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Pinus Densiflora Bark Extract (PineXol) Decreases Adiposity in Mice by Down-Regulation of Hepatic De Novo Lipogenesis and Adipogenesis in White Adipose Tissue

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PineXol, extracted from Korean red pine bark, has beneficial effects, such as antioxidant, anti-inflammatory, and antilipogenic activities in vitro. We tested the hypothesis that PineXol supplementation could have anti-obesity effects on mice fed a high-fat diet (HFD). Four-week-old male C57BL/6 mice were fed normal chow (18% kcal from fat) or a HFD (60% kcal from fat). HFD-fed animals were also subjected to PineXol treatment at a dose of 10 or 50 mg/kg body weight (BW) (PX10 or PX50, respectively) body weight. The body weight and body fat mass in the PX50 group were statistically lower than those in the HFD group ($p < 0.05$ and $p < 0.001$, respectively). The concentration of hepatic triglycerides, total cholesterol, and low-density lipoprotein cholesterol were reduced in the PX50 group compared with the HFD group ($p < 0.01$). Acetyl CoA carboxylase ($p < 0.01$), elongase of very long chain fatty acids 6 ($p < 0.01$), stearoyl CoA desaturase 1 ($p < 0.05$), microsomal triglyceride transfer protein ($p < 0.01$), and sterol regulatory element-binding protein 1 ($p < 0.05$) were significantly decreased in the PX50 group compared with that in the HFD group. In white adipose tissue, CCAAT-enhancer-binding protein alpha ($p < 0.05$), peroxisome proliferator-activated receptor gamma ($p < 0.001$), and perilipin ($p < 0.01$) were decreased in the PX50 group compared with those in the HFD group. Therefore, the current study implies the potential of PineXol for the prevention and/or amelioration of obesity, in part by inhibition of both hepatic lipid synthesis and adipogenesis in white adipose tissue.

Keywords: PineXol, anti-obesity, hepatic lipogenesis, adipogenesis

Introduction

Obesity is a critical health issue, not only in Western countries but also in Asia-Pacific countries [1]. Major causes of obesity are related to high-energy food intake and a decline in physical activities [2]. Increases in the rates of obesity have led to higher rates of chronic diseases, such as cardiovascular disease, various cancers, type 2 diabetes, and hyperlipidemia [3, 4]. Thus, public attention and knowledge about the biology of energy balance and body weight (BW) have improved in recent years [5]. Anti-obesity medications, including orlistat, phentermine, and sibutramine, were developed to treat this growing epidemic [6]. The benefits of these drugs, including weight loss, lower body fat mass, and a reduction in the waist circumference, emerged as consequences of their activities, such as the inhibition of gastrointestinal lipase and neurotransmitter reuptake [7, 8]. However, later studies reported that these medications were associated with side effects, including dry mouth, insomnia, and fecal incontinence [6, 9]. Strategies for obesity changed from treatment to prevention, resulting in the search for natural anti-obesity ingredients [10].

Extracts of pine bark are known to contain phenolic and polyphenolic flavonoids [11]. Pycnogenol, a bark extract from the maritime pine *Pinus pinaster*, was trademarked by Horphag Research (Geneva, Switzerland) [12]. It has been used worldwide as a nutritional supplement to

prevent various chronic diseases [13–15]. Pycnogenol has numerous beneficial properties, including anti-inflammatory, antioxidant, and anti-obesity activities [16–18]. Pycnogenol supplementation, in particular, inhibits adipocyte differentiation and stimulates selective lipolysis in small lipid droplets within differentiated 3T3-L1 cells [19, 20]. Its anti-obesity effects, including increased levels of lipase and decreased levels of the enzyme that regulate hepatic de novo lipogenesis, were also demonstrated in *ob/ob* mice [21].

PineXol (Nutrapharm Co., Korea), extracted from the bark of Korean red pine (*Pinus densiflora*), has been reported to have similar beneficial effects, including antioxidant, anti-inflammatory, and antilipogenic activities in vitro [22, 23]. The composition of PineXol is similar to that of Pycnogenol, with comparable phenolic compounds (catechin and taxifolin) and flavonoids (procyanidin B1 and B2), according to HPLC analyses [24, 25]. However, the anti-obesity effects of PineXol in vivo have not yet been studied. The current study tested the hypothesis that PineXol supplementation would have anti-obesity effects mediated by a reduction in de novo lipogenesis and adipogenesis in mice fed a high-fat diet (HFD).

Materials and Methods

Animals and Diets

The use of animals followed the protocol approved by IACUC at Kookmin University (KMU-2015-2). Four-week-old male C57BL/6 mice were purchased from Dae-han Bio Link (Korea). The animal facility was maintained at 22 ± 1°C and 50 ± 10% relative humidity, with a 12-h light (7 am to 7 pm)/dark cycle. Four mice were placed in each individually ventilated cage. Animals were adapted to the environment for 1 week prior to experimentation. Mice were fed either normal chow (2018S; Harlan, Switzerland) or a HFD (D12492; Research Diets, Inc., USA). The experimental groups consisted of the following: (i) normal diet (ND, 18% kcal from fat), (ii) control HFD (CTL, 60% kcal from fat), (iii) HFD + PineXol (10 mg/kg BW, PX10), and (iv) HFD + PineXol (50 mg/kg BW, PX50). Food and water were fed ad libitum for 12 weeks, during which PineXol was administrated daily by oral gavage. The BW and food intake were measured weekly. Mice were sacrificed after 12 weeks. Blood was withdrawn from the retro-orbital cavity, and serum was collected by centrifugation at 2,000 ×g for 15 min at 4°C. Liver samples were collected and snap frozen, followed by storage at –80°C for further analysis.

Body Composition Assessment Using Dual-Energy X-Ray Absorptiometry (DEXA)

Animals were fasted overnight for DEXA imaging and then intraperitoneally injected with ketamine (100 mg/kg BW) and xylazine (10 mg/kg BW) for anesthesia. The BW, lean body mass, body fat mass, and body fat percentage were measured at 0, 4, 8, and 12 weeks by an InAlyzer (Medikors, Korea).

Hepatic Triglyceride (TG) Synthesis Using Poloxamer 407 (P407)

Mice were fasted for 4 h prior to the analysis of hepatic TG synthesis. P407, a lipoprotein lipase inhibitor, was purchased from Sigma Aldrich (USA). Animals were intraperitoneally injected with P407 at 1 g/kg BW to observe hepatic TG production. Serum was collected 0, 1, 2, 4, and 24 h after injection. Serum levels of TG at each time point were measured using a commercial kit (Wako, Ltd., Japan) [26].

Blood Biomarker Assay

Serum levels of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were assessed with a commercial kit (Asan Co., Korea) to determine liver function [27]. Levels of TG, total cholesterol (TC), and LDL cholesterol (LDL-C) were evaluated using a commercial kit (Wako Ltd.) [28].

mRNA Expression Analysis

Total RNA was extracted from animal tissues using the Isol-RNA Lysis Reagent (5PRIME, USA), and 5 µg total RNA was reverse-transcribed into cDNA using the iScript Supermix (Bio-Rad Co., USA). Twenty microliters of the total reaction volume, including cDNA, forward and reverse primers, and 10 µl iQ SYBR Green Supermix (Bio-Rad Co.), was used for real-time reverse-transcription PCR (RT-PCR). Reactions were performed in duplicates with an 18S internal control. The cycle threshold value was utilized for quantification of mRNA expression levels.

Immunoblotting

Cytoplasmic proteins were prepared in 4× Laemmli sample buffer (Bio-Rad Co.) and 1× RIPA buffer (CellNest; Minato, Japan), containing a phosphatase inhibitor (Cell Signaling, USA) and a protease inhibitor (Cell Signaling). Lysates were subjected to 20% SDS-PAGE and transferred to a PVDF membrane. The membrane was blocked with 5% bovine serum albumin (GenDEPOT, Inc., USA) and immunoblotted using primary antibodies. Suitable HRP-conjugated secondary antibodies were applied, and the blots were quantified by Image Lab (Bio-Rad Co.). Antibodies to acetyl CoA carboxylase 1 (ACC1), fatty acid synthase (FAS), stearoyl CoA desaturase 1 (SCD1), CCAAT/enhancer-binding protein alpha (C/EBPα), peroxisome proliferator-activated receptor gamma (PPARγ), perilipin, and β-actin were purchased from Cell Signaling. Antibodies to microsomal TG transfer protein (MTP), elongase of very long chain fatty acids 6 (ELOVL6), and sterol regulatory element-binding protein 1 (SREBP1) were purchased from Santa Cruz Biotechnology (USA).

Statistical Analysis

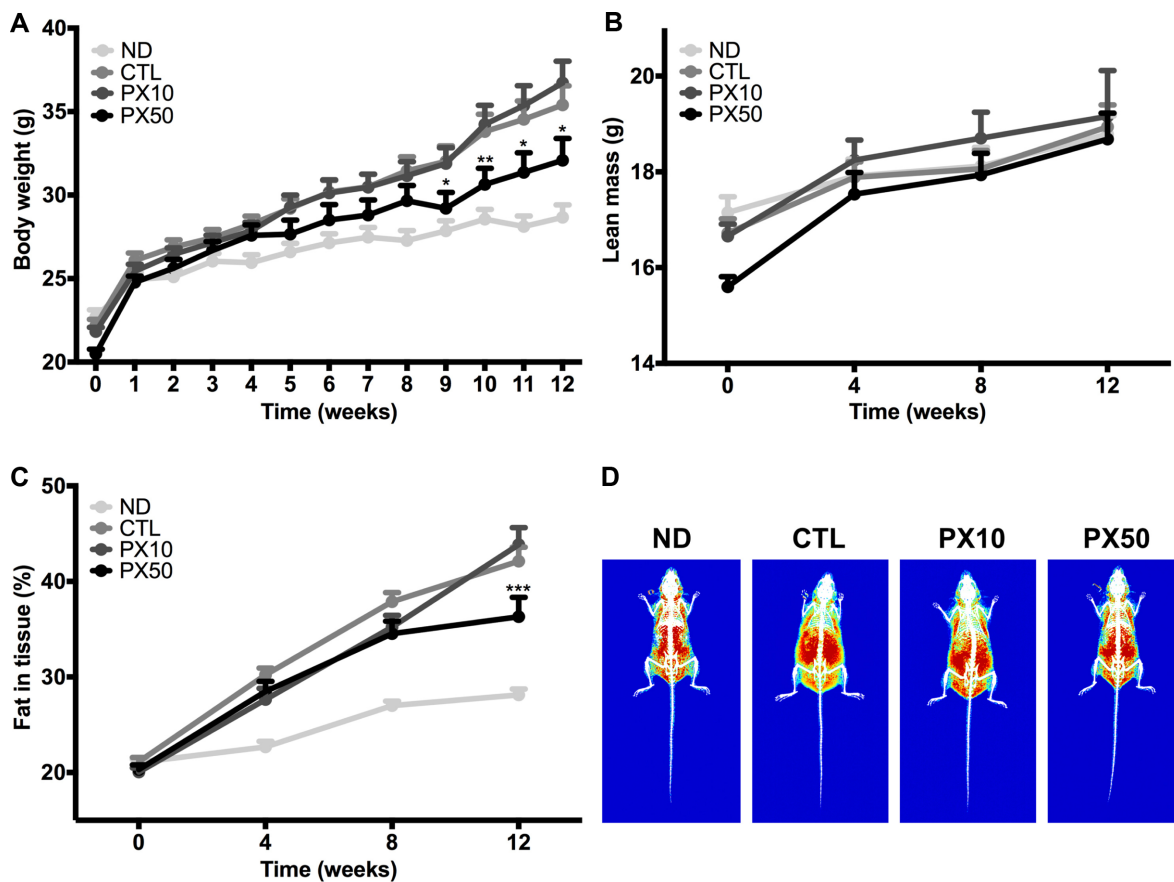All values are presented as the mean ± standard error. The

Fig. 1. Effects of PineXol on growth performance and body composition in mice.
(**A**) Body weight, (**B**) lean mass, (**C**) fat in tissue, and (**D**) representative image of dual-energy X-ray absorptiometry. Fat mass is shown as red in the images. ND, normal diet; CTL, high-fat diet (HFD, 60% kcal from fat); PX10, HFD with PX 10 mg/kg/day; PX50, HFD with PX 50 mg/kg/day. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$. Error bars represent the standard error ($n = 12$).

statistical analysis was performed using Prism 6 (GraghPad Software, USA). Mouse growth performance (BW, feed intake, and fat mass) and hepatic TG synthesis were analyzed using a two-way analysis of variance (ANOVA). Other data were analyzed using a one-way ANOVA. A Dunnett's multiple comparisons test was used to compare means between the CTL, PX10, and PX50 groups, excluding the ND group. *P* values less than 0.05 were considered statistically significant.

Results

Effects of PineXol on Mouse Growth Performance and Body Composition

The BW and body composition were measured for 12 weeks, as shown in Fig. 1. There were no differences in BW between the CTL and PX10 groups during the study period. The BW in the PX50 group was statistically lower than that in the CTL group from nine weeks to the end of

the dietary intervention ($p < 0.05$) (Fig. 1A). DEXA results include lean body mass, fat in the tissue, and X-ray image of mice. The lean body mass did not differ among the groups (Fig. 1B). Strikingly, the PX50 group showed significantly lower body fat at 12 weeks compared with the CTL group ($p < 0.001$). Body fat in the CTL and PX10 groups was not significantly different at 12 weeks (Fig. 1C). X-ray images show the fat distribution in red. In the PX50 group, the fat distribution was dramatically decreased compared with the CTL group (Fig. 1D). BW gain, food intake, and animal food efficiency ratios are shown in Table 1. None of these values of the PineXol-treated groups were significantly different from those of the CTL group. These results suggest that oral administration of PineXol does not induce a loss of appetite or an increase in food intake.

Effects of PineXol on Hepatic Function in Mice

Serum AST and ALT activities are shown in Table 2 as an

Table 1. Weight gain, food intake, and food efficiency ratio in mice fed PineXol.

Group	ND[a]	CTL	PX10	PX50
Weight gain (g/week)	0.50 ± 0.05	1.11 ± 0.06	1.23 ± 0.09	0.99 ± 0.07
Food intake (g/week)	20.59 ± 0.29	14.44 ± 0.21	15.77 ± 0.64	14.56 ± 0.12
FER[b]	2.42 ± 0.23	7.67 ± 0.43	7.79 ± 0.46	6.76 ± 0.46

[a]ND, normal diet; CTL, high-fat diet (HFD, 60% kcal from fat); PX10, HFD with PineXol (10 mg/kg/day); PX50, HFD with PineXol (50 mg/kg/day).

[b]Food efficiency ratio (FER) = (weight gain/food intake) × 100.

Values are expressed as the mean ± standard error (n = 12).

Table 2. Effects of PineXol on hepatic function in mice.

Group	ND[a]	CTL	PX10	PX50
AST (IU/l)	21.83 ± 1.62	22.82 ± 0.76	32.53 ± 3.85	33.31 ± 1.18
ALT (IU/l)[b]	8.74 ± 0.05	10.99 ± 0.34	19.09 ± 0.74***	16.39 ± 0.83**
AST/ALT	2.62 ± 0.25	2.04 ± 0.19	2.00 ± 0.08	2.07 ± 0.16

[a]ND, normal diet; CTL, high-fat diet (HFD, 60% kcal from fat); PX10, HFD with PineXol (10 mg/kg/day); PX50, HFD with PineXol (50 mg/kg/day).

[b]ALT, alanine aminotransferase; AST, aspartate aminotransferase.

**$p < 0.01$; ***$p < 0.001$ compared with CTL. Values are expressed as the mean ± standard error (n = 4, sera were pooled).

analysis of liver function. There were no significant differences in AST values between the CTL and PineXol-treated groups. ALT values in the PineXol-treated groups were increased compared with those of the CTL group ($p < 0.01$). However, the ALT and AST values of all groups stayed within normal limits (0–40 IU/l). In addition, the AST/ALT ratio was used as an indicator of a functional liver disorder; this ratio was within the normal range ($\geq$1) in all groups, with no significant differences among them.
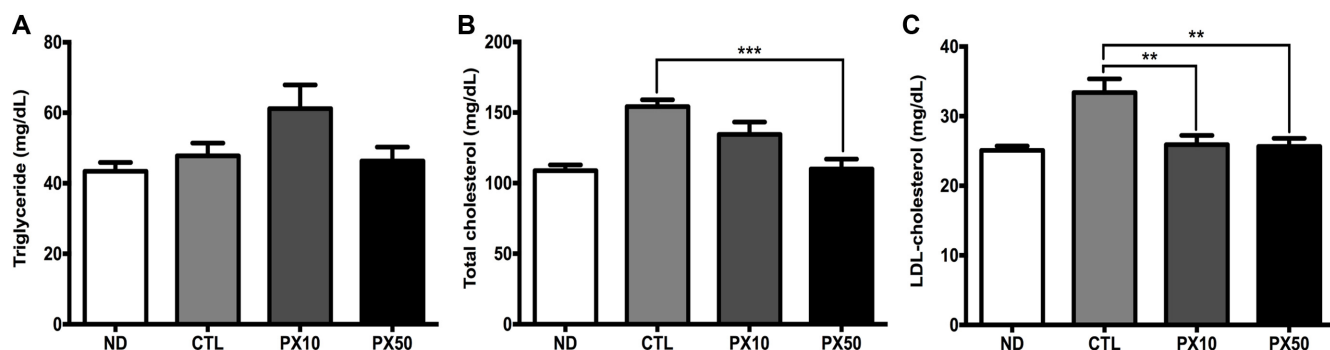
Effects of PineXol on Serum Lipid Profiles in Mice

Serum lipid profiles are shown in Fig. 2. There were no significant differences in serum levels of TG at 12 weeks between any groups (Fig. 2A). Serum levels of TC in the PX50 group were approximately 25% lower than that of the CTL group ($p < 0.01$) (Fig. 2B). Serum levels of LDL-C were significantly decreased in both the PX10 and PX50 groups by 25% compared with the CTL group ($p < 0.01$) (Fig. 2C).

Effects of PineXol on Hepatic TG Production in Mice

Fig. 3 shows hepatic production of TG after P407 injection, an inhibitor of lipoprotein lipase. Initial TG production was not significantly different between any of the groups. TG production in the PX50 group was suppressed by approximately 40% compared with the CTL group at 24 h ($p < 0.001$). On the other hand, the PX10 group produced appoximately 17% more TG at 24 h compared with the CTL group ($p < 0.001$).

Fig. 2. Effects of PineXol on serum lipid profiles in mice.
(**A**) Serum triglyceride, (**B**) serum total cholesterol, and (**C**) serum LDL-cholesterol levels. ND, normal diet; CTL, high-fat diet (HFD, 60% kcal from fat); PX10, HFD with PX 10 mg/kg/day; PX50, HFD with PX 50 mg/kg/day.**$p < 0.01$; ***$p < 0.001$. Data represent the mean ± standard error (n = 8).
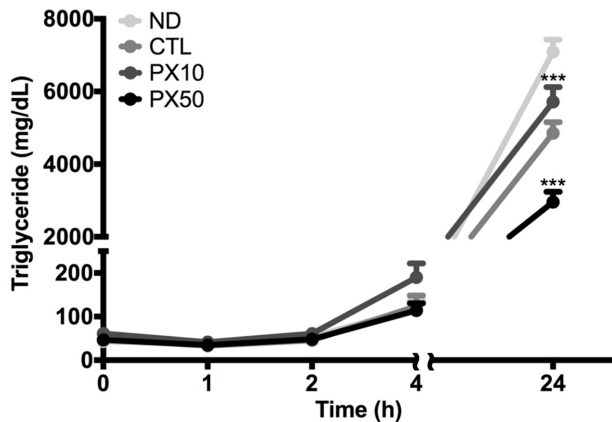
Fig. 3. Effect of PineXol on hepatic triglyceride production with poloxamer 407 in mice.

ND, normal diet; CTL, high-fat diet (HFD, 60% kcal from fat); PX10, HFD with PX 10 mg/kg/day; PX50, HFD with PX 50 mg/kg/day. ***$p < 0.001$ compared with CTL group. Error bars represent the standard error ($n = 12$).

Effects of PineXol on De Novo Lipogenesis in the Liver

In Fig. 4, protein levels of ACC1, FAS, SCD1, ELOVL6, and MTP, which are the regulatory enzymes of de novo lipogenesis and adipogenesis, were examined by western blotting. SREBP1, a transcriptional regulatory factor, was also investigated. Expression levels were normalized to that of β-actin. Levels of ACC1, which synthesizes malonyl CoA, were decreased by 61% in the PX50 group compared with the CTL group ($p < 0.01$). Levels of SCD1, which

desaturates fatty acids, were diminished by 40% in the PX50 group compared with the CTL group ($p < 0.05$). Levels of ELOVL6, which converts palmitic acid into stearic acid, were decreased by 56% in the PX50 group compared with the CTL group ($p < 0.05$). Levels of MTP, which transports TG in the plasma, were reduced by 74% in the PX50 group compared with the CTL group ($p < 0.001$). Levels of SREBP1, which stimulates regulatory enzymes for de novo lipogenesis, declined by 64% compared with the CTL group ($p < 0.01$). To summarize, the expression of each protein examined, except for FAS (which synthesizes palmitic acid from malonyl CoA), decreased considerably in the PX50 group compared with the CTL group. There were no significant differences in protein levels between the PX10 and CTL groups; additionally, mRNA expression levels were not significantly different between the CTL and PineXol-treated groups (data not shown).

Effects of PineXol on Adipogenesis in Mouse White Adipose Tissue

C/EBPα, PPARγ, and perilipin, which function in adipocyte differentiation in the white adipose tissue, were analyzed, and the results are shown in Fig. 5. C/EBPα, a transcriptional factor, enhances early cellular differentiation, whereas PPARγ is preferentially expressed in adipocytes to stimulate adipogenesis. Perilipin serves as a protective coating against lipases and is localized at the periphery of lipid droplets. These factors were analyzed by RT-PCR and western blotting. Expression levels were normalized to that
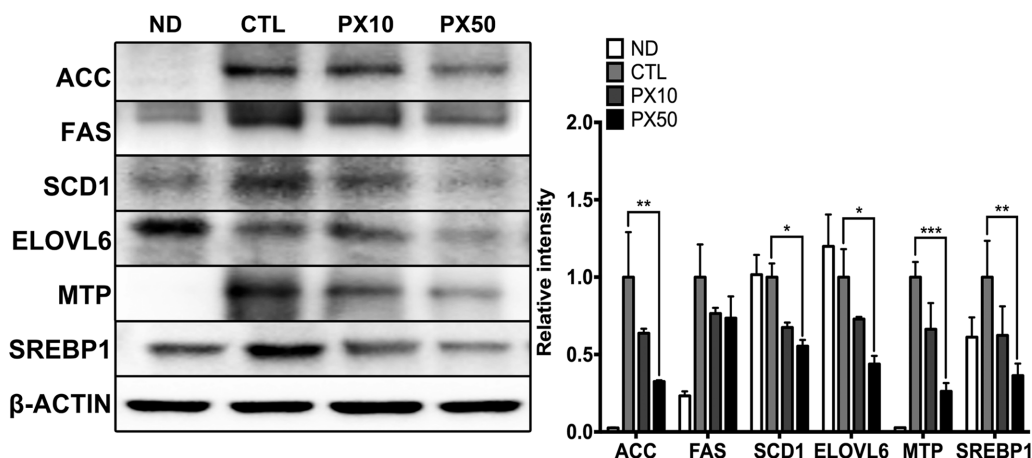

Fig. 4. Effect of PineXol on de novo lipogenesis in mice liver.

The relative intensities by densitometry are shown. Expression levels were normalized to that of β-actin. ND, normal diet; CTL, high-fat diet (HFD, 60% kcal from fat); PX10, HFD with PX 10 mg/kg/day; PX50, HFD with PX 50 mg/kg/day. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$. Data represent the mean ± standard error ($n = 3$). ACC1, acetyl-CoA carboxylase1; FAS, fatty acid synthase; SCD1, stearoyl-CoA desaturase 1; ELOVL6, elongase of very long chain fatty acids 6; MTP, microsomal triglyceride transfer protein; SREBP1, sterol regulatory element-binding protein 1.
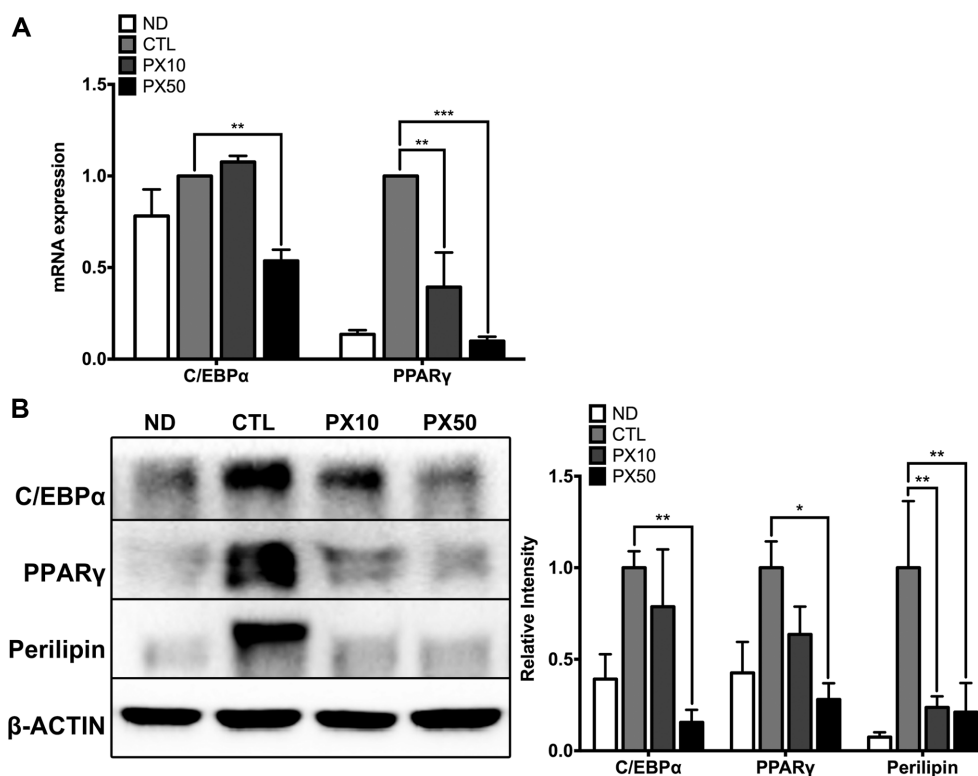
Fig. 5. Effect of PineXol on adipogenesis in mice white adipose tissue.
(**A**) mRNA expression of C/EBPα and PPARγ. (**B**) Representative western blots of C/EBPα, PPARγ, and perilipin. The relative intensities by densitometry are shown. Expression levels were normalized to that of β-actin. ND, normal diet; CTL, high-fat diet (HFD, 60% kcal from fat); PX10, HFD with PX 10 mg/kg/day; PX50, HFD with PX 50 mg/kg/day. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$. Data represent the mean ± standard error ($n = 6$). C/EBPα, CCAAT/enhancer-binding protein alpha; PPARγ, peroxisome proliferator-activated receptor gamma.

of β-actin. Reduced mRNA expression levels of C/EBPα and PPARγ were observed in the PX50 group compared with the CTL group ($p < 0.01$ and $p < 0.001$, respectively). Levels of PPARγ were significantly decreased by 65% in the PX10 group compared with the CTL group ($p < 0.01$) (Fig. 5A). These findings are consistent with the inhibition of protein expression. Protein levels of C/EBPα and PPARγ were clearly decreased in the PX50 group compared with the CTL group ($p < 0.01$ and $p < 0.05$, respectively). Levels of perilipin in the PineXol-treated groups were also significantly lower (approximately 80%) than that in the CTL group ($p < 0.01$) (Fig. 5B).

Discussion

The composition of PineXol is similar to that of Pycnogenol. The main components of both are phenolic compounds and flavonoids [24, 25]. Pycnogenol and PineXol have been reported to possess comparable antioxidant effects [29]. However, the role of PineXol in obesity or lipid

homeostasis is unclear. The degree of BW and body fat are generally used to define obesity. These values were both significantly decreased in the PX50 group compared with the CTL group. Likewise, BW and body fat mass were reduced significantly after nine weeks of 30 mg/kg Pycnogenol administration in C57BL/6J *ob/ob* mice [21]. Hsu and Yen [30] discovered that the phenolic compounds in Pycnogenol decreased the BW in mice fed a HFD. Sato *et al.* [31] established that the BW and white adipose tissue weight were remarkably diminished in ApoE$^{-/-}$ mice fed a pine bark extract for 9 weeks. Thus, PineXol supplementation in vivo reduces both BW and body fat mass.

Increased levels of serum ALT and AST are indicators of hepatic damage. Levels of ALT in PineXol-supplemented groups were significantly higher than those in the CTL group. However, these levels were all within the normal range (0–40 IU/l) [32]. Trevaskis *et al.* [33] reported ALT levels of 18 U/l in wild-type C57BL/6 mice and greater than 500 U/l in Lep^{ob}/Lep^{ob} mice. Additionally, Chen *et al.* [34] measured ALT levels greater than 500 IU/l in mice in

which liver injury was induced by polyinosinic:polycytidylic acid treatment. The AST/ALT ratio is known to be associated with functional liver impairment [35]; there were no significant differences in the AST/ALT ratio between any groups examined in this study. Therefore, PineXol supplementation did not cause significant liver damage.

Blood lipid profiles, including TG, TC, and LDL-C, are widely used to diagnose the metabolic syndrome. The current study showed that serum levels of TC and LDL-C were significantly decreased in the PineXol-treated groups. A reduction in LDL-C is known to help prevent cardiovascular disease (CVD). Sato *et al*. [31] showed that serum and liver levels of TC, but not serum levels of TG, were dramatically lowered in pine bark extract-fed ApoE$^{-/-}$ mice after 9 weeks. Devaraj *et al*. [36] reported that Pycnogenol supplementation (150 mg/day) reduced plasma levels of LDL-C in healthy humans after 6 weeks. Furthermore, after 1 month, Pycnogenol supplementation (360 mg/day) significantly reduced serum levels of TC and LDL-C in 40 patients diagnosed with chronic venous insufficiency [37]. Therefore, PineXol would likely help prevent CVD in both mice and humans by improving levels of cholesterol.

Hepatic TG production was significantly inhibited in the PX50 group but not in the PX10 group. These results are consistent with the levels of lipogenic enzymes present in the liver as determined by western blotting. Expression levels of ACC, SCD1, ELOVL6, MTP, and SREBP1 were lower in the PineXol-treated groups than in the CTL group, suggesting an anti-obesity effect via the down-regulation of de novo lipogenesis. Guerrero *et al*. [38] studied the effects of Pycnogenol on de novo lipid synthesis in HepG2 cells (hepatic human cells), finding that at a dose of 25 mg/l, Pycnogenol caused a significant reduction in cellular lipid levels. Taken together, PineXol may decrease de novo lipogenesis both in vivo and in vitro; however, additional studies must be performed to confirm these results.

In addition, we analyzed several enzymes present in the adipose tissue. Levels of C/EBPα, PPARγ, and perilipin were clearly suppressed in the PineXol-treated groups, suggesting that PineXol could be used to prevent obesity. Previous studies have reported similar antilipogenic effects in vitro: PineXol and Pycnogenol, for example, both inhibit lipid accumulation in 3T3-L1 adipocytes [16, 23]. Pycnogenol decreases the levels of perilipin A and H89 (a PKA inhibitor) in both *ob/ob* mice and primary adipocytes [21]. Yang *et al*. [39] also reported that Pycnogenol inhibited adipogenesis in 3T3-L1 cells via the suppression of PPARγ

and adiponectin. Therefore, PineXol could block both adipogenesis and adipocyte differentiation.

In conclusion, PineXol supplementation in vivo down-regulates hepatic de novo lipogenesis and inhibits adipogenesis in body fat. PineXol also improves levels of TC and LDL-C, thereby reducing BW and adiposity. These findings suggest that PineXol has the potential to improve conditions of hypercholesterolemia and obesity.

Acknowledgments

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