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ORIGINAL PAPER

Silkworm pupae powder ingestion increases fat metabolism in swim-trained rats

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Sung Pil Ryu. Silkworm pupae powder ingestion increases fat metabolism in swim-trained rats. *JENB*., Vol. 18, No. 2, pp.141-149, 2014 **[Purpose]** Many researchers are trying to solve the metabolic syndrome by utilizing a variety of nutritional control and exercise. Of those, silkworm pupae peptides are known to inhibit the synthesis of fat. Therefore, we examine the effect of fat metabolism by supplying silkworm pupae (SP) for 5-week in swim-trained rats. **[Methods]** Animals were divided into four groups as a group (n = 32) fed a normal diet (CO) with exercise training (CE); a group fed a silkworm pupa diet (SPC) with an exercise training (SPE), respectively. **[Results]** Abdominal fat pads (abdominal and epididymal) weight were lowest in SPE. The serum triglyceride, total cholesterol concentrations were lower in the SP and the SPE. HDL-cholesterol, however, was not different between groups. Liver AMPK (AMP-activated protein kinase) was increased in the CE and the SPE. Liver PPAR-α (Peroxisome proliferator-activated receptor alpha) was increased in the SPC and SPE. L-FABP (liver fatty acids binding protein) was increased by SP ingestion. Liver CPT-1 (carnitine palmitoyltransferase-1) protein expression was increased by exercise training only. **[Conclusion]** In the present study showed that the silkworm pupae intake and/or swimming exercise training activates fat metabolism to reduce the concentration of serum lipids. Thus, the silkworm pupae intake leads to a reduction in fat storage, this is considered to be effective in the inhibition of the metabolic syndrome. **[Keyword]** silkworm pupae, stored fat, blood lipids, exercise training, rat

INTRODUCTION

In order to increase fat metabolism during exercise, a variety of nutrition-related supplements have been studied [1-3]. Increasing economic growth has made eating habits westernized. Therefore, a lot of research for a high-fat diet has been conducted [4-6]. Also, there have been studies for increasing fat metabolism by adding nutritional supplements to everyday diet. Both animals [7] and people [8] have been subjects in those studies. Increasing fat metabolism during exercise is to intensify the utilization of nutrients that last for a longer period within the body. For this, diverse nutrition-related supplements need to be utilized in exercise and nutrition studies.

In recent years, since the value of insects started to be reevaluated as the last untapped biological resource, the growth of the insect industry has been accelerating [9]. South Korea has also been actively engaging in a trend to use insects commercially. They have been used to be the natural enemies of insect pests, pollination workers, the main character of the events like Hampyeong Butterfly Festival, and pets. They have enjoyed popularity [10]. On the other hands, other countries began to use insects as a new source of nutrition. An array of international conferences on edible insects led by the United Nations has been held. The subject of forest insects as food was discussed at the 2008 International Workshop of the United Nations Food and Agriculture Organization held in Chiang Mai, Thailand [11].

Edible insects have been also eaten in South Korea since a long time ago and what is the most common is silkworm pupae. This research team reported the effect of increasing muscle mass by using silkworm pupae [12] and the improvement of inflammatory cytokines [13]. Silkworm pupae includes a variety of amino acids [12] as well as diverse fatty acids such as palmitic acid, oleic acid, linoleic acid, and stearic acid. Among them, oleic acid and linoleic acid, which are unsaturated fatty acids, are reported to account for above 30 percent [14]. In addition, its crude fat is about 36 percent,

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which is higher than that of beef. Palmitoleic acid, oleic acid, and linoleic acid which are fatty acids of dried silkworm pupae account for about 70 percent [15]. As a result of having rats take silkworm pupae oil for 18 weeks, total cholesterol in blood and triglycerides were significantly reduced as well as high-density lipoprotein cholesterol was notably increased [16]. Peptide, which is not a fatty acid of silkworm pupae, is also reported to be effective in inhibiting the synthesis of fat [17]. The silkworm pupae intake has been reported not to cause hepatotoxicity or any mutation [18,19]. Scientific validation for the health benefits of silkworm pupae, which Koreans have eaten for a long time, must be conducted in diverse perspectives. In particular, studies on silkworm pupae in connection with proper exercise and nutrition are needed.

Edible insects are being considered as new food sources both from an economic, as well as eco-friendly perspective. In effect, this study is aimed at examining whether silkworm pupae as a food source, when combined with exercise, may increase fat metabolism. This is to evaluate the effect of silkworm pupae as a new health supplement. This study can also enhance the value of the health effects of Korean silkworm pupae as well as provide a material which increases utilization of it.

METHODS

Animal care

This study used a total of 32 male Sprague Dawley rats aged 4 weeks. After they were brought into the lab, the experimental rats were kept in preliminary breeding for 2 weeks to help them adapt to the environment. The rats were reared in individual in a separate cage to minimize their stresses. The internal environment of the breeding room was maintained at a temperature of 23-25℃, with a relative humidity of 60% and a dark period from 08:00 to 20:00. They were divided into 4 groups; Control Diet (CO), Control Diet + Exercise (CE), Silkworm Pupae Diet 3% (SPC) and Silkworm Pupae Diet 3% + Exercise (SPE). When the preliminary breeding was finished, the mean body weight of randomly selected rats was calculated, and after that, 8 of them were assigned to each of the four groups.

Dietary composition

The diet was formulated by mixing the silkworm pupae powder with casein at a ratio of one to one in accordance with AIN-76, and 20 g of the mixture was fed to the animals at 8:00 in the morning and evening for one hour, with no limit to water intake. The feed quantify of the silkworm pupae powder was the same as the previous studies [12,13]. The composition of the experimental diet was as shown in (Table 1).

Table 1. The composition of experimental diet (g/kg diet)

Constituents	Control diet	Silkworm pupae diet
Casein	200	-
Silkworm pupae powder	-	200
Sucrose	200	200
Corn starch	400	400
Corn oil	60	60
Lard	40	40
Cellulose	50	50
Mineral mix:AIN-76*	35	30
Vitamin mix:AIN-76*	10	5
D,L-methionine	3.5	3.5
Choline	1.5	1.5

* AIN-76 formula

MATERIALS

Silkworm pupae were directly purchased from Yeongdeok (Gyeongbuk, Korea). To add it to the diet, it was freeze-dried and was processed into powder. The freeze-drying was carried out using the vacuum-freeze dryer installed in the Regional Innovation Center at Kyungpook National University (SFDKS 10K, Samwon Freezing Engineering Co., Korea).

Exercise training

The exercise started at the age of 6 weeks after the preliminary breeding was finished and lasted 5 weeks. Two grounding-type heaters were used to keep the temperature of the water in the pool (30 cm in length × 80 cm in width) at 32.0 ± 1.0℃, under which condition the rats were left to do swimming exercise 5 times per week. To allow the rats to adapt to water, the first exercise lasted for 10 minutes, which was gradually increased on a daily basis. Staring from the third week, the animals swam for 60 minutes. During the exercise, the waves were artificially made to prevent the rats from floating and to force them to move the legs continuously.

Body weight measurement

The body weights were measured at the same time under the same environment twice every week. That is to say that to maintain the same experimental environment, the experimental animals did exercise on a regular basis at 3 pm on Mondays and Thursdays.

Sample collection

After 12 hours of fasting, the rats were put into anesthesia with ether. And their abdomen was cut open to collect blood samples from the abdominal aorta to analyze the blood lipid concentration, and the collected blood samples were stirred in a centrifugal separator at a rate of 700 × g, which was then kept in a freezer until the analysis. And after removing the liver, it was frozen with liquid nitrogen to terminate its activation, and then it was stored in a freezer at a temperature of -80℃ until the analysis. In addition, the retroperitoneal and epididymal fat containing fascia were removed from each of their connected parts in order to weigh the fat pads. After that, they were rinsed with a saline solution and were measured with a scale with a readability of 1/10,000 (XT-220, Precia, Swiss).

Analysis items and method

The total cholesterol (TC) and triacylglycerol (TG) among the blood lipid contents were measured using the quantitative kit (Asan Pharm, Korea) and the spectrophotometer, while the HDL cholesterol was analyzed with the auto biochemical analyzer (BS-220, Mindray, China). To analyze the appearance of the proteins, the liver tissues were homogenized by adding 0.5 M EDTA (Duksan, Korea), lysis buffer (Gendepot, R4200-100, USA) and phosphatase inhibitor 100 × (Gendepot, P3200-001, USA) to the samples. After the homogenization was finished, each sample was stirred in a centrifugal separator at a rate of 1,200 × g for 10 minutes to extract the supernatants from the sample, and then 1 µl of the supernatant was mixed with 1 ml of brad-ford, 200 µl of which was placed in the ELISA plate and was analyzed to measure the absorbance rate. After that, it was mixed with the Laemmli sample buffer to prepare samples. Then, each 20 µl protein sample was added onto the 10% SDS-PAGE gel, which underwent electrophoresis at 100 V to separate the proteins. After the separation of proteins, the proteins were transferred onto polyvinylidene difluoride (PVDF) membranes by electro-blotting at 100 V for 1 h. After the transfer was over, they went through blocking for one and half hours in the BAS-containing blocking buffer (TBST buffer; 50 mM Tris-HCl, 150 mM NaCl and 0.05%, Tween 20). The primary antibodies were diluted in accordance with their respective method - AMPK 1:1,000 (Cell Signaling Technology, Inc., CA NO. #2532, USA), PPAR-α 1:1,000 (Abcam, CA NO. ab8934, UK), L-FABP: 1: 5000 (Abcam, CA NO. ab7847, UK), CPT-1 1:5,000 (Abcam, CA NO. ab128568, UK) - and were incubated overnight at 4℃ before being rinsed for 10 minutes 5 times with the TBST (5% tween-20). The secondary antibodies were diluted in accordance with their respective method-AMPK: 1:3,000 (goat-anti rabbit, Santa Cruz Biotechnology, Inc, CA NO. SC-2004, USA), PPAR-α 1:2,000 (goat-anti rabbit, Santa Cruz Biotechnology, Inc, CA NO. SC-2004, USA), L-FABP 1:3,000 (goat-anti rabbit, Santa Cruz Biotechnology, Inc., CA NO. SC-2004, USA), CPT-1 1:10,000 (goat-anti mouse, Santa Cruz Biotechnology, Inc., CA NO. SC-2005, USA)-and were reacted for one hour. After that, they were rinsed with the TBST (5% tween-20) for 10 minutes 5 times. These subsequently induced band movements were observed by highlighting the membranes with the ECL kit and having them developed on the X-ray film. The revealed bands were calculated and expressed as the band/β-actin by using Image J (NIH, Ver. 1.47t, USA).

Statistics

The data resulting from this study was analyzed using the SPSS/PC + 19.0, a statistical program designed for Window-based PCs, and all test data were presented in average and standard deviation. The one-way ANOVA analysis was conducted to verify significant differences among groups. In case of an item with a significant difference, the post verification was carried out using the Least Significant Different method. All significance levels were set at $p < .05$.

RESULTS

This study analyzed body weight and blood lipid concentration changes as well as the appearance of fat metabolism-related proteins, which occurred after having male Sprague Dawley rats do swimming exercise for 5 weeks and concurrently providing them with the feed mixed with the silkworm pupae which were freeze-dried.

Body weight changes

In terms of body weight changes, all test groups were found to gain weight as the experiment progressed (Fig. 1). At the time when the test animals were divided into the four groups, the average body weight was measured at 180 g, but when the 5 week-long breeding was finished, the average body weight of the CO and the CE were recorded at 304 g and 282 g, respectively, whereas those of the CE and the SPC

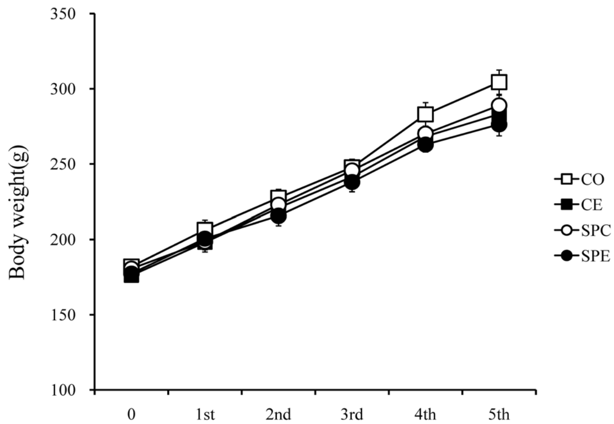

Fig. 1. Body weight changes during the experimental periods

Table 2. Fat pads weight (g)

	CO	CE	SPC	SPE
Abdominal	10.5 ± 1.6^{a}	8.3 ± 1.1^{ab}	7.4 ± 1.4^{b}	6.2 ± 1.1^{b}
Epididymal	9.8 ± 1.9^{a}	7.8 ± 1.1^{ab}	7.3 ± 1.4^{ab}	6.1 ± 1.3^{b}

Values are M ± SE. CO: control group; CE: control-exercise group; SPC: silkworm pupae intake group; SPE: silkworm pupae intake and exercise group; different letters within the weighed item means significance at $p < .05$, respectively.

were 280 g and 268 g. When it comes to body weight, the SPE showed the most suppressed body weight increase. However, there were no significant differences among the groups.

In regard to differences in the fat pad weight, the CO showed a significantly higher level of abdominal fat compared with the SPC and the SPE ($p < .05$), but no significant difference among other groups were observed (Table 2). In terms of the epididymal fat, the SPE alone showed a significantly lower level than the CO, but no other significant difference between groups were observed.

Changes in blood lipid concentration

As shown in Table 3, the SPC and the SPEC showed a significant lower level ($p < .05$) than the CO in terms of TG. When comparing the groups that did swimming exercise, the SPE showed a significantly lower concentration than the CE ($p < .05$). In comparison to the SPE, the TG levels of the CO and the CE were lower by about 41% and 34%, respectively. In terms of the TC concentrations in the blood, the SPC and SPE showed significantly lower levels than the CO and CE ($p < .05$), and the CE, which did swimming exercise, showed a significantly lower degree than the CO ($p < .05$). In addition, the SPE, which also did swimming exercise, indicated remarkably lower level concentrations than

Table 3. Blood lipids profile (mg/dl)

	CO	CE	SPC	SPE
Triglyceride	62.3 ± 7.0^{a}	55.5 ± 4.0^{ab}	45.1 ± 3.5^{bc}	36.6 ± 8.5^{c}
Total-cholesterol	176.5 ± 22.0^{a}	130.1 ± 13.6^{b}	100.0 ± 9.1^{c}	96.0 ± 9.2^{c}
HDL-cholesterol	51.5 ± 12.2^{a}	54.3 ± 10.0^{a}	52.2 ± 4.1^{a}	58.0 ± 3.4^{a}

Values are M ± SE. CO: control group; CE: control-exercise group; SPC: silkworm pupae intake group; SPE: silkworm pupae intake and exercise group; different letters within the analyzed item means significance at $p < .05$, respectively.

the CE ($p < .05$). Especially, the concentration level of the SPE was lower than the CO and CE by 46% and 26%, respectively. The study did not observe any significant differences between the four groups in terms of the blood level of HDL-C.

Differences of fat metabolism-related protein expression

In terms of the levels of AMPK (AMP-activated protein kinase) protein expression in the liver, the CE and SPE groups which did swimming exercise showed significantly higher degrees than the other groups ($p < .05$).

In regard to PPAR-α (Peroxisome proliferator-activated receptor alpha), there was no difference between the CO and CE groups. The SPC and SPE groups which were fed with silkworm pupae showed significant PPAR-α expression levels compared with the other groups ($p < .05$). In addition, the SPE group indicated a higher appearance level than any other groups ($p < .05$). Especially, the appearance rate of the SPE was higher than the CO, CE and SPC groups by 48%, 40%

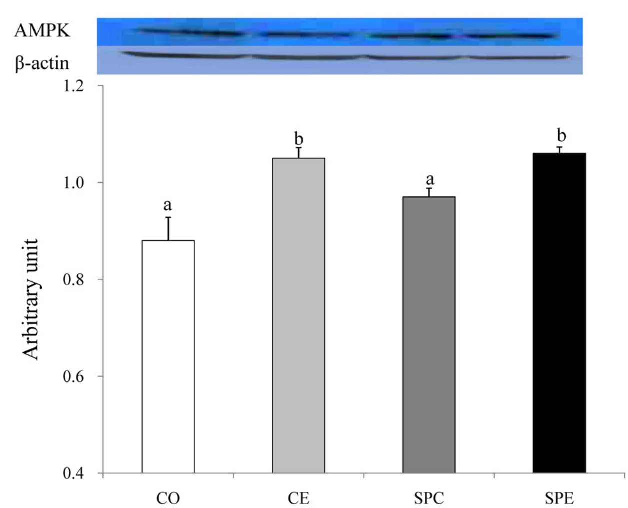

Fig. 2. AMPK protein expression after 5 weeks of swimming exercise training with/without silkworm pupae powder ingestion. Bars are M ± SE. CO: control group; CE: control-exercise group; SPC: silkworm pupae intake group; SPE: silkworm pupae intake and exercise group; different letters among the group means significance at $p < .05$.

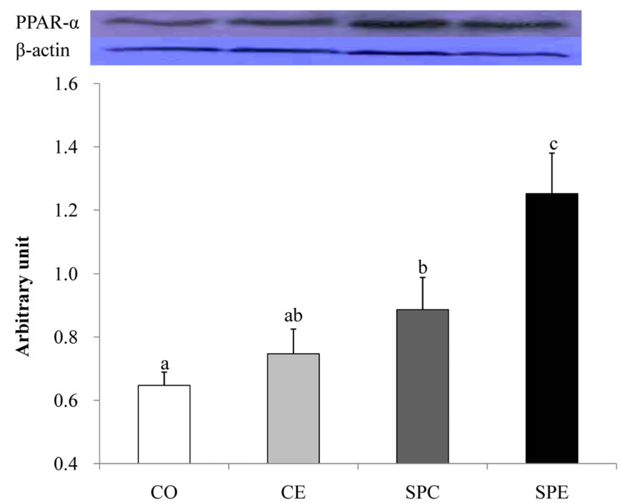

Fig. 3. PPAR-α protein expression after 5 weeks of swimming exercise training with/without silkworm pupae powder ingestion. Bars are M ± SE. CO: control group; CE: control-exercise group; SPC: silkworm pupae intake group; SPE: silkworm pupae intake and exercise group; different letters among the group means significance at $p < .05$.

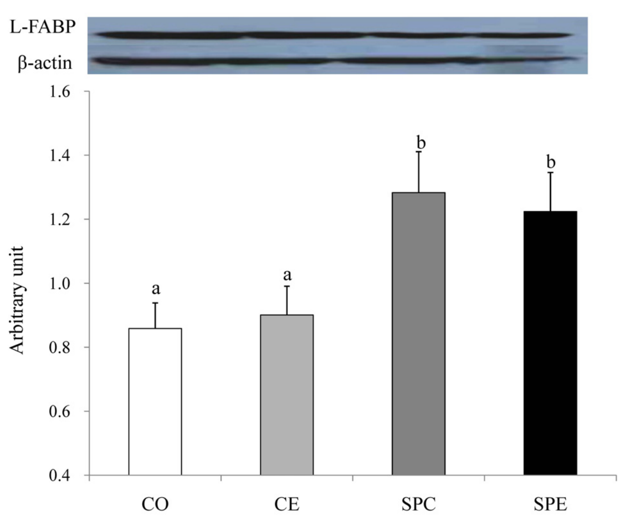

Fig. 4. L-FABP protein expression after 5 weeks of swimming exercise training with/without silkworm pupae powder ingestion. Bars are M ± SE. CO: control group; CE: control-exercise group; SPC: silkworm pupae intake group; SPE: silkworm pupae intake and exercise group; different letters among the group means significance at $p < .05$.

and 29%, respectively.

In regard to L-FABP (liver fatty acid binding protein), there was no difference between the CO and CE. However, the SPC and SPE which were fed with silkworm pupae showed significantly higher expression levels than the other groups ($p < .05$). There were no remarkable changes between the groups with or without swimming.

In terms of CPT-1 (carnitine palmitoyltransferase-1), the SPC and SPE groups, which were fed with silkworm pupae,

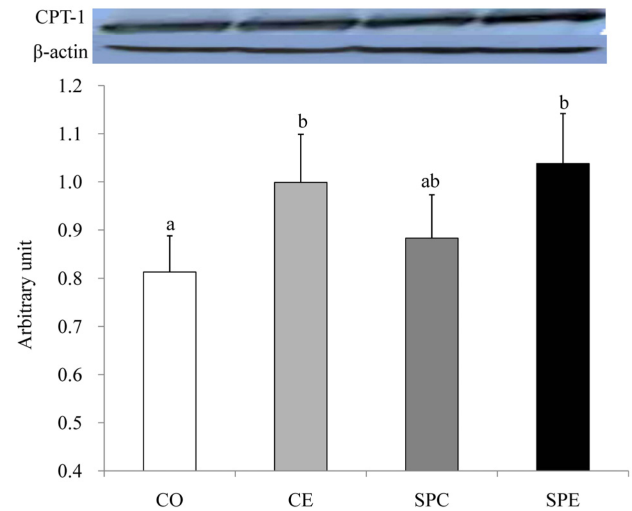

Fig. 5. CPT-1 protein expression after 5 weeks of swimming exercise training with/without silkworm pupae powder ingestion. Bars are M ± SE. CO: control group; CE: control-exercise group; SPC: silkworm pupae intake group; SPE: silkworm pupae intake and exercise group; different letters among the group means significance at $p < .05$.

showed significantly higher protein expression levels than the CO and SPC groups ($p < .05$). Likewise, there was no distinctive change induced by swimming exercise even among the groups which were fed with the silkworm pupa powder.

DISCUSSION

This study aimed to identify changes in lipid metabolism when a combination of silkworm pupae intake and swimming exercise was carried out. Recently, edible insects are grabbing much attention as an alternative food and especially as a source of protein [12,13]. The silkworm pupa is a kind of edible insects which are rich in amino acids and fatty acids, and has an unsaturated fatty acid level of more than 30% [14]. It was reported that the unsaturated fatty acid level in the total fatty acids accounted for approximately 70% based on the dry weight [15]. This study was carried out on the hypothesis that the amino acids and various fatty acids enriched in the silkworm pupae could improve the blood lipid content and also activate the lipid metabolism, when taken in combination with swimming exercise.

The 5 week-long combination of silkworm pupae intake and swimming exercise suppressed an increase in body weight and was found to be effective in controlling body weight increases [13]. Although it failed to find out other previous studies which could be directly compared with this study, similar studies reported that when fed with a high protein diet, the control group showed a higher degree of body weight gain while the silkworm pupae intake groups indicated decreases in the size of epididymal fat, presented lower levels of the total fat pads and fat cells, and reduced the body weight growth rates [17]. In response to these study results, some researchers said that in case of obesity induced by a high protein diet intake, the supply of silkworm pupae peptides could inhibit the synthesis of fatty tissues by modulating signal transduction pathways, which the study suggested could subsequently reduce fat accumulation and the size of fatty cells [17]. In addition, another study by Lee *et al*. [20] reported that it observed a reduction in the formation of fat droplets after the treatment of hydrolysates from silkworm proteins to 1 mg/ml of silkworm pupa peptides in the 3T3-L1 fibroblast of rats. This study also observed that the combination of silkworm pupa powder intake and swimming exercise could reduce the fat pads. Such a reduction in the fat pads was believed to contribute to suppressing an increase in body weight. Also, the study which caused the formation of alcohol-induced fatty livers in rats and then treated them with a fermented silkworm pupae powder for 4 weeks reported that the fatty droplets in the fatty livers were diluted [21]. Given this, it was expected that similar reductions in fat pads could occur in various tissues.

In the meantime, this study found that the level of blood TG was decreased by a substantial degree in case of the combination of silkworm pupae powder intake and swimming exercise, and that the silkworm pupae powder group which did not do swimming exercise showed a lower level of TC concentration than the control group and control-exercise group. This was believed to be thanks to α-linolenic acids which are rich in silkworm pupae [16,22]. That is to say that a high protein diet such as silkworm pupa oil could significantly reduce the TC concentrations in the blood, TG, low-density lipoprotein cholesterol (LDL-C), atherogenic indices, platelet aggregability, etc., compared with the control group, whereas it showed an significantly increased level of high-density lipoprotein cholesterol (HDL-C) [22]. In another similar study which treated white rats with the pupae oil extracted from Eri silkworms for 18 weeks, no toxicity was observed, and the TC concentration in the blood and the TG were significantly reduced whereas the HDL-C was increased [16]. However, this study observed no difference between different groups in terms of the high-density lipoprotein cholesterol. Meanwhile, some study results reported an improvement in the blood lipid content, which was induced by fatty acids in silkworm pupae, while others suggested the suppression of an increase in the blood lipid due to a supply of silkworm protein and an improvement in the blood TG and TC concentration thanks to a supply of peptide from silkworm protein [17,23]. Therefore, it was thought based on the results of this study that the intake of silkworm pupae was effective in improving the blood lipid content and that when it was done in combination of an exercise, it could generate synergy effects.

It is widely known that an increase in AMPK leads to a surge in fat oxidation and a suppression of the synthesis of TG [24]. An increase in AMPK can be prompted by both long-period and short-period endurance exercises [25,26]. In this study, it was also observed that the swimming exercise contributed to increasing the AMPK protein expression. This indicates that fat oxidation was effectively activated by exercise. However, the study failed to observe synergy effects induced by the silkworm pupae powder intake. This is believed to be due to the fact that silkworm pupae contain large quantities of unsaturated fatty acids including oleic acid and linoleic acid [14]. In other words, as the previous studies suggested that oleic acid and linoleic acid do not induce the phosphorylation of the AMPK [27], this was believed to the result of the intake of silkworm pupae enriched in unsaturated fatty acids, and the exercise contributed only to increasing the appearance rate. In addition, it was reported that PPAR-α could activate fat oxidation like AMPK. Some studies reported that a short-period exercise alone could enhance the appearance rate [28] while others claimed that the appearance rate could be boosted only by a long-period exercise [29]. This study observed that the control-swimming exercise group showed an increasing tendency and that the expression rate of the PPAR-α protein was boosted by the combination of silkworm pupae powder intake and swimming exercise. In contrast to what was suggested in the AMPK results, this was believed to be thanks to the intake of silkworm pupae which are rich in unsaturated fatty acids. In other words, it was reported that the 4 week-long treatment of unsaturated fatty acids to rats increased the level of PPAR-α [30] by a significant degree, which could be explained by a report that claimed that the α-linoleic acid enhanced the appearance rate of the PPAR-α [31,32]. Especially, the silkworm pupae powder intake control group which did not exercise also showed an increased appearance rate of the PPAR-α, which was thought to be a meaningful result. Therefore, this suggested that when done in combination with exercise, the intake of silkworm pupae could generate synergy effects in enhancing fat oxidation. In affiliation with this, the study found that the swimming exercise group has a limited effect in enhancing the appearance rate of L-FABP, whereas those groups which were fed with the silkworm pupa powder all showed a significant increase in the L-FABP appearance rate. Considering the reports that when a mouse was fed with a diet enriched in α-linoleic acid, the L-FABP grew significantly [33] and that oleic acid could increase the L-FABP in rats [34], this indicates that a variety of fatty acids contained in silkworm pupae can boost fat metabolism in the liver. In addition, it is thought that the reason why this study failed to observe any exercise-induced increases was because that the study did not directly analyze the skeletal muscles. Given the reports claiming that if an acute moderate intensity exercise was carried out equally, there was no increase in the L-FABP after exercise compared with before exercise, that when a single bout exercise was conducted, the FABP3 (heart and muscle type FABP) was boosted [36], and that the FABP3 was recorded to be remarkably high before and after muscle strengthening exercise [37], any exercise-induced changes in the L-FABP were considered insignificant. Future studies need to be conducted to analyze the skeletal muscles. Actually, it was found that even when a high intensity exercise is carried out, it can increase the FABP in the gastrocnemius [38]. In the meanwhile, the CPT-1, which plays a critical role in transferring long chain fatty acids into mitochondria, was increased only by exercise. The silkworm pupae powder intake control group showed an increasing tendency. Considering the study results that after having obese rats exercise, the CPT-1 was increased by a significant degree [39] and that a high intensity intermittent exercise can also increase the CPT-1 [38], some of this study's results can be explained. In addition, there emerged studies that reported a subsequent increase in the CPT-1 after feeding rats with an oil enriched in α-linoleic acid [31], and this can explain why the two groups that were fed with the silkworm pupae powder showed elevated levels of CPT-1.

Taking all the above results together, it is thought that fatty acids contained in large quantities in the silkworm pupae, especially unsaturated fatty acids, can inhibit the fat storage by accelerating the lipid metabolism. In addition, its increasing effect on the appearance rate of fat metabolism-related proteins indicates that it can be utilized as a nutritional supplement. Also, due to the subsequently elevated lipid metabolism, it can be recommended as a dietary product to prevent metabolism syndromes. Therefore, it is concluded that a more variety of related follow-up studies need to be conducted.

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