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PREVENTING EFFECT ON DIET-INDUCED OBESITY OF WATER-EXTRACT FROM MASSON PINE POLLEN ON MICE

Yue Geng, Han Zhang, Hang Song, Liang Zhang, Jimin Xu, Ming Liu, Shanshan Chen, Lirong Han

From Key Laboratory of Animal Resistance Biology of Shandong Province College of Life Science, Shandong Normal University, Jinan 250014,China)

E-mail*:* gengy@sdnu.edu.cn

Abstract

Background: This study was carried out in order to investigate the preventing effect of water-extract from masson pine pollen (PWE) on obesity in mice.
Materials and Methods: Sixty male KM mice (bodyweight was 19±1g) were divided randomly into six groups: normal control group (NC), high fat group (FC), positive control group (Orlistat, 0.05g/kg bw·d, PC), low-dose PWE group (0.0625g/kg bw·d, PWEL), medium-dose group (0.125g/kg bw·d, PWEM) and high-dose group (0.25g/kg bw·d, PWEH). Mice were treated by intragastric administration daily for 6 weeks.
Results: The body weight changes of three PWE groups decreased obviously. In serum, TC of PWEH group reduced remarkably ($P<0.05$); TG of three PWE groups had significantly declined ($P<0.01$); HDLC of PWEM and PWEH groups had significantly increased ($P<0.05$). Leptin level of PWEM and PWEH groups had significantly increased ($P<0.01$). Adiponectin, resistin level and the activity of CPT-I of PWE groups increased remarkably ($P<0.01$). Orexigenic peptides NPY and AgRP in serum of PWE groups had significantly declined ($P<0.01$). Anorexigenic peptides POMC had no difference with each other, and CART had declined.
Conclusion PWE could not only promote the absorption and synthesis of fat but also promote the oxidation of fat. Critical was that the oxidation was stronger than the synthesis.

Key words: Pine pollen; water extract; obesity; adipokines; CPT-I

Introduction

The increasing prevalence of obesity has been extensively reported in recent times (Chen, 2008; Flegal et al., 2010; Howard et al., 2008). Severe obesity is associated with a substantial increase in the risk of morbidity and mortality from chronic health conditions, such as diabetes, hypertension, cardiovascular disease and some kinds of cancers (Friedman, 2003; Must et al., 1999; Shirai, 2004).

There are a variety of drugs currently in preclinical development for the treatment of obesity. Anti-obesity drugs currently in development generally fall into two broad classes: those that reduce energy intake and those that stimulate energy expenditure. But drugs always have adverse effects; common side-effects include insomnia, nervousness, nausea, dry mouth, vomiting and constipation, etc. It had been reported that sibutramine would elevate blood pressure and the heart rate (Halford, 2006). Orlistat would cause gastrointestinal adverse reactions and vitamins deficiency (McDuffie et al., 2002), etc. So, it has been an attractive object to finding natural healthy products which can cause effective weight loss. Pine pollen, named Song Huang in Chinese traditional pharmacy, has functions in losing weight and tonifying recorded in ancient medical book. Recently, numerous studies about pine pollen on lipid metabolism showed that pollen of different species can cure or prevent more than 60 kinds of human diseases. However, the specific mechanism has not been scientifically understood. Fan et al. observed that the pine pollen is effective in preventing the formation of high blood-fat in the experimental mice (Fan et al., 2005). And Zhao et al., also found that masson pine pollen significantly increased the fecal triglycerides, cholesterol and total fatty acid excretion amount of piglets, and accelerate the bile acids excretion, and it had important role in regulation of energy metabolism, lipid metabolism and inhibition of fat absorption (Zhao et al., 2007).

In our study, we used PWE from broken pollen of masson pine (*Pinus massoniana*) to investigate the preventing effect on diet-induced obesity of PWE on mice, which may provide basis mechanism in the development and application of the pine pollen in the field of weight loss.

Materials and Methods

Preparation of Water-extract from Masson Pine Pollen

Masson pine pollen powder was provided by New Era Health Industry Group in Yan Tai. The pollen wall-broken rate was above 95%. Masson pine pollen was extracted by ultrasonic extraction in water for three times. Extracting solution was merged and supernatant was collected, and then vacuum freeze-drying. 1g PWE was equivalent to 2.84g masson pine pollen.

Animals and Diets

Sixty male KM white mice (average bodyweight was 19±1g) were used in the present study. Four weeks old mice were purchased from Center of Experimental Animal in Shandong Traditional Chinese Medicine University. The mice were housed 5 per cage at 23 ^{o}C+1 ^{o}C, 50% ± 10% relative humidity, and 12h light-dark cycle and were allowed ad libitum access to water. The mice were divided into six groups (NC; PC;

FC; PWEL; PWEM; PWEH). The mice were fed normal diet for 1 week to stabilize their metabolic condition. NC group was fed normal diet, and other groups were maintained at high fat diet (75% normal diet, 15% lard oil, 10% whole milk powder). Meanwhile, mice were treated by intra-gastric administration daily by water-extract (PWEL 0.0625g/kg bw·d、PWEM 0.125g/kg bw·d and PWEH 0.25g/kg bw·d) or orlistat (PC 0.125g/kg bw·d, purchased from WEDOCHEM company, 98.2% purity) at 3~4 pm everyday, both were dissolved in soybean oil. Treatment lasted for 6 weeks. All procedures were conducted in accordance with the regulations on the administration of experimental animals in China.

Body Weight and Body Fat

Body weight was recorded every 3 days throughout the study. Parameters like weight net increase (final body weight－initial body weight) and weight gain rate (weight net increase / initial body weight×100%) were calculated. Adipose tissues around kidney and testis were stripped of after dissection, combined with the data of body weight to figure out body fat content and rate of body fat (body fat content / final body weight×100%).

Blood Analysis

At 42nd day of the experiment, blood was collected via eyeball after anesthetization and was centrifuged at 10000r/m for 10 min after standing. Serum were used to test lipid profiles (TC, TG and HDLC; tested by kits purchased from Beijing BHKT clinical reagent Co. Ltd), adipokines (LEP, ADP and Resistin; tested by elisa kits purchased from Shanghai Yaji biological technology Co. Ltd) and neuropeptides (NPY, AgRP, POMC and CART; tested by elisa kits purchased from Shanghai Yaji biological technology Co. Ltd) levels.

CPT-I

At 42nd day of the experiment, liver and adipose tissue was stripped off after dissection and triturated by homogenizer, we centrifuged homogenate and collected supernatant to measure CPT-I concentration in liver and adipose tissue (measured by Tianrun biological technology Co. Ltd).

Measurement of Fecal Lipids

Feces of each group were collected together every 6 days, after freeze-drying, the feces of each group were equally divided into three repeats. Total lipids of each group were extracted by mixed solvent (consisted of carbinol and petroleum ether and chloroform ratio was 1:1:2) and then dissolved by isopropanol, The TC and TG concentration were tested by kits (purchased from Beijing BHKT clinical reagent Co. Ltd).

Gene Expression

At the end of the experiment, liver and hypothalamus were rapidly dissected and survived at -80℃. Total RNA was isolated using RNA simple Total RNA Kits (purchased from TIANGEN biochemical technology Co. Ltd) according to the manufacturer's instructions and used for reverse transcription at once. And then we choose three samples which had the highest purity in each group to test mRNA expression levels of FAS, CPT-I in liver and NPY, AgRP, POMC, CART in hypothalamus by RT-PCT (tested by Jinan YINGLUN biochemical technology Co. Ltd). Information of Gene Primer is shown in table 1.

Table1: Information of Gene Primer

Target Genes	Primer Pairs for Real-time PCR Forward (5' to 3')	Backward (5' to 3')	Product Length(bp)
FAS	TTCCAAGACGAAAATGATGC	AATTGTGGGATCAGGAGAGC	131
L- CPT-I	CTTCCAAGGCAGAAGAGTGG	GAACCTTGGCTGCGGTAAGAC	107
NPY	TACTCCGCTCTGCGACACTA	TCTTCAAGCCTTGTTCTGGG	134
AgRP	GAGTTCCCAGGTCTAAGTCTGAATG	ATCTAGCACCTCCGCCAAAG	102
POMC	GAGGCCACTGAACATCTTTGTC	GCAGAGGCAAACAAGATTGG	76
CART	CGAGAAGAAGTACGGCCAAGTC	CCGATCCTGGCCCCTTT	75
β-actin	GGACTCCTATGTGGGTGACG	CTTCTCCATGTCGTCCCAGT	103

Statistical Analysis

Statistical comparisons of the groups were made by ANOVA and all values are presented as mean±SD.

Table 2: Bodyweight and adipose tissue weight of the mice (n=10)

Groups	Body weight (g)	Body weight increment (g)	The rate of weight increment (%)	Adipose weight (g)	Adipose weight /bodyweight (%)
NC	$39.10\pm4.04^{c\#}$	$11.39\pm0.97^{\#\&}$	$41.23\pm2.48^{\#\&}$	1.24 ± 0.32	3.31 ± 1.09
FC	$51.64\pm2.75^{*\&}$	$20.50\pm4.11^{*\&}$	$59.29\pm17.17^{*\&}$	1.91 ± 0.84^{c}	3.63 ± 1.92
PC	$33.22\pm1.74^{a\#}$	$5.71\pm1.02^{*\#}$	$20.93\pm4.40^{*\#}$	1.11 ± 0.46^{b}	3.42 ± 0.50
PWEL	$37.16\pm3.29^{\#}$	$6.72\pm1.05^{*\#}$	$21.99\pm2.00^{*\#}$	1.42 ± 0.41	3.76 ± 0.77
PWEM	$37.84\pm3.59^{\#}$	$8.05\pm0.68^{*\#c}$	$27.13\pm2.01^{*\#}$	1.10 ± 0.36^{b}	3.25 ± 1.47
PWEH	$33.78\pm5.20^{a\#}$	$5.09\pm2.79^{*\#}$	$17.76\pm9.52^{*\#}$	$0.89\pm0.20^{\#}$	2.51 ± 0.52

a: P<0.05, *: P<0.01 *vs.* NC group, b: P<0.05, #: P<0.01 *vs.* FC group, c: P<0.05, &: P<0.01 *vs.* PC group, the same in table 3-6.

Results

Body Weight and Body Fat

Mice in all experimental groups were apparently healthy, showing no pathological signs or abnormalities during the entire experimental period. The changes in body weight and fat are shown in table 2. It meant that the diet-induced obese mice model was established successfully. Orlistat has an obvious effect on weight control. PWE experimental groups showed significantly lower than FC group on the body weight, weight increment, and the rate of weight increment. With the increase of PWE concentration, adipose weight gradually decreased.

Blood Lipid

The changes in blood lipid are shown in table 3. The TC and TG levels of FC group were significantly higher, but PWE experimental groups were very low. Especially, the TG level of the experimental groups showed significant reduction ($P<0.01$, *vs.* FC group). HDLC level of PWE experimental groups were higher than the FC group, especially PWEM group.

Table 3: Lipid profile in mice serum (n=10)

Groups	TC (mg/dl)	TG (mg/dl)	HDLC (mg/dl)
NC	130.47 ± 17.32^{b}	$87.83\pm23.71^{\#}$	104.00 ± 8.27^{c}
FC	173.97 ± 49.90^{a}	$167.88\pm27.13^{*\&}$	$94.19\pm6.58^{\&}$
PC	141.70 ± 53.41	$78.92\pm3.87^{\#}$	$127.73\pm28.29^{a\#}$
PWEL	139.31 ± 17.13	$71.75\pm6.03^{\#}$	$94.40\pm9.40^{\&}$
PWEM	146.62 ± 15.38	$65.27\pm12.82^{\#}$	123.20 ± 26.00^{b}
PWEH	122.65 ± 10.03^{b}	$61.56\pm23.37^{a\#}$	119.47 ± 13.91^{b}

Adipokines

The levels of adipokines in serum were shown in table 4. The LEP, ADP and resistin levels in mice serum of FC group all lower than other groups, and the PWE experimental groups mostly had significantly increased compared with FC group.

Table 4: Adipokines concentration in the mice serum (n=10)

Groups	LEP (pg/ml)	ADP (ng/ml)	Resistin (ng/ml)
NC	$818.33\pm123.27^{\&}$	$115.71\pm9.87^{\#}$	$103.80\pm4.28^{\#}$
FC	$738.33\pm92.50^{\&}$	$40.00\pm21.07^{*\&}$	$21.24\pm4.07^{*\&}$
PC	$1241.67\pm280.53^{*\#}$	$136.67\pm23.45^{\#}$	$101.40\pm12.80^{\#}$
PWEL	$845.00\pm147.86^{\&}$	$129.05\pm49.24^{\#}$	$78.60\pm6.54^{*\#\&}$
PWEM	978.33 ± 168.49^{bc}	$111.90\pm10.59^{\#}$	$76.68\pm9.65^{*\#\&}$
PWEH	955.00 ± 110.93^{bc}	$96.67\pm15.92^{\#c}$	$79.08\pm7.12^{*\#\&}$

CPT-I Concentration and Gene Expression of FAS and CPT-I in Liver

Table 5 showed that CPT-I concentration of PWE experimental groups had significantly increased both in liver and adipose tissue ($P<0.01$, *vs.* FC group), and related to the dose of PWE.

Table 5: CPT-I concentration in mice liver and adipose tissue (n=10)

Groups	Liver (IU/mg.pr)	Adipose (IU/mg.pr)
NC	$1.81\pm0.21^{\#}$	11.84 ± 0.76
FC	$1.13\pm0.16^{*\&}$	15.28 ± 4.04
PC	$1.76\pm0.11^{\#}$	16.90 ± 2.64
PWEL	$1.99\pm0.368^{\#}$	21.58 ± 5.15^{a}
PWEM	$2.16\pm0.14^{a\#\&}$	$32.67\pm10.43^{*\#\&}$
PWEH	$1.66\pm0.22^{\#}$	$34.65\pm11.83^{*\#\&}$

The tissue homogenates were manufactured by the phosphate buffer solution (pH=7.4), and then tested by the elisa kits and BCA kits, the results were showed by the quotients of the above two. Just like CPT-I concentration, gene expression levels of L-CPT-I and FAS mRNA in liver of PWE experimental groups had significantly increased ($P<0.01$, *vs.* FC group). The results showed in figure 1.

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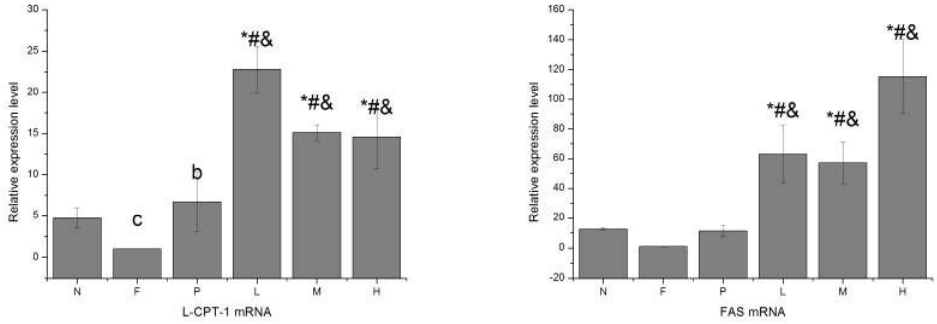

Figure 1: L-CPT-I and FAS mRNA expression in mice liver (n=3)
N: NC, F: FC, P: PC, L: PWEL, M: PWEM, H: PWEH; The same in figure 2-4.
a: P<0.05, *: P<0.01 *vs.* NC group, b: P<0.05, #: P<0.01 *vs.* FC group, c: P<0.05, &: P<0.01 *vs.* PC group, the same in figure 4.

Lipids in Feces

As shown in figure 2, TC concentration of PWE experimental groups has no significant difference compared with FC group, but it had a little lower than FC group at the later time (Day 36, Day 42). But in figure 3, we saw TG concentration of PC group was significantly higher than other groups (P<0.01). FC group was also significantly higher than experimental groups.

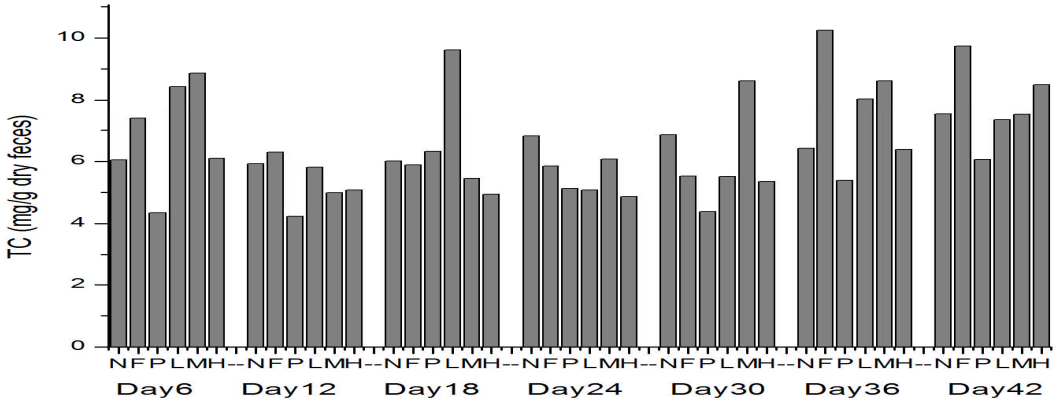

Figure 2: The concentration changes of TC in mice feces (n=3)

Feces of each group were collected together every 6 days, after freeze-drying, the feces of each group were divided into three repeats. Total lipids of each group were extracted by mixed solvent (consisted of carbinol and petroleum ether and chloroform ratio was 1:1:2) and then dissolved by isopropanol, get the solution density as A (Unit: g. dry feces/ml). Lastly tested by kits, and get TC concentration in the solution as B (Unit: mg/ml) and TG concentration in the solution and C (Unit: mg/ml). The result showed by B/A and C/A in figure 2 and 3, respectively.

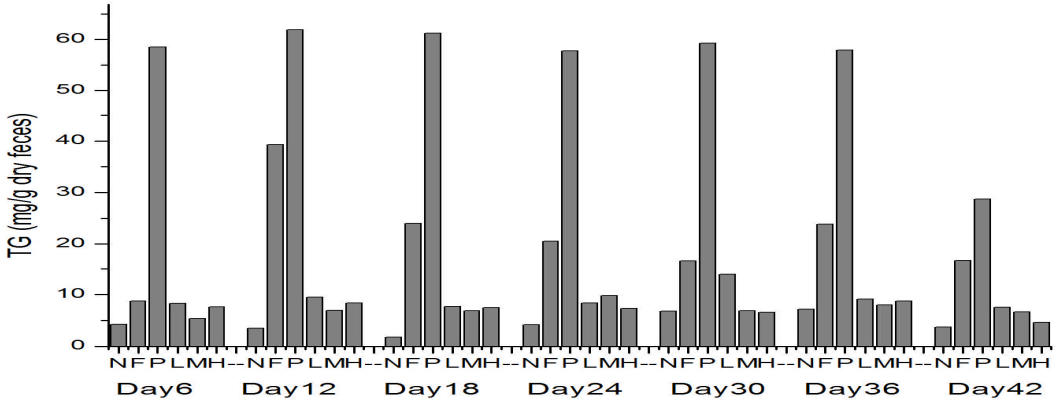

Figure 3: The concentration changes of TG in mice feces (n=3)

Concentration of Neuropeptides in Blood and Gene Expression in Hypothalamus

In table 6, NPY and AgRP concentration of PWE experimental groups significantly reduced (P<0.01, *vs.* FC group).

Table 6: Neuropeptide concentration in mice serum (n=10)

Groups	NPY(pg/ml)	AgRP(pg/ml)	POMC(U/ml)	CART(pg/ml)
NC	287.75±26.02$^{\#}$	54.73±0.80$^{\#}$	52.73±4.44	24.60±2.46$^{\#\&}$
FC	710.00±155.72$^{*\&}$	108.18±11.77$^{*\&}$	49.17±15.70	14.75±6.94$^{*\&}$
PC	360.75±35.65$^{\#}$	57.41±4.75$^{\#}$	49.02±6.09	36.93±6.38$^{*\#}$
PWEL	247.75±38.67$^{\#c}$	53.84±11.14$^{\#}$	47.99±4.31	27.80±8.10$^{\#c}$
PWEM	275.25±34.21$^{\#}$	50.80±10.76$^{\#}$	43.04±2.54	23.93±3.12$^{b\&}$
PWEH	245.25±53.05$^{\#c}$	50.45±10.94$^{\#}$	43.25±2.86	19.67±2.74$^{\&}$

As figure 4 showed, PWEL, PWEM and PWEH groups had different effects on the NPY and AgRP mRNA gene expression levels in hypothalamus. The NPY and AgRP gene expression levels of PWEL and PWEH groups both increased, but PWEM group reduced. In another side, PWEM group showed significant increase in POMC mRNA gene expression level (P<0.01, *vs.* FC group). Conversely, CART mRNA gene expression level was significantly lower than FC group (P<0.01).

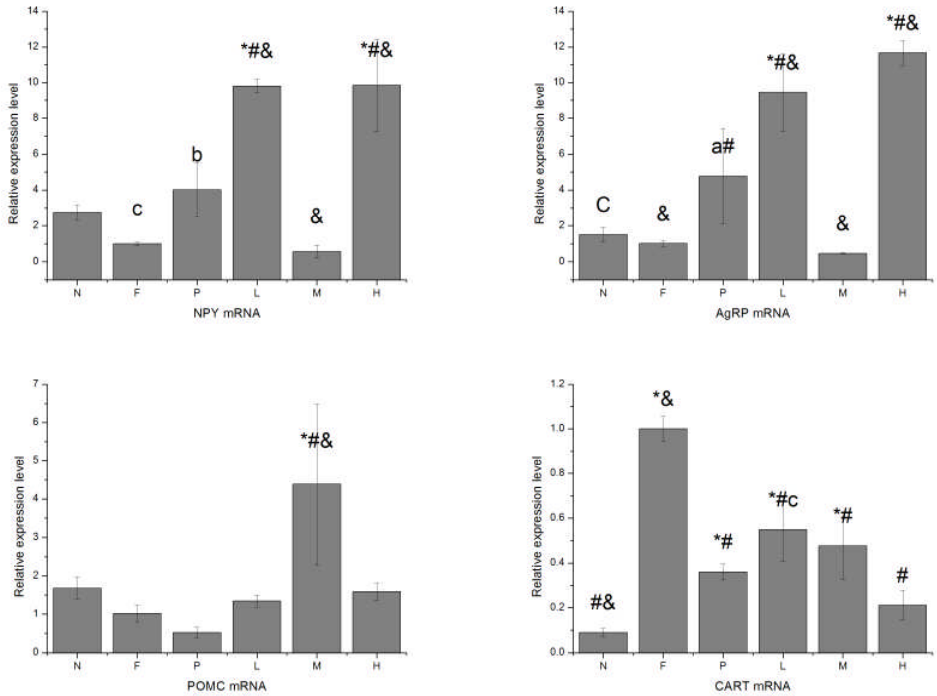

Figure 4: Neuropeptide mRNA expression in mice hypothalamus (n=3)

Discussion

The Effect of PWE on Mice Body Weight, Body Fat Content, Lipid Excretion and Lipid Levels of Serum

As previously mentioned, Fan (Fan et al., 2005) and Zhao (Zhao et al., 2007) had observed that the pine pollen had effect on fat metabolism, but they just used the broken pine pollen as substrate; here, we investigated the role of the PWE in diet-induced obese mice. In our study, we found that PWE could effectively control body weight and fat weight, and significant reduction of cholesterol and triglycerides in serum could also be observed. High density lipoprotein cholesterol in serum had significantly increased too. So, we can conclude that PWE can control the lipid levels at normal levels. In another side, TG concentration of PC group was significantly higher than other groups (P<0.01), which exactly explained that orlistat could inhibit the digestion and absorption of fat by inhibiting the activity of pancreatic lipase (Sternby et al., 2002). And the TC and TG concentration of FC group in mice feces was mostly higher than water-extract groups. So, we thought that the PWE could promote fat absorption rather than inhibiting the activity of pancreatic lipase as orlistat did.

The Effect of PWE on Carnitine Palmitoyl Transferase I of Mice

In mammals, β-oxidation of long-chain fatty acids is the major metabolic pathway for energy utilization in most organs and tissues. From the result of our study, we can find that the PWE significantly elevated the CPT-I concentration in liver and adipose tissue. And it also

significantly promoted the L-CPT-I mRNA expression. In another side, PWE also promoted the FAS mRNA expression which played an important role in the synthesis process of fatty acid. We speculated that PWE could promote LCFA synthesis continuously. And then, the LCFA were transported to the mitochondria by CPT-I, and supplied energy through β-oxidation.

The Effect of PWE on the Adipokines Levels of Serum on Mice

Leptin is an adipose-derived hormone that acts on hypothalamic leptin receptors to regulate energy balance (Fulton et al., 2006). The leptin hormone is critical for normal food intake and metabolism (Hommel et al., 2006). The ability of leptin to activate STAT3 via ObRb has been most extensively studied in hypothalamus, and critical circuits involving leptin signals within POMC and NPY/AgRP neurons in the arcuate nucleus have been defined through functional Neuroanatomy and genetic gain- and loss-of-function approaches (Balthasar et al., 2004; Elias et al., 1999; Elmquist et al., 2005; Erickson et al., 1996; Luquet et al., 2005)**.** From table 4, we found that PWE can increase the leptin concentration in serum; thus conducive to weight control. Adiponectin is an adipocyte-secreted hormone that improves lipid and glucose metabolism (Yamauchi et al., 2003; Yamauchi et al., 2002). In addition to the insulin sensitizing effect, adiponectin directly enhances fatty acid oxidation in skeletal muscle through its own signaling or other pathways including p38 MAPK/PGC-1α (Lee and Shao, 2012). From the table 4, we also found that PWE significantly increased adiponectin concentration *vs.* FC group ($P<0.01$). PWE has an effect on fatty acid oxidation in mice. Resistin is also produced by adipocytes (Holcomb et al., 2000; Kim et al., 2001; Steppan et al., 2001) and circulates at increased levels in obesity (Steppan et al., 2001). And chronic hyperresistinemia could impair normal glucose metabolism (Rangwala et al., 2004). In our study, the resistin concentration of PWE groups all have significant increase than FC group ($P<0.01$), but lower than NC group. So, we speculated that the PWE did not impair normal glucose metabolism.

The Effect of PWE on the Neuropeptides Levels of Mice

The AgRP, NPY, POMC and CART are key neuropeptides that have been associated with energy balance functions (Schwartz, 2001; Schwartz et al., 2000). Two of these - AgRP and NPY - are orexigenic neuropeptides (Broberger et al., 1998; Gropp et al., 2005; Hahn et al., 1998; Luquet et al., 2005) whereas the other two - POMC and CART are anorexigenic neuropeptides (Elias et al., 1998; Germano et al., 2007; Kristensen et al., 1998; Valassi et al., 2008). Increased NPY levels or its enhanced activity increases food intake in animal studies (Mercer et al., 2011). The processing of POMC involves proteolytic cleavage by proteases into several biologically active peptides/hormones including ACTH, β-endorphin and α-MSH. Among these the α-MSH can decrease food intake through its receptors MC3R and MC4R (Cowley et al., 2001). We can find that PWE reduced the NPY and AgRP concentration in serum, and increased the CART concentration, but we did not find an increase in POMC concentration. PWEM could significantly improve POMC mRNA expression and reduce NPY and AgRP mRNA expression. In our study, PWE groups showed significant decrease in NPY and AgRP concentration in serum ($P<0.01$, *vs.* FC group), but almost had no statistically significant difference with FC group in POMC and CART concentration. Judging from the genetic level, only the PWEM could decrease the mRNA expression of NPY and AgRP, but had no statistically significant difference with FC group. PWE groups all could increase the POMC mRNA expression. Just the PWEM group showed statistically significant difference compared with FC group. In another side, PWE groups showed significant decrease in CART mRNA expression ($P<0.01$, *vs.* FC group). The result showed that the CART level can be elevated by the PWE, but had negative correlation with dose. So we speculate that PWE could suppress the appetite in the orexigenic peptides way.

The Role of PWE in Mice

In summary, we can use the figure 5 to illustrate the role of PWE in mice: Firstly, the basic nutrients were absorbed into the small intestine, and then transported via the blood to the cells in liver. The basic nutrients synthesized to acetyl-CoA by ACS and then acetyl-CoA was synthesized to malonyl-CoA catalyzed by ACC. Malonyl-CoA was the main substrate to synthesize the LCFA which was catalyzed by FAS. FAS are the key enzyme in the procedure of fatty acids synthesis. When the activity of FAS was strong, malonyl-CoA was continuously catalyzed to LCFA. Otherwise, the malonyl-CoA which accumulated in cells could affect on the hypothalamus and inhibit the appetite. The excess LCFA could form the fat by esterification in animal, thus increasing the fat deposition. In our study, the TC and TG concentration of FC group in mice feces mostly higher than water-extract groups, so there were sufficient malonyl-CoA catalyzed, and FAS activity in PWE groups significantly higher than FC group, and so the LCFA synthesis more than FC group. Secondly, the LCFA oxidized into ATP by CPT-I in mitochondria. CPT-I is a key enzyme in fatty acid β oxidation which is the most important way for fat to provide energy. It had been reported that adiponectin could promote the role of the CPT-I's activity. In another side, the PWE could suppress the appetite by decreasing NPY and AgRP concentration and mRNA expression level.

In a word, PWE cannot only promote the absorption and synthesis of fat but also promote the oxidation of fat. Critical is that the oxidation was stronger than the synthesis.

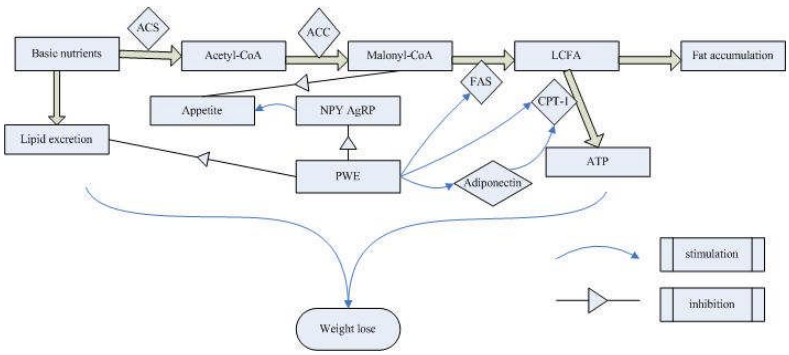

Figure 5: The speculated role of PWE in mice

The basic nutrients are catalyzed by ACS and generate acetyl-CoA, which will become malonyl-CoA under the catalysis of ACC. Then malonyl-CoA will generate LCFA with the help of catalysis of FAS. The LCFA can be oxidized to supply ATP catalyzed by CPT-I, excess LCFA can form the fat by esterification in animals. The basic nutrients which have not been absorbed will be excreted. In our study, firstly, PWE can stimulate the activity of FAS and CPT-I; secondly, PWE can promote the secretion of adiponectin, which can stimulate the activity of CPT-I; thirdly, PWE can inhibit the generation of NPY and AgRP, which are benefit for appetite, and the malonyl-CoA has the opposite effect; lastly, PWE can promote the absorption of nutrients and inhibit nutrients excretion. Based on the above role, PWE exhibits a beneficial effect on weight control.

Conclusions

In this study, we investigated the effects of water-extract of masson pine pollen on the development of obesity in multiple aspects. As composed of complex compounds, water-extract of masson pine pollen showed multiple effects on control nerve system, hormone secretory, lipid metabolism and lipid digestion and has pleiotropy. The results provide scientific basis for the development of health food and weight-reducing medicine.

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