observed in the RM-493 group given the normal diet, cholesterol levels significantly decreased in the western-type diet group ($p<0.0001$) shown in Figure 1A. Serum triglyceride levels increased on the western-type diet compared to the normal diet groups, but a significant increase was observed only among the RM-493-treated groups ($p<0.01$) demonstrated in Figure 1B. Serum HDL levels significantly increased in the western-diet group compared to the control as a result of Western-type diet ($p<0.001$). RM-493 injection

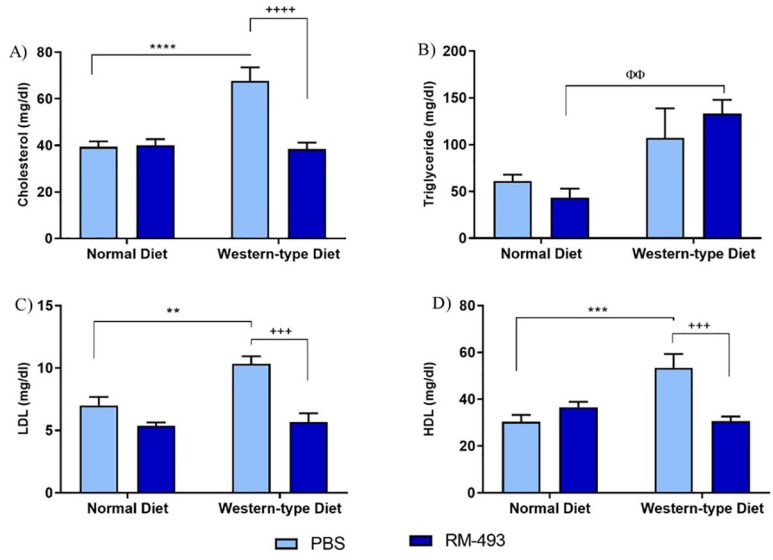

Figure 1: Levels of serum lipids in all the groups. Western-type diet increased the levels of the lipids, and RM-493 reduced this rise in the groups fed with WD, but there was no significant difference between the groups fed with ND. **ND vs. WD (p<0.01), ***ND vs. WD (p<0.001), ****ND vs. WD (p<0.0001), ΦΦRM-493 vs. WD+RM-493 (p<0.01), +++WD vs. WD+RM-493 (p<0.001), and ++++WD vs. WD+RM-493 (p<0.0001). LDL: Low-density lipoprotein, HDL: High-density lipoprotein, PBS: Phosphate buffered saline, RM-493: Setmelanotide

significantly reduced these levels in groups fed a Western-type diet (p<0.01) illustrated in Figure 1C. Serum LDL levels increased in the western-type diet compared to the normal diet groups. A significant increase was observed in the Western-type diet group as a result of the fatty diet compared to the control group (p<0.01). Injection of RM-493 significantly reduced these levels in the Western-type diet groups (p<0.001) displayed in Figure 1D.

Serum leptin levels tended to increase in the western-type diet groups, but were not statistically significant. There was no significant difference between the groups given PBS and agonists as shown in Figure 2A. Serum serotonin levels were lower in western-type diet group when compared with normal diet group, but there was no significant difference. RM-493 injections did not affect these levels significantly as presented in Figure 2B. Western-type diet group were found to have lower levels of GABA content than their control groups. Treatment with RM-493 increased these levels in both diet groups. This increase was significant only in the normal diet group (p<0.01) as exhibited in Figure 2C.

Western-type diet showed a significant increase in POMC expression when compared to normal diet (p<0.005). Likewise, it was significantly higher in the western-type diet in the group given RM-493 than in the control group (p<0.0001). POMC expression was found to be significantly increased in the group fed the western-type diet and injected with RM-493 in comparison to the control (p<0.0001) as demonstrated in Figure 2D. RM-493 injection caused an increase in BDNF expression in normal diet group and western-type diet group (p<0.001, p<0.0001, respectively). Similarly, a significant increase was observed in the western-type diet when compared to the control (p<0.05). In the Western-type diet group, the group given RM-493 showed a significant increase in comparison to the control group (p<0.0001) as illustrated in Figure 2E. Compared to the normal diet group, MC4R expressions significantly increased in the normal diet group given RM-493 and in the western-type diet group (p<0.0001, p<0.01, respectively). RM-493 injection significantly increased in the Western-type diet group when compared to the control (p<0.0001). MC4R expression significantly increased in the Western-type diet and RM-493 injection group in comparison to the normal diet group (p<0.0001) as presented in Figure 2F.

The significant difference between the groups given normal diet increased with RM-493 injection (p<0.05). Among the groups treated with RM-493, it was observed that eating a western diet significantly reduced this time in open arm (p<0.05) as exhibited in Figure 3A. The number of open arms did not differ significantly according to the type of diet the animals were fed and the injections given to the animals as indicated in Figure 3B. The number of closed arms increased in the western-type diet group when compared to the normal diet group, but it was not significant. Likewise, treatment with RM-493 was not significant between the groups as illustrated in Figure 3C. Feeding with a western-type diet and treatment with RM-493 did not produce significant positive or negative results on memory as mentioned in Figure 3D.

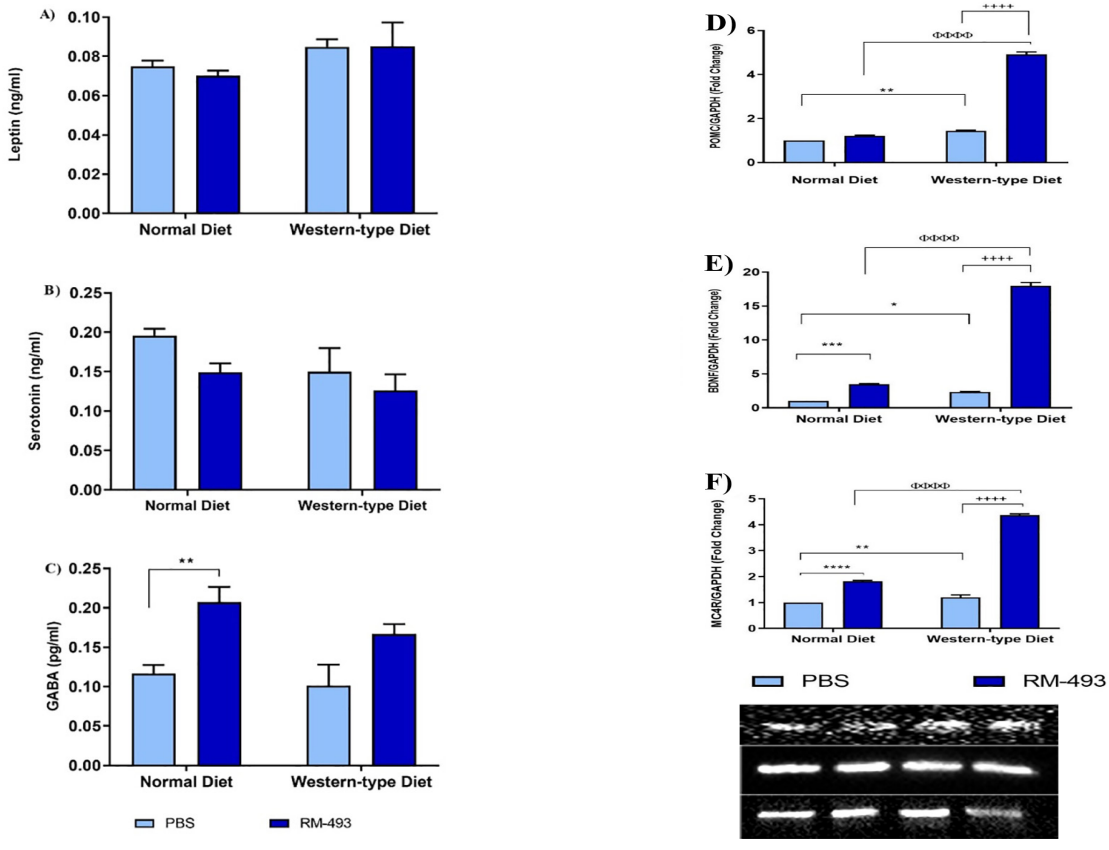

Figure 2: Levels of serum leptin, serotonin and GABA in all groups. There was no significant difference between the groups of these parameters except the level of GABA. RM-493 increased the GABA level in the group fed with normal diet (ND). **ND vs. RM-493 (p<0.01). GABA: Gamma aminobutyric acid, PBS: Phosphate buffered saline, RM-493: Setmelanotide. Determination of hipocampal POMC, BDNF and MC4R expressions in all groups. Western-type diet and RM-493 increased these parameters when compared to their controls. *ND vs. WD (p<0.05), **ND vs. WD (p<0.005), ***ND vs. RM-493 (p=0.0005), ****ND vs. RM-493 (p<0.0001), ΦΦΦΦRM-493 vs. WD+RM-493 (p<0.0001), ++++WD vs. WD+RM-493 (p<0.0001). PBS: Phosphate buffered saline, RM-493: Setmelanotide

In light microscopic examination, normal morphological structure was visible in hippocampal CA3 region, dentate region, and neocortex in normal diet+PBS group (Figure 4a, e, i, m) and normal diet+RM-493 group (Figure 4b, f, j, n). Vacuolization in dentate hilus region and neocortex, edema in CA3 stratum lucidum layer, degeneration in CA3 pyramidal neurons were observed in 50% of the animals in the Western type diet+PBS group (Figure 4c, g, k, o). Vacuolization was not noticed in neocortex and decreased in dentae hilus region in Western type diet+RM-493 group (Figure 4d, l, p).

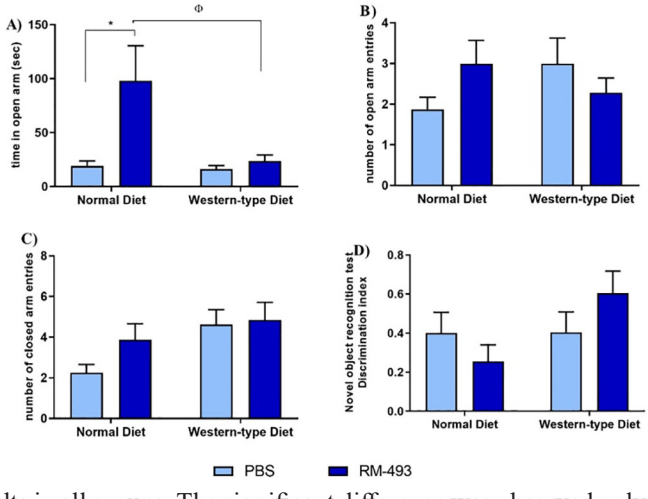

Figure 3: Behavioral test results in all groups. The significant difference was observed only in the open arm time variable. RM-493 increased the time the group fed with normal diet. Western-type diet reduced the time when compared to its control. There was no significant difference in other parameters. *ND vs. RM-493 (p<0.05), ΦRM-493 vs. WD+RM-493 (p<0.05). PBS: Phosphate buffered saline, RM-493: Setmelanotide

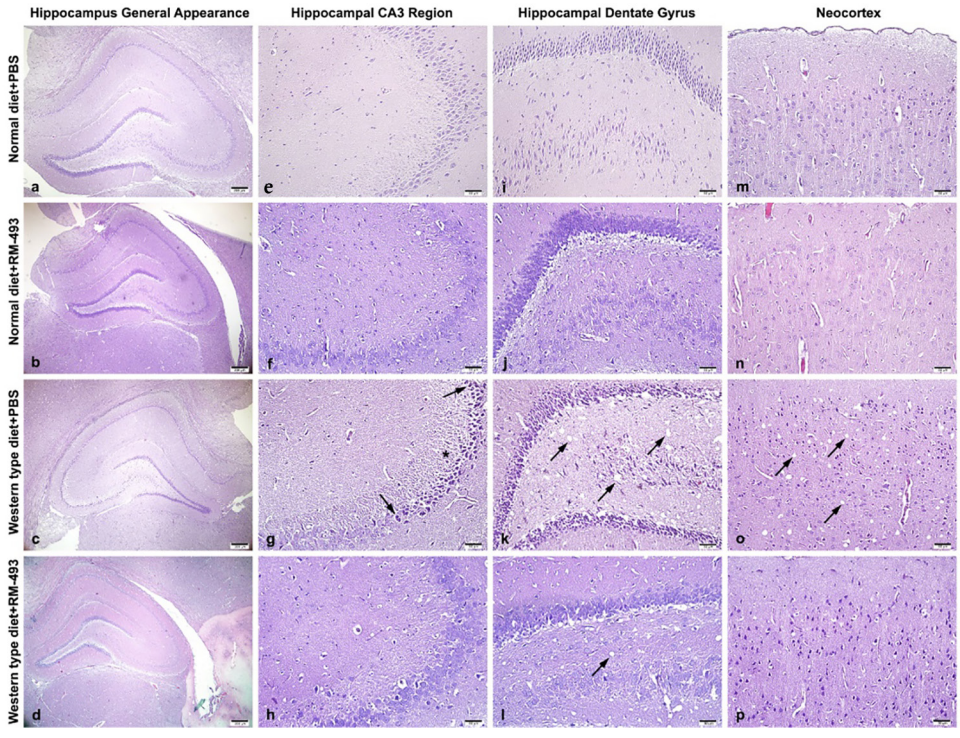

Figure 4: General appearance of hippocampus (a), hipokampal CA3 region (e), dentate gyrus (i), and neocortex (m) showing normal morphology in normal diet+PBS group and general appearance of hippocampus (b), hipocampal CA3 region (f), dentate gyrus (j), and neocortex (n) demonstrating normal morphology in normal diet+RM-493 group. General appearance of hippocampus (c) in Western type diet group. Edema (*) in CA3 stratum lucidum layer (g), vacuolization (arrow) in dentate hilus region (k) and neocortex (o), and degeneration (region between arrows) in pyramidal neurons in Western type diet+PBS group. General appearance of hippocampus (d) and neocortex (p) illustrating normal morphology in Western type diet+RM-493 group. Decreased vacuolization in dentate hilus region (arrow) (l), stratum lucidum layer and pyramidal neurons in CA3 region displaying normal morphology (h) in Western type diet+RM-493 group.

Pyramidal neurons and stratum lucidum layer showed normal morphology in the same group (Figure 4h).

Discussion

Metabolic disorders that can be caused by consuming this diet for a long time also cause disruptions in brain signal pathways and affected brain signaling pathways leads to memory disorders and anxiety (3). In our study, serum cholesterol, triglyceride, HDL and LDL levels were measured in order to observe the effects of the given diet on metabolism within 4 weeks. According to these results, at the end of 4 weeks, serum cholesterol, HDL and LDL levels significantly increased in the western-type diet group when compared to the normal diet group, respectively. Although the triglyceride increased in comparison to the control, it did not show a significant change. In a study with a 16-week feeding period, a significant increase was observed in serum lipid levels, excluding triglyceride levels, as in our study (21).

In the study by Clemmensen *et al.*, serum cholesterol and triglyceride levels in animals of the group treated with MC4R agonist were evaluated, but the treatment with RM-493 did not cause a significant difference in cholesterol and triglyceride levels (18). In our study, there was no significant change in the group fed with a normal diet and treated with RM-493 when compared to the control group, while cholesterol, HDL and LDL levels significantly decreased in the groups fed with a western-type diet, respectively. According to the results of the study by Clemmensen *et al.*, RM-493 treatment was found to treat diet-induced obesity. This weight loss has been reported to be from fat body mass, not lean body mass. The reason for this is based on the knowledge that the MC4R agonist increased fat breakdown and energy expenditure in the body (22).

In a study given MC3R and MC4R agonists, serum leptin levels significantly decreased when compared to the control group (23). According to our results, the results of serum leptin levels in our groups were close to each other. However, serum leptin levels in the normal diet group given MC4R agonist were not significant, but tended to decrease when compared to the control. While this agonist, which caused a decrease in leptin level, was not a selective agonist for MC4R (23), the agonist used in our study was a selective agonist for MC4R (18).

Therefore, it is thought that the agonist in our study may not have significantly altered serum leptin levels.

The effects of leptin are mediated by stimulation of POMC-expressing neurons in the arcuate nucleus, which in turn stimulates MC4Rs (24, 25). In a study, groups fed a high fat diet for 12 weeks showed a significant increase in POMC mRNA expression when compared to their controls (26). In our study, POMC protein expressions increased significantly in groups fed with a western-type diet for 4 weeks in comparison to the group fed a normal diet. The reason is that leptin from a high fat and high sugar diet activates the neuron groups. According to the results of 2 and 5 days injections of RM-493, POMC increased significantly in the RM-493 group when compared to the control group. In another study in contrast (18), our results with a 5 day injection of RM-493, the expression of this protein significantly increased in the western-type diet when compared to the control. It was shown that activation of MC4R activated POMC neurons and there was a positive feedback circuit between them. In other words, this circuit had a critical importance in alleviating the deterioration in synaptic plasticity. According to the results of the other study, RM-493, also known as the α-MSH analog, has been reported to have drug status for POMC deficiency (27). These results suggest that both the western-type diet and the MC4R agonist activated the pathway.

Previous studies have shown that POMC-derived peptides act on MC4R-expressing neurons to reduce body weight (28). According to the results of our study, a significant increase in MC4R expression was observed in the western-type diet-fed group when compared to the control group. It is thought that high fat content in the diet activated this pathway and increased receptor activation. When the receptor agonist was given, significant increases were noticed in both diet groups in comparison to the control. Again, according to the findings of the study by Clemmenson *et al.*, MC4R mRNA expressions significantly increased in the MC4R agonist injection group when compared to the control (18). The results of our study were also in agreement with this study.

There are studies showing that western-type and high fat diets reduce BDNF levels (29-31). In several previous studies, BDNF levels significantly declined in the high fat diet group in comparison to the control group (32, 33). According to our findings, it was found that the BDNF protein expressions of the western-type diet group significantly increased when compared to those of the normal diet-fed group. BDNF levels were also found to be significantly increased in the groups given RM-493 in comparison to the control group. It is thought that the MC4R agonist may prevent possible brain damage caused by long-term high fat and high carbohydrate diets by increasing BDNF expressions. GABA is the major inhibitory neurotransmitter in the mammalian brain and is involved in controlling excitability, information processing, plasticity, and synchronization of neuronal activity. In a previous study, GABA level in the hippocampus and frontal cortex significantly reduced in groups fed a high fat diet when compared to the control group (34). The results of our study also support this finding, but the differences in the reductions were not significant.

There are a number of tests developed to evaluate cognitive functions in animals. One of the most used is the elevated plus maze test. It is used to assess anxiety in animals. The increase in the time spent by the animals in the closed arms of the maze is interpreted as an increase in anxiety. In a study, it was found that the high fat diet compared to the low fat diet in the elevated plus maze test decreased the time of staying in the open arm and the entering numbers of open arms (35, 36). In another study, longer-term high oil feeding when compared to low oil feeding could decrease the duration of stay in the open arm, while the duration of staying in the closed arm increased. At the same time, the numbers of entering the open arm and the numbers of entering the closed arm decreased (37). In our study, the time to stay in the open arm significantly increased in the normal diet group given RM-493 when compared to the control group. This time significantly decreased in the western-type diet group given RM-493 in comparison to the normal diet group given RM-493. In another study, in which melanocortin receptor agonists were given, the time of stay in the open arm and the numbers of entering the open arm significantly decreased in comparison to the control group (17). This suggests that the differences in results may be due to variations in the amount of agonist administered and the duration of treatment with the agonist.

Another parameter evaluated besides anxiety was memory. Because it has been shown that eating a western-type diet causes hippocampal dysfunction (38). Namely, the most studied tissue to evaluate memory has been the hippocampus. α-MSH secretion by POMC neurons in CA3 of the hippocampus activates MC4R in CA1. This shows the POMC/MC4R circuit in the CA3-CA1 region, while the blockage of this circuit causes synaptic strengthening and deterioration in synaptic plasticity, which is called long-term potentiation (39). In order to test circuit, the novel object recognition test was assessed. An index was used to evaluate this test, taking a sample from a previous study. This index

was evaluated as the ratio of the time spent on the new object to the total time spent on both the familiar and the new object. An increase in this rate has been interpreted as a better functioning of memory (20).

According to this study, the results in the behavioral tests were not significant between the groups. When the literature is surveyed, significant results were generally seen in models with obesity. Since we evaluated the behavioral changes that may occur in the process leading to obesity, the feeding times of the animals and gender may also have an impact on these results. In the structural examination of the brain tissues, normal morphology was observed in the neocortex and CA3 regions of the hippocampus and dentate gyrus regions in the groups fed with a normal diet and injected with PBS and RM-493. Neuropil vacuolization in the neocortex and dentate gyrus hilus regions, edema in the stratum lucidum layer of the CA3 region, and degeneration of the pyramidal neurons of the CA3 region were observed in 50% of the animals in the western-type diet group. In a previous study, it has shown that feeding a high fat and high sugar diet had negative effects on synaptic plasticity (40). The results of this study also supported the findings of our study. In addition, our results showed that RM-493 injection increased BDNF level and improved the brain that are in consistent with the results of histological evaluation in male rats.

Conclusion

In conclusion, feeding a western-type diet and treatment with RM-493 showed that BDNF, which has an important role in brain plasticity, increased, which indicated an increase in the open-arm time in the elevated plus maze test used to evaluate cognitive functions. The results of this study will shed light on studies that will investigate receptor-agonists or antagonists that can be used to determine therapeutic treatment methods on both physical and cognitive disorders caused by a high fat and high sugar western-type diet. However, our findings revealed only the results in male rats. It seems that comparison of female rats results is needed to make an objective comment about RM-493 as an therapeutic treatment in cognitive funcitons.

Acknowledgment

The authors would like to thank Marmara University of Medical School for providing facilities for this work. This work was supported by Marmara University Scientific Research Projects Commission with the project number SAG-C-YLP-120418-0154.

Conflict of Interest

None declared.

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