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Antioxidant and anti-adipogenic activities of chestnut (*Castanea crenata*) byproducts

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Abstract The antioxidant and anti-adipogenic activities of chestnut byproducts were evaluated. At 100 μg/mL, the methanol extract (ME) scavenged 34.2% of DPPH and 78.8% of ABTS radicals. The DPPH and ABTS radical scavenging activity of the water extract (WE) was found to be low (13.7 and 33.1%, respectively) compared with controls. WE and ME dose-dependently inhibited lipid accumulation of 3T3-L1 adipocytes. WE and ME at 100 μg/mL suppressed 3T3-L1 adipogenesis by 71.0 and 96.5%, respectively, when compared with mature adipocytes. The results indicated that WE and ME inhibited adipocyte differentiation by down-regulating the mRNA expression levels of CCAAT/enhancer binding protein (C/EBP)-β, C/EBPα, and peroxisome proliferator-activated receptor (PPAR)-γ in 3T3-L1 cells. Our study also revealed that WE and ME inhibited pre- and early stage adipogenesis in 3T3-L1 cells. The results suggest that chestnut byproducts are a promising source of antioxidant and anti-obesity molecules.

Keywords: chestnut, byproducts, antioxidant activity, gallic acid

Introduction

Chestnut, a member of the Fagaceae family, is cultivated worldwide, especially in Asia, Europe, North America, and North Africa, and more than 60,000 tons are produced annually in South Korea (1). The fruit is popular in Korea for its distinctive, rich taste and high nutritional value, as well as for its use in traditional customs such as wedding ceremonies and other ancestral rites.

Chestnut contains phenols with hydroxyl groups that have antioxidant activity (2), and act as reducing agents, donate hydrogen, quench free radicals, and chelate metals (3). In addition, the fruit is also rich in hydrolysable tannins that are concentrated in the inner shell (4-7), including gallotannins and ellagitannins (8-11). Tannin is a distinctive group of high molecular weight phenolic polymers with an astringent, bitter taste that reduces palatability, but also prevents aging, hypertension, arteriosclerosis, and adipogenesis (12).

Chestnut is widely processed at industrial scale, and approximately 10% (w/w) of each fruit is generated as byproducts. These byproducts are mainly composed of inner and outer shells, and are either discarded or underutilized. It has been reported that chestnut inner shell showed significant antioxidant activity (6,7), and outer shell (pericarp) and inner shell (integument) contained lots of phenolic compounds (5), however, little information is available on chestnut byproducts after processing as a dye or metal chelating agents. Thus, in this study, we evaluate the potential of these byproducts as a source of high-value antioxidant and anti-obese materials. We analyzed methanol and water extracts for total phenols, tannins, and ability to scavenge DPPH and ABTS, and anti-adipogenic activity in 3T3-L1 cell model.

Materials and Methods

Reagents DPPH, ABTS diammonium salt, dimethyl sulfoxide (DMSO), gallic acid, hydrogen peroxide, sodium chloride, sodium carbonate, sodium phosphate, ascorbic acid, and a methanol solution of tetramethylammonium hydroxide (TMAH) for a methylating reagent in GC/MS, Oil Red O (ORO), 3-isobutyl-1-methylxanthine (IBMX), dexamethasone (Dex), insulin, and MTT were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). Folin-Ciocalteu reagent was obtained from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Ethanol and methanol were provided from Duksan Pure Chemical Co. (Ansan, Korea). Dulbecco's modified Eagle's medium (DMEM), penicillin-streptomycin, and bovine calf serum (BCS) were obtained from Welgene Inc. (Daegu, Korea). Fetal bovine serum (FBS) was purchased from GE Healthcare Bio-Sciences Co. (Piscataway, NJ, USA). TRIzol

reagent was purchased from Life Technologies (Carlsbad, CA, USA). CycleScript RT PreMix was obtained from Bioneer (Daejeon, Korea). The water used in this study was prepared using a super purity water system (Purite Ltd., Oxon, UK). All the other organic solvents and chemicals used in this study were of analytical grade.

Preparation of extracts from chestnut byproducts Chestnut (*Castanea crenata, Okkwang*) byproducts were kindly supplied by Isaac Food Ltd. (Gongju, Korea) on November 2014. The chestnut byproducts were dried in a dry oven (Sangwoo Co., Incheon, Korea) at 50°C for 12 h, then the dried byproducts were ground using a blender (Samyang Co., Gimpo, Korea). The powder with particle diameter smaller than 710 μm (25 mesh) was used in the following extraction preparation. To prepare water extract (WE), 1 gram of chestnut byproducts was extracted with 100 mL deionized water at 100°C for 30 min. Similarly, methanol extract (ME) was also obtained through extraction of one gram of chestnut byproducts with 100 mL methanol at 25°C for 12 h. The extracts were followed by filtration through a Whatman No. 1 filter paper (Advantec, Tokyo, Japan). The solvents of the filtrates were then removed by a rotary evaporator (Eyela N-1000; Tokyo Rikakikai Co., Tokyo, Japan) under reduced pressure at 37°C. Extraction yields of WE and ME were 7.10 and 11.35% (w/w powder), respectively. The extracts were then dissolved in DMSO to a concentration of 50 mg/mL, and were stored in a deep freezer (Operon Co., Seoul, Korea) at 70°C for further experiments.

DPPH radical scavenging activity (RSA) To determine the DPPH RSA of the WE and ME, each extract (0.1 mL) was mixed with 0.9 mL of 0.041 mM DPPH dissolved in ethanol for 30 min, and then its absorbance was measured at 517 nm using a spectrophotometer (UV-1601; Shimadzu, Kyoto, Japan) (13). Ascorbic acid was used as a positive control and all tests were carried out in triplicate. RSA was expressed as percentage inhibition and calculated by the following formula:

$$\% \text{ DPPH RSA} = [1(A_{sample}/A_{control})] \times 100$$

where, $A_{control}$ is the absorbance of the control reaction (the mixture of 0.1 mL of distilled water and a solution of 0.9 mL of DPPH was used as the control), and A_{sample} is the absorbance of the reaction under the presence of extracts.

ABTS RSA The ABTS RSA of the WE and ME was determined as described by Re *et al*. (14). Each extract (0.1 mL) was mixed with sodium phosphate buffer (0.1 mL, 0.1 M, pH 5.0) and hydrogen peroxide (20 μL, 10 mM), and then pre-incubated at 37°C for 5 min. After pre-incubation, ABTS (30 μL, 1.25 mM, in 50 mM phosphate-citrate buffer, pH 5.0) and peroxidase (30 μL, 1 unit/mL) were added to the mixture and then it was incubated at 37°C for 10 min. The absorbance was measured at 405 nm using a multiplate reader (Sunrise RC/TS/TS Color-TC/TW/BC/6Filter; Tecan Austria GmbH, Grödig, Austria). Ascorbic acid was used as a positive control and all tests were carried out in triplicate; the ABTS RSA was calculated by the following formula:

$$\% \text{ ABTS RSA} = [1(A_{sample}/A_{control})] \times 100$$

where, A_{sample} is the absorbance in the presence of extracts and $A_{control}$ is the absorbance of the control reaction (DMSO was used instead of sample).

Total phenolic content (TPC) The TPC of the WE and ME were determined according to the method of Gutfinger (15). Each extract (1.0 mL) was mixed with 1.0 mL of 2% (w/v) Na_2CO_3, and the mixture was then allowed to stand at room temperature for 3 min. After the addition of 0.2 mL of 50% (w/v) Folin-Ciocalteu reagent, the mixture was kept for 30 min in a dark room, followed by centrifugation at 13,400× *g* for 10 min. The absorbance of supernatant was measured at 750 nm using a spectrophotometer (UV-1601; Shimadzu Co.), and the TPC were expressed as gallic acid equivalents (GAE).

Tannin content (TC) The TC of chestnut byproducts were determined according to the method of Yonemori *et al*. (16). Powder of dried chestnut byproducts (5 g) was homogenized with 80% (v/v) methanol. The homogenate was centrifuged at 3,600× *g* for 10 min and pellet was washed again with the same solvent. The combined supernatant, containing soluble tannins, was made up to 100 mL with 80% (v/v) methanol. The pellet containing insoluble tannins was then suspended in 1% (v/v) hydrochloric acid in methanol (1% HCl-MeOH) and left standing for 30 min at room temperature. The extract was centrifuged and the pellet was washed again with 1% (v/v) HCl-MeOH. The combined supernatant was adjusted to 100 mL with solvent. Each supernatant (1.0 mL) was mixed with 1.0 mL of 2% (w/v) Na_2CO_3, and then the mixture was allowed to stand at room temperature for 3 min. After the addition of 0.2 mL of 50% (w/v) Folin-Ciocalteu reagent, the mixture was kept for 30 min in a dark room, followed by centrifugation at 13,400× *g* for 10 min. The absorbance of supernatant was measured at 750 nm using a spectrophotometer (UV-1601; Shimadzu Co.), and the TC was expressed as GAE.

3T3-L1 cell culture and differentiation The 3T3-L1 preadipocytes were obtained from Korean Cell Line Bank (KCLB, Seoul, Korea) and were maintained in DMEM supplemented with 10% (v/v) BCS and 100 unit/mL of penicillin-streptomycin at 37°C in a humidified 5% (v/v) CO_2 atmosphere, and the medium was replaced every 2 days. Two-day post-confluent 3T3-L1 cells were treated with each extract (WE and ME, 10 μL/well) during Day −2 to Day 6. At day 2 after reaching confluency, designated as Day 0, the preadipocytes were cultured in DMEM supplemented with 10% (v/v) FBS and 100 unit/mL antibiotics (FBS-media) containing 500 μM IBMX, 5.2 μM Dex, and 167 nM insulin (differentiation medium; DM). After Day 2, the medium was changed to FBS-media with 167 nM insulin added for another 2 days (post-differentiation medium; Post-DM). Thereafter, the 3T3-L1 adipocytes were cultured in FBS medium.

Table 1. Primer sequences for PCR analysis

Primer	Species	Primer sequence (5′→3′)
C/EBPβ	*Mus musculus*	GCAAGAGCCGCGACAAG (sense) GGCTCGGGCAGCTGCTT (antisense)
PPARγ	*Mus musculus*	CCATTCTGGCCCACCAACTT (sense) CCTTCTCGGCCTGTCGATCC (antisense)
C/EBPα	*Mus musculus*	CCAGAGGATGGTTTCGGGTC (sense) TCCCCAACACCTAAGTCCCT (antisense)
β-Actin	*Mus musculus*	AGGGAAATCGTGCGTGACAT (sense) AGCTCAGTAACAGTCCGCCT (antisense)

Oil Red O (ORO) staining To examine the anti-adipogenic activity of WE and ME in 3T3-L1 cells, intracellular lipid accumulation in differentiated adipocytes was assessed using ORO staining through Day 6. The medium was removed and the cells were fixed with 3.7% (v/v) formaldehyde for 30 min at room temperature after phosphate buffered saline (PBS) washing. The fixed adipocytes were then washed three times with distilled water. The lipid droplets were stained with 3 mg/mL ORO dissolved in isopropanol and the plates were gently shaken for 15 min. After ORO staining, the cells were washed with distilled water, and the images of stained 3T3-L1 cells were obtained. The stained lipid droplets were dissolved in DMSO and transferred at 100 μg/wells to a 96-well plate. The absorbance of each well was quantified using microplate reader at a wavelength of 510 nm.

Isolation of total RNA and RT-PCR analysis The mRNA expression levels of transcription factors were estimated by RT-PCR analysis. Total RNA was extracted from 3T3-L1 cells treated with WE and ME by using the TRIzol reagent. The total RNA was dissolved in 40 μL by RNase-free water. Two micrograms of cDNA was then subjected to reverse transcription using the CycleScript RT PreMix for cDNA synthesis through 12 cycle reactions at 48°C. The mRNA expression levels were analyzed by electrophoresis using agarose gel and β-actin was used as a control. For RT-PCR, the primers for C/EBPβ, PPARγ, and C/EBPα are shown in Table 1.

Statistical analysis All data were expressed as mean±SD. Statistical analysis was performed using the Statistical Package for Social Society (version 14; SPSS, Inc., Chicago, IL, USA) and the significance of each group was verified by one-way analysis of variance followed by Duncan's or Student *t*-test. A *p*-value <0.05 was considered statistically significant.

Results and Discussion

DPPH and ABTS RSAs Free radicals are associated with the development of arteriosclerosis, diabetes, rheumatoid arthritis, inflammation, cancer, and aging (17). Free radical scavenging is commonly used as a measure of antioxidant activity (18). WE and ME of chestnut byproducts (50-250 μg/mL) scavenged DPPH radicals in a concentration-dependent manner, although ME had approximately 2.3-fold higher activity (Fig. 1A). The DPPH radical scavenging activity of ME and WE at 100 μg/mL was 34.2 and 13.7%, respectively. In comparison, DPPH RSA of ascorbic acid, a positive control, was 17.1% at 20 μg/mL, which was much higher than that of either extract. This might be because ascorbic acid was highly pure, while chestnut extracts were crude preparations. DPPH RSA of 100 μg/mL boiling water extracts of Portugal chestnut inner shell and outer shell was previously reported to be 95.6 and 93.8%, respectively (2). However, direct comparison with this published result is not straightforward, because the analytical method and positive control are different.

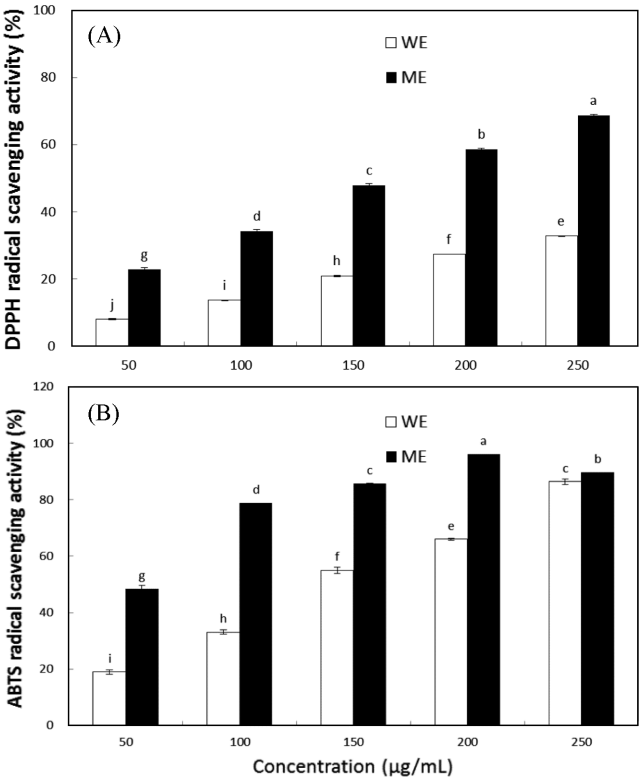

Fig. 1. DPPH and ABTS radical scavenging activities (RSA) of water extract (WE) and methanol extract (ME) from Chestnut Shells. Each value is mean±SD. Values with different letters on the bars in each figure are significantly different by Duncan's multiple range test at $p<0.05$.

ABTS has been widely used to evaluate the relative RSA of plant extracts that contain hydrogen-donating and chain-lysing antioxidants, which scavenge aqueous-phase and lipid peroxyl radicals, respectively (19). As shown in Fig. 1B, ME of chestnut byproducts scavenged ABTS in dose-dependent fashion, and at 2.8-fold higher levels than WE. However, the scavenging activity against ABTS was stronger in both extracts than against DPPH. Thus, at 100 μg/mL, the scavenging activity against ABTS was 78.8 and 33.1% in ME and WE, respectively. Accordingly, IC_{50} values were calculated to be 145.6 and 52.3 μg/mL. In contrast, 20 μg/mL ascorbic acid scavenged 70.9% of ABTS. As

Table 2. Total phenolic content (TPC) of water extract (WE) and methanol extract (ME), and tannin content (TC) of chestnut byproducts (Unit: mg GAE/g)

TPC		TC	
WE	ME	Insoluble	Soluble
173.1±2.5[1)]	205.0±2.5	35.8±0.2	36.4±0.1

[1)]The values were expressed as gallic acid equivalent (GAE). Each value is mean±SD.

noted, the purity of ascorbic acid may account for the significantly stronger activity. In comparison, Kim *et al*. (20) reported 57.4% scavenging activity against ABTS in chestnut inner skin extracted with 60% (v/v) methanol at 250 μg/mL. Remarkably, this activity nearly doubled to 98.8% after extracts were fractionated in ethyl acetate. The difference in scavenging activity against DPPH and ABTS may be due to the nature of the free radical, which is uncharged stable in DPPH, but cationic in ABTS. Alternatively, the difference may be due to the selectivity of experiments, as assays based on ABTS measures hydrophilic and hydrophobic antioxidants, while those based on DPPH only tests hydrophobic antioxidants (21). Therefore, it is possible that some molecules that strongly scavenge ABTS are undetectable in a DPPH assay (22).

TPC and TC Polyphenolic compounds are the most abundant antioxidants in the diet, and confer protection against cancer, cardiovascular disease, diabetes, osteoporosis, and neurodegeneration (23). At 100 μg/mL, WE and ME contained 173.1 and 205.0 mg GAE/g of total phenols, respectively (Table 2). In comparison, Vázquez *et al*. (4) reported higher yields of 55.8 and 32.5 g GAE in 100 g water and methanol extract. Presumably, the discrepancy in yield is due to differences in the extraction process. In the study by Vázquez *et al*. (4), water extracts were prepared with 2.5% (w/v) Na_2SO_3, and methanol extracts were prepared in a Soxhlet apparatus. In contrast, we extracted chestnut byproducts in methanol at room temperature. Indeed, higher extraction temperatures boost yield, and as much as 411.11 mg GAE/g may be obtained by extracting in water at 90°C (11).

Tannin, a polyphenol, is a key agent of chemical defense in plants against rot and herbivores (24). The compound also has strong antioxidant activity due to the abundance of hydroxyl groups, and thereby provides healthful benefits (24,25). We found dried chestnut byproducts to contain 35.8 and 36.4 mg GAE/g (w/w), soluble and insoluble tannins, respectively (Table 2). Of note, Ham *et al*. (7) reported that yields of condensed tannin increased with extraction temperature, and that as much as 100.1 mg catechin equivalents/g may be obtained by extracting inner shells in water at 90°C.

Anti-adipogenic activity Adipose tissue is found mainly beneath the skin, around internal organs, and in breast tissue. Adipose tissue has many beneficial effects such as insulation against heat and cold, and maintaining energy balance and homeostasis (26). However, an

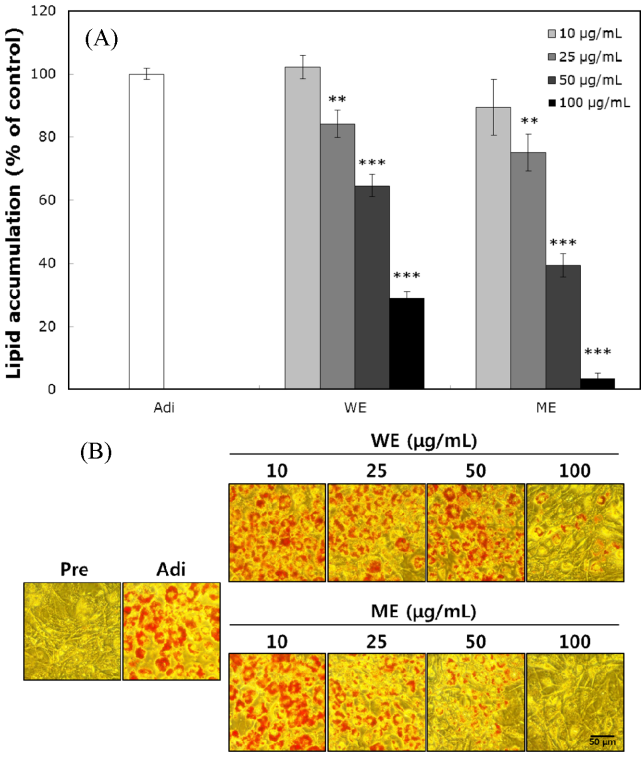

Fig. 2. Effects of water extract (WE) and methanol extract (ME) treatments on the 3T3-L1 adipogenesis. 3T3-L1 preadipocytes were subjected to adipogenesis in the presence of 10, 25, 50, and 100 μg/mL WE and ME during Day -2 to Day 6. The levels of lipid accumulation in mature 3T3-L1 adipocytes were quantified by ORO staining at Day 6 (A). Microscopic image analysis (B). Pre: preadipocytes, Adi: nature adipocyles at Day 6. Corresponding letters indicate significant differences by Student's *t*-test (**p<0.01 and ***p<0.001).

excessive accumulation of fat in adipose tissue can be a cause of obesity, which is the result of an imbalance between energy intake and its expenditure leading to the pathological growth of adipocytes (27). The cytotoxicities of WE and ME were evaluated by MTT assay. The treatments of 3T3-L1 cells with WE and ME up to a concentration of 100 μg/mL did not affect their viability (data not shown). Thus, 10, 25, 50, and 100 μg/mL of WE and ME were chosen for subsequent experiments. To examine whether WE and ME treatments could inhibit 3T3-L1 adipogenesis, the 3T3-L1 cells were exposed to adipogenic differentiation medium to initiate adipogenesis in the presence or absence of WE and ME. The results showed that WE and ME inhibited 3T3-L1 adipogenesis in a dose-dependent manner. WE and ME at concentrations of 10, 25, 50, and 100 μg/mL inhibited 3T3-L1 adipogenesis by 0, 15.7, 35.4, and 71.0% and by 10.6, 24.8, 60.8, and 96.5%, respectively (Fig. 2A). The anti-adipogenic activity of ME was found to be stronger than that of WE. In addition, the number and size of intracellular lipid droplets dramatically decreased upon WE and ME treatments (Fig. 2B). These results indicate that the WE and ME inhibit intracellular lipid accumulation through inhibition of 3T3-L1 adipogenesis. Recent studies have tried to find natural functional materials with anti-adipogenic effects (28,29). Several

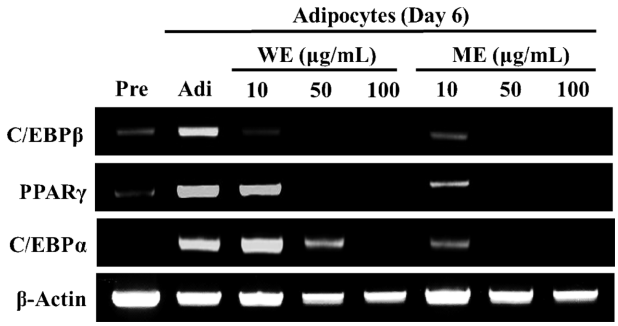

Fig. 3. Effects of water extract (WE) and methanol extract (ME) treatments on the mRNA expression levels of adipogenic transcriptional factors. β-Actin was used as a control. Pre: 3T3-L1 preadipocytes, Adi: Mature adipocytes at Day 6

researchers have shown the anti-adipogenic effects of proanthocyanidins from seed shells of Japanese horse chestnut (*Aesculus turbinate* Blume) (30), and the antioxidant activity of chestnut (*Castanea crenata*) in *t*-BHP treated HepG2 cells (6). However, the precise role and molecular mechanisms in anti-adipogenic activity of chestnut shells are still unclear.

Expression levels of adipogenic transcriptional factors To examine whether WE and ME inhibited adipogenesis by down-regulating the expressions of transcription factors, the mRNA levels of C/EBPβ, PPARγ, and C/EBPα were analyzed by RT-PCR. The 3T3-L1 adipocytes were exposed to 10, 50, and 100 μg/mL WE and ME from Days −2 to 7. The mature adipocytes showed a marked increase in the mRNA expression levels of adipogenic marker genes (Fig. 3). However, 3T3-L1 adipocytes treated with WE and ME exhibited a dose-dependent decrease in the expression levels of C/EBPβ, PPARγ, and C/EBPα. The 3T3-L1 mature adipocytes treated with WE (50 and 100 μg/mL) and ME (10, 50 and 100 μg/mL) exhibited similar levels of C/EBPβ, PPARγ, and C/EBPα in comparison with preadipocytes. Previous studies have reported that the anti-adipogenic activities of *Rumex crispus* L. and *Aster scaber* Thunb. were associated with down-regulated mRNA expression levels of transcription factors such as C/EBP and PPAR families (31,32). Thus, based on the results, it was possible to conclude that 3T3-L1 adipogenesis was inhibited by WE and ME treatments through suppression of transcriptional factors including C/EBPβ, PPARγ, and C/EBPα levels. Activated transcriptional factors subsequently coordinate the expressions of adipogenic hormones and adipocyte-specific genes including fatty acid synthase (FAS) and adipocytes protein 2 (aP2), which crucial lipogenic enzymes modulating lipid metabolism (33).

The WE and ME-inhibited 3T3-L1 adipogenesis is limited to pre- and early stages We further investigated whether the inhibition of lipid accumulation by WE and ME was limited to certain stage of 3T3-L1 adipogenesis. The treatments of WE and ME were divided into four stages such as pre-adipogenic stage (Days −2~0), early stage (Days

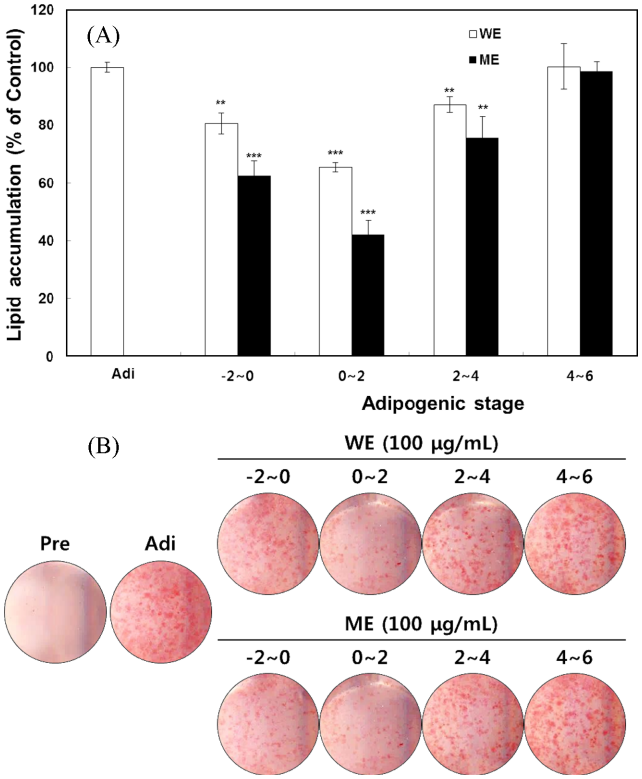

Fig. 4. The water extract (WE) and methanol extract (ME)-inhibited adipogenesis are strongly limited in pre- and early stages. The 3T3-L1 cells were treated with 100 μg/mL of WE and ME during indicated periods (A). The inhibited adipogenesis was observed after ORO staining (B). Corresponding letters indicate significant differences by Student's *t*-test (***p*<0.01 and ****p*<0.001). Pre: Preadipocytes, Adi: Mature adipocytes at Day 6.

0~2), intermediate stage (Days 2~4), and terminal stage (Days 4~6). The WE and ME treatments in the terminal stage of adipogenesis were no significant differences when compared with 3T3-L1 mature adipocytes (Fig. 4). However, 100 μg/mL WE and ME treatments significantly suppressed 3T3-L1 adipogenesis in order of early, pre- and intermediate stages. Similar with anti-adipogenic activities, the inhibitory effect of ME was stronger than that of WE during whole stages. It was also observed that inhibition of 3T3-L1 adipogenesis upon WE and ME treatments is limited to the pre- and early stages of 3T3-L1 adipogenesis. Previous studies have shown that the activation or maintenance of preadipogenic genes such as Wnt6, Wnt-10b, GATA2, GATA3, EGFR, Mmp3, and α-SMA resulted in the suppression of adipogenesis (34,35). It is also known that above preadipogenic genes are specifically expressed during pre- and early stage of adipogenesis (36). In addition, previous study showed that activated preadipogenic genes could inhibit adipogenic transcriptional factors (37). Thus, it is possible to speculate that WE and ME treatments could suppress 3T3-L1 adipogenesis through regulation of transcriptional factors such C/EBPβ, PPARγ, and C/EBPα, particularly in the pre- and early adipogenic stages. This is the first report to elucidate the mechanism of action of anti-adipogenic activity of chestnut.

The data suggest that chestnut byproducts, which consist of outer and inner shells generated after processing, contain significant antioxidant and anti-adipogenic activities. Methanol extracts contained higher amounts of phenols, and are therefore more active than water extracts. However, a suitable process to remove methanol, or an alternative extraction process with a safe solvent, is required to maximize usability of extracts.

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Disclosure The authors declare no conflict of interest.

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