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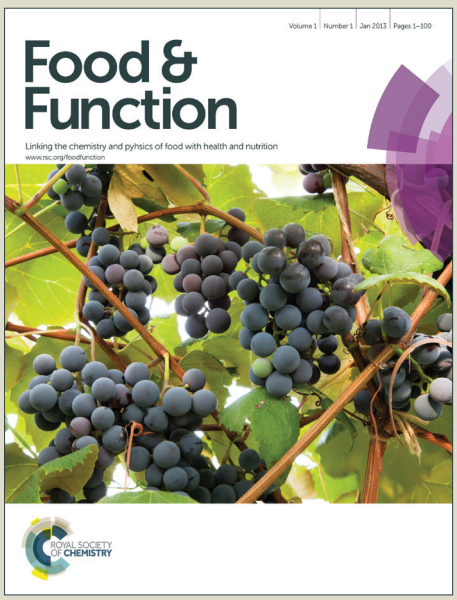

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WNT/β-catenin pathway is involved in the anti-adipogenic activity of cerebroside from the sea cucumber *Cucumaria frondosa*

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Abstract: Both adipocyte hypertrophy and hyperplasia lead to obesity. Here, we isolated cerebrosides from the sea cucumber *Cucumaria frondosa* (*CF*C) and examined its anti-adipogenic activity *in vitro*. *CF*C inhibited the lipid accumulation of 3T3-L1 cells, suppressed PPARγ and C/EBPα expressions, proving its anti-adipogenic activity. Furthermore, *CF*C suppressed lipogenesis in mature adipocytes. WNT/β-catenin pathway acts as an anti-adipogenic factor. *CF*C enhanced β-catenin expression, promoted its nuclear translocation and up-regulated the expression of CCND1 and c-myc, two target genes of β-catenin. Moreover, after cells were treated with β-catenin inhibitor 21H7, β-catenin nuclear translocation and transcription activity can be recovered by *CF*C. These findings suggested that *CF*C promoted the activation of WNT/β-catenin pathway. Besides, *CF*C enhanced expressions of Fz1, LRP5 and LRP6, while had no effect on that of Wnt10b and GSK3β. These findings indicated that *CF*C exhibit anti-adipogenic activity through enhancing the activation of WNT/β-catenin pathway, which was mediated by FZ and LRPs.

Introduction

Adipose tissue plays an important role in whole-body energy homeostasis.[1] It can respond rapidly and dynamically to nutrient deprivation and excess through two ways: adipocyte hypertrophy and hyperplasia.[2] Although adipocyte hypertrophy plays dominant part in adult obesity, adipocyte hyperplasia is now also proved to be responsible for the adipose tissue mass increase in adults.[3] Furthermore, adipocyte can secret various adipocytokines to regulate glucose and lipid metabolism, while disordered adipogenesis deregulates the adipocytokines secretion, leading to the development of obesity related metabolism diseases, such as insulin resistance and type 2 diabetes.[4-6] Therefore, controlling the adipocyte differentiation, namely adipogenesis, may be a strategy against the obesity.[7]

Adipogenesis is controlled by a cascade of transcriptional factors, among which CCAAT/enhancer binding protein-α (C/EBPα) and peroxisome proliferator-activated receptor-γ (PPARγ) play vital roles.[8] PPARγ and C/EBPα are controlled by a series of pathways and one of the most important one is the wingless-type MMTV integration site (WNT)/β-catenin pathway.[9] When the pathway is inactivated, cytoplasmic β-catenin is recruited to the destruction complex, where β-catenin is phosphorylated by GSK3β and subsequently degraded.[10] However, when Wnt10b binds to the frizzled (FZ) receptors and LRP5/6 coreceptors in the membrane of the preadipocytes, the destruction complex cannot be formed, so β-catenin was accumulated in the cytoplasm and translocated into the nucleus.[10] Nuclear β-catenin binds to the T cell factor/lymphoid enhancer factor (TCF/LEF) family to mediate the

Wnt response genes, such as CCND1 and c-myc, which then inhibit the expression of PPARγ and C/EBPα.[11]

Cerebroside is one of the simplest members in the family of glycosphingolipids.[12] Studies showed that cerebroside possesses anti-tumor, immunomodulatory and anti-bacterial activities.[13] Because of the special environment, marine-derived cerebroside may have better bioactivities.[14] Therefore, attention has been paid to the cerebroside isolated from sea cucumber (SCC), one of the most importance sources of cerebroside in marine organisms. SCC consists of three different structural units: a monosaccharide (such as glucose and galactose), an amide-linked fatty acid and a sphingoid base, also called long-chain base.[15] It is reported that SCC exhibited anti-tumor and anti-oxidant activities.[13,16] SCC also regulated the lipid metabolism in liver by inhibiting the hepatic lipogenic process.[17,18] However, the effect of SCC on adipose lipid metabolism and adipogenesis were still unknown. In the present study, we isolated the cerebroside from the sea cucumber *Cucumaria frondosa* (*CF*C) and investigated its anti-adipogenic activity *in vitro* for the first time. The underlying mechanism was further clarified.

Materials and methods

Preparation of *CF*C

Dried *Cucumaria frondosa* was purchased from a sea food market in Qingdao, China. *CF*C was extracted from the sea cucumber *Cucumaria frondosa* and analyzed using the previous described methods.[17] The content of *CF*C in dried *Cucumaria frondosa* was about 4.17±0.11‰. Its molecular weight was in the range of 785.7~870.1

analyzed by the ESI-MS method. The main chemical structure of *CF*C was shown in Fig. 1.

Cell lines and cell culture

3T3-L1 cells were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA). They were cultured with Dulbecco's modified eagle medium (DMEM, Gibio, Gaithersburg, MD, USA) and fetal bovine serum (FBS, Hyclone, Logan, UT, USA) at 37 ºC in a humidified atmosphere of 5% CO_2 and subcultured every two or three days.

Adipocyte differentiation

3T3-L1 cells were induced to mature adipocytes using the standard cocktail method.[19] Briefly, 3T3-L1 cells were seeded into 24-well plates (2×10^4/well) and cultured to be confluent. Having been confluent for 48h, cells were treated with 0.5mM isobutylmethylxanthine (IBMX), 1μM dexamethasone (DEX), 10μg/ml insulin (Sigma, St. Louis, MO, USA) in DMEM containing 10% FBS for 48h (marked as day 0). The cells were then maintained in DMEM containing 10μg/ml insulin and 10% FBS for another two days. The culture medium were then changed with the complete medium (DMEM with 10% FBS) every two days until day 8.

Oil-red O staining

3T3-L1 cells were induced to mature adipocytes with the method described above. Different concentrations of *CF*C (62.5, 125, 250μg/ml) were added from day 0, day 2 or day 4. At day 8, cells were fixed with 10% fresh formaldehyde for 1h at room temperature. After washed with PBS, cells were then stained with filtered 0.5%

Oil-red O solution (Sigma, St. Louis, MO, USA) for 0.5h, washed with 60% isopropanol and photographed with an inverted microscope (IX51, Olympus, Tokyo, Japan). To quantify the effect of *CF*C on adipocyte differentiation, the combined Oil-red O dye was dissolved with isopropanol after images were collected and the absorbance value was measured at 570nm wavelength.

Triglyceride (TG) and free fatty acid (FFA) measurement

Differentiated to mature adipocytes with the method described in 2.3, 3T3-L1 cells were treated with 0.5% BSA for 12h. Then different concentrations of *CF*C were added. Twenty-four hours later, the culture medium was collected and the content of FFA was measured with an ELISA kit. At the same time, cells were lysed and the cell lysate were collected after centrifugation. The intracellular TG content was examined using the triglyceride assay kit (Aibio, Beijing, China) and protein content by a bicinchoninic acid kit (Beyotime, Jiangsu, China) to revise the results. TG content is expressed as TG content / protein content (mg/mg).

Real-time PCR (qRT-PCR) analysis

The expressions of genes that regulate the adipocyte differentiation, such as Wnt10b, FZ1, LRP5, LRP6, GSK3β, β-catenin, CCND1 (cyclin D1), c-myc, C/EBPα, PPARγ were examined by qRT-PCR. 3T3-L1 cells were induced to mature adipocytes with the method described in 2.3. 250μg/ml *CF*C and 50μM 21H7 (Sigma, St. Louis, MO, USA) were added from day 0 and total RNA was extracted using TRIzol method at day 0, day 2, day 4 and day 8. RNA (1μg) was transcribed to cDNA with M-MLV (Takara, Otsu, Japan). The cDNA was amplified in a 25μl system containing SYBR

Green mix (Roche, Basle, Switzerland) using a quantitative real-time PCR thermocycler (iQ5, Bio-Rad, Hercules, CA, USA). The amplification conditions were as follow: pre-denatured at 95ºC for 10min, denatured at 95ºC for 15s, annealed at 60ºC for 10s and extended at 72ºC for 45s of 45 cycles. β-Actin was used as an internal control. The relative mRNA expression of different genes was expressed as the ratio of target gene expression to that of β-actin. Prime sequences of genes examined were listed in Table 1.

Western blotting analysis

The protein expressions of the key factors related to the WNT/β-catenin pathway, such as LRP6, β-catenin, C/EBPα and PPARγ were measured by western blotting. To get the total protein, cells were lysed with the RIPA lysis buffer, centrifuged and proteins were collected. To obtain the nuclear protein, adipocytes were digested by trypsin and then nuclear extracts were got using a nuclear extract kit (Solarbio, Beijing, China). The proteins were then separated by SDS-PAGE and transferred to PVDF membrane. The membrane was blocked with non-fat milk, incubated with antibodies against LRP6, β-catenin, β-actin, TBP (Cell Signaling, Danvers, MA, USA), C/EBPα and PPARγ (Abcam, Burlingame, CA, USA) and then with horseradish peroxidase-conjugated secondary antibody. β-Actin was used as a control for total protein expression and TBP for nuclear protein level. The protein banks were visualized using an ECL kit and quantified with Image J program (Version 1.41o).

Statistical analysis

All data were present as mean ± standard deviation (S.D.) of at least three independent experiments. One-way analysis of variance (ANOVA) followed by the Turkey's test was used to assess the differences between individual groups. Differences were considered significant at $P<0.05$.

Results

***CF*C inhibited the adipogenesis of 3T3-L1 cells**

Lipid accumulation was examined by Oil-red O staining. As shown in Fig.2A, *CF*C significantly inhibited the adipocyte differentiation, as the number of lipid droplets in the *CF*C-treated cells is much lesser than that of the control group. That opinion was further confirmed by the Oil-red O absorbance result (Fig. 2B). Since *CF*C had no effect on the activity of lactate dehydrogenase (LDH) in the culture supernatant of 3T3-L1 cells, it had no cytotoxicity on 3T3-L1 cells (data not show). Adding *CF*C from the early stage (0d), middle stage (2d) or later stage (4d) both had significant inhibitory effect on the lipid accumulation, suggesting that *CF*C may regulate all the stage of adipocyte differentiation.

Adipogenesis is accompanied by both lipogenesis and adipocyte-specific gene expression, for this reason it is difficult to distinguish lipogenesis and adipogenesis merely according to the final lipid accumulation.[20] C/EBPα and PPARγ, the most important transcriptional factors, cooperate with each other to induce the expression of genes leading to mature adipocyte phenotype.[21] Their expressions were drastically elevated with the proceeding of adipocyte differentiation (Fig. 3A and B).

Therefore, the raised expression of C/EBPα and PPARγ is a better marker of adipogenesis. Fig. 3 indicated that *CF*C markedly down-regulated the expression of C/EBPα and PPARγ both at the level of transcription and translation, which further demonstrated the anti-adipogenic activity of *CF*C.

_CF_C regulated the lipid metabolism in mature adipocytes

Since *CF*C suppressed the lipid accumulation during adipogenesis, we then investigated the effect of *CF*C on lipid metabolism in mature adipocytes. As shown in Fig. 4, TG content was significantly reduced after adipocytes were treated with *CF*C for 24h. Meanwhile the content of FFA in the culture supernatant was increased by *CF*C (Fig. 4B), which indicated that *CF*C may accelerate the lipidolysis process. To further explore how *CF*C regulates the lipid metabolism, the expressions of the lipogenesis and lipidolysis related genes were measured. *CF*C markedly reduced the protein expression of the lipogenesis related factors, GPAT and FAS (Fig. 4C and D), and increased that of the lipidolytic genes, HSL and CPT-1 (Fig. 4E and F). In summary, *CF*C reduced the lipid accumulation in mature adipocyte by suppressing the lipogenesis process and accelerating the lipidolysis process.

_CF_C enhanced the WNT/β-catenin related factors during adipogenesis

WNT/β-catenin pathway is a well-researched negative regulator of adipogenesis through promoting β-catenin transferring into the nuclear.[22] To investigate the effects of *CF*C on the upstream members of the pathway, expressions of the key regulators of WNT/β-catenin pathway, namely Wnt10b, LRP5, LRP6, FZ and GSK3β were measured in the presence or absence of *CF*C. The mRNA expression of

Wnt10b, a WNT ligand that inhibits adipose tissue formation, was rapidly suppressed in the process of adipogenesis, but *CF*C had no effect on it (Fig. 5A). However, *CF*C significantly up-regulated the mRNA expressions of its receptor FZ and coreceptors, LRP5 and LRP6 and the protein expression of LRP6. Involved in the degradation of β-catenin, GSK3β is known as a negative regulator of the WNT/β-catenin pathway and its mRNA expression is slightly enhanced during the adipogenesis, but *CF*C cannot affect it. These results indicated that *CF*C may mediate the WNT/β-catenin pathway by up-regulating the expression of FZ1 and LRP5/6, with no change in Wnt10b.

***CF*C promoted the nuclear translocation of β-catenin**

Once the WNT/β-catenin pathway is activated, β-catenin transfers to the nucleus to regulate the expression of genes related to adipocyte differentiation, such as C/EBPα and PPARγ.[23] To investigate whether β-catenin is involved in *CF*C-mediated anti-adipogenesis, its expression and nuclear translocation was measured. The mRNA expression of β-catenin was reduced during adipogenesis in the absence of *CF*C, while *CF*C treatment markedly enhanced it at day 4 and day 8 (Fig. 6A). *CF*C also up-regulated the β-catenin protein levels both in the total cell lysate and in the nuclear extract (Fig. 6D and E), indicating the elevated nuclear translocation of β-catenin. Furthermore, the mRNA expressions of CCND1 and c-myc, two transcriptional products of β-catenin, were increased by *CF*C, which demonstrated the improved transcriptional activity of nuclear β-catenin.

_CF_C exhibited anti-adipogenic activity under β-catenin inhibitor 21H7 treatment

To further evaluate the role of β-catenin in the inhibitory effect of *CF*C on adipocyte differentiation, 3T3-L1 cells were treated with the β-catenin inhibitor 21H7. The mRNA expression of β-catenin was not affected by 21H7 (Fig. 7A), but 21H7 markedly reduced its total protein level, producing the reduction of 44.22% (Fig. 7B). The nuclear β-catenin level and the mRNA expression of CCND1 and c-myc were also significantly suppressed by 21H7 (Fig. 7C-E). These data suggested that 21H7 reduce the stability, nuclear translocation and transcriptional activity of β-catenin.

However, *CF*C retarded the inhibition effect of 21H7 on β-catenin, as the protein levels of total β-catenin or nuclear β-catenin and the mRNA expressions of CCND1 and c-myc were enhanced by *CF*C under the treatment of 21H7 (Fig. 7B-E). Notably, *CF*C inhibited the lipid accumulation, which was elevated by 21H7 (Fig. 7 F and G). The 21H7 induced up-regulation of C/EBPα and PPARγ expressions were also significantly recovered by *CF*C (Fig. 7H-K). Taken together, these results further proved that *CF*C exhibit anti-adipogenic activity by activating the WNT/β-catenin pathway.

Discussion

Obesity is characterized by the growth of adipose tissue mass.[24] Although a balance of energy intake and consume by food and physical activity is recommend therapies for obesity, inhibition of adipogenesis and lipogenesis is an additional strategy for combating obesity.[25] In the present study, we obtained the cerebroside from sea cucumber *Cucumaria frondosa* (*CF*C) and found that it inhibited the adipogenesis of

3T3-L1 cells, suppressed the lipogenesis process and accelerated the lipidolysis process in adipocytes. These findings suggested that *CF*C exhibited potent anti-obesity activity.

We showed that *CF*C significantly inhibited the adipogenesis by suppressing induction of the adipogenic transcription factors, such as C/EBPα and PPARγ. C/EBPα and PPARγ, mainly found in adipose tissue, are the two most important transcriptional factors in the terminal adipocyte differentiation.[26] Overwhelming evidence has shown that the activation of C/EBPα is sufficient to induce the adipogenesis without exogenous hormonal stimuli.[27] Meanwhile, PPARγ is necessary and sufficient to induce adipocyte differentiation.[28] After activated, C/EBPα and PPARγ induce the expression of genes that are involved in lipogenesis and lipolysis to maintain the mature adipocyte phenotype, including Glut4, GPAT and ap2.[29] Our results showed that *CF*C suppressed the expression of lipogenic genes, GPAT and FAS, and promoted that of lipidolytic factors, HSL and CPT-1, which demonstrated the anti-obesity activity of *CF*C from another point of view.

*CF*C was observed to activate the WNT/β-catenin pathway, which explains the mechanisms of its anti-adipogenic activity. Although WNT/β-catenin pathway has multiple roles in cell growth and differentiation depending on the cell type, it is well-proved that the WNT/β-catenin acts as an inhibitor of adipogenesis in normal adipocytes.[9,30] The activation of WNT/β-catenin pathway in adipose tissue suppressed the adipocyte differentiation and even leaded to the adipocytes dedifferentiated.[31] This was proved by the evidence that exogenous addition of

Wnt3a markedly decreased the number and size of the lipid droplets in adipocytes.[31]

Once Wnt/β-catenin pathway is activated, cytoplasmic β-catenin was released from the destruction complex and translocated into the nucleus.[32] As the central of WNT/β-catenin pathway, high levels of β-catenin could be important in preventing the adipogenesis.[33] We found that β-catenin expression was down-regulated during the adipogenesis and the nuclear translocation was also inhibited. However, treatment with *CF*C up-regulated the expression of β-catenin and promoted its nuclear translocation. Moreover, when β-catenin was degraded by 21H7, *CF*C still inhibited the adipogenesis and enhanced the activation of WNT/β-catenin pathway, suggesting the important role of WNT/β-catenin pathway in *CF*C-mediated anti-adipogenic activity. In the nucleus, β-catenin binds to TCF4, modulating the expression of its target genes, including CCND1 and c-myc.[34] Those target genes then inhibit the adipocyte differentiation by suppressing the expression of C/EBPα and PPARγ.[35] In the present study, the expression of CCND1 and c-myc was enhanced by *CF*C, which further demonstrated the elevated transcription activity of β-catenin.

WNT10b binding to the FZ receptors and LRPs receptors initiates the WNT/β-catenin pathway.[36] Wnt10b is only expressed in preadipocyte and is rapidly down-regulated when preadipocyte undergo adipogenesis,[37] which is in keeping with our results. However, not all the modulation of WNT/β-catenin pathway is accompanied by regulating Wnt10b. It is reported that IL-6 can activates the pathway with no change in the expression of Wnt10b.[38] We found that *CF*C had no significant influence on Wnt10b expression, which may similar to the mechanism of

IL-6. The inhibitory Wnt signals on adipogenesis are also mediated by regulating FZ and LRPs.[39] It is reported that LRP6-deficent mouse embryonic fibroblasts spontaneously induce adipogenic differentiation.[40] Exogenous addition of FZ inhibitor can induce the 3T3-L1 preadipocyte to adipocyte without extra stimulus.[41] Our results showed that *CF*C markedly enhanced the expression of FZ1, LRP5 and LRP6. These findings indicate that *CF*C may activate the WNT/β-catenin pathway through increasing the expression of FZ and LRPs instead of Wnt10b.

In conclusion, the present study provides evidence that *CF*C exhibited marked anti-adipogenic activity. Moreover, that effect was modulated by activating the WNT/β-catenin pathway through enhancing the FZ and LRPs. To our knowledge, this is the first report that provides a scientific basis for the application of cerebroside from sea cucumber against obesity.

Abbreviations

ACC, Acetyl-CoA carboxylase; ANOVA, One-way analysis of variance; ATGL, Adipose triglyceride lipase; C/EBPα, CCAAT/enhancer binding protein α; *CF*C, cerebroside from the *cucumaria frondosa*; CCND1, cyclin D1; DMEM, dulbecco's modified eagle medium; CPT-1, Carnitine palmitoyl transderase-1; Dex, dexamethasone; FBS, fetal bovine serum; FFA, free fatty acid; FZ, frizzled; GPAT, Glycerol-3-phosphate acltransferase; HSL, Hormone sensitive lipase; IBMX, isobutylmethylxanthine; LRP, Lipoprotein receptor-related protein; PPARγ, Peroxisome proliferators-activated receptor γ; qRT-PCR, real-time PCR; TG, triglyceride.

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Figure legends:

Fig. 1 Main chemical structure of *CF*C.

Fig. 2 Effects of *CF*C on adipocyte differentiation. 3T3-L1 cells were induced to mature adipocytes and stained with oil-red O at day 8. Data are represented as mean ± S.D. of three independent experiments. Multiple comparisons were done using one-way ANOVA analysis. **P* < 0.05 *versus* control; ***P* < 0.01 *versus* control.

Fig. 3 Effects of *CF*C on C/EBPα and PPARγ expression. The mRNA expressions of C/EBPα and PPARγ were measured by RT-PCR and protein expression by western blotting. β-Actin was used as an internal control. Data are represented as mean ± S.D. of three independent experiments. Multiple comparisons were done using one-way ANOVA analysis. ***P* < 0.01 *versus* control.

Fig. 4 Effect of *CF*C on the lipid metabolism in adipocytes. 3T3-L1 cells were induced to mature adipocytes and incubated with different concentrations of *CF*C for 24h. TG and FFA content were examined by the ELISA kits. Protein expression of GPAT, FAS, HAL and CPT-1 were measured by western blotting. β-Actin was used as an internal control. Data are represented as mean ± S.D. of three independent experiments. Multiple comparisons were done using one-way ANOVA analysis. ***P* < 0.01 *versus* control.

Fig. 5 Effects of *CF*C on the Wnt/β-catenin pathway. The mRNA expressions of Wnt10b, FZ1, LRP5, LRP6 and GSK3β were measured by RT-PCR and protein expression of LRP6 by western blotting. β-Actin was used as an internal control. Data are represented as mean ± S.D. of three independent experiments. Multiple comparisons were done using one-way ANOVA analysis. ***P* < 0.01 *versus* control.

Fig. 6 Effects of *CF*C on β-catenin. The mRNA expressions of β-catenin, CCND1 and c-myc were measured by RT-PCR and protein expression of β-catenin by western blotting. β-Actin was used as an internal control for total protein test and TBP for nuclear protein test. Data are represented as mean ± S.D. of three independent experiments. Multiple comparisons were done using one-way ANOVA analysis. **P* < 0.05 *versus* control; ***P* < 0.01 *versus* control.

Fig. 7 Effects of *CF*C on adipogenesis under 21H7. 3T3-L1 cells were induced to mature

adipocytes, incubated with 50μM 21H7 and stained with Oil-red O at day 8. The mRNA expressions of β-catenin, CCND1 and c-myc, C/EBPα and PPARγ were measured by RT-PCR and protein expression of β-catenin, C/EBPα and PPARγ by western blotting. β-Actin was used as an internal control for total protein test and TBP for nuclear protein test. Data are represented as mean ± S.D. of three independent experiments. Multiple comparisons were done using one-way ANOVA analysis. $^{*}P < 0.05$ *versus* control; $^{**}P < 0.01$ *versus* control; $^{\#}P < 0.05$ *versus* 21H7 group; $^{\#\#}P < 0.01$ *versus* 21H7 group.

Table 1 Primer sequences of different genes examined

Genes	Forward primer (5' to 3')	Reverse primer (5' to 3')
WNT10b	ATGCGGATCCACAACAACAG	TTCCATGGCATTTGCACTTC
FZ1	CAGCAGTACAACGGCGAAC	GTCCTCCTGATTCGTGTGGC
LRP5	ACCCGCTGGACAAGTTCATC	TCTGGGCTCAGGCTTTGG
LRP6	ACCTCAATGCGATTTGTTCC	GGTGTCAAAGAAGCCTCTGC
GSK3β	ACCCTCATTACCTGACCTT	CTCAACTTAACAGACGGCT
β-catenin	GATTTCAAGGTGGACGAGGA	CACTGTGCTTGGCAAGTTGT
CCND1	GCGTACCCTGACACCAAT	ATCTCCTTCTGCACGCACTT
c-myc	TGATGACCGAGTTACTTGGAG	GGCTGGTGCTGTCTTTGC
PPARγ	CGCTGATGCACTGCCTATGA	AGAGGTCCACAGAGCTGATTCC
C/EBPα	GAACAGCAACGAGTACCGGGTA	GCCATGGCCTTGACCAAGGAG
β-actin	TGTCCACCTTCCAGCAGATGT	AGCTCAGTAACAGTCCGCCTAGA

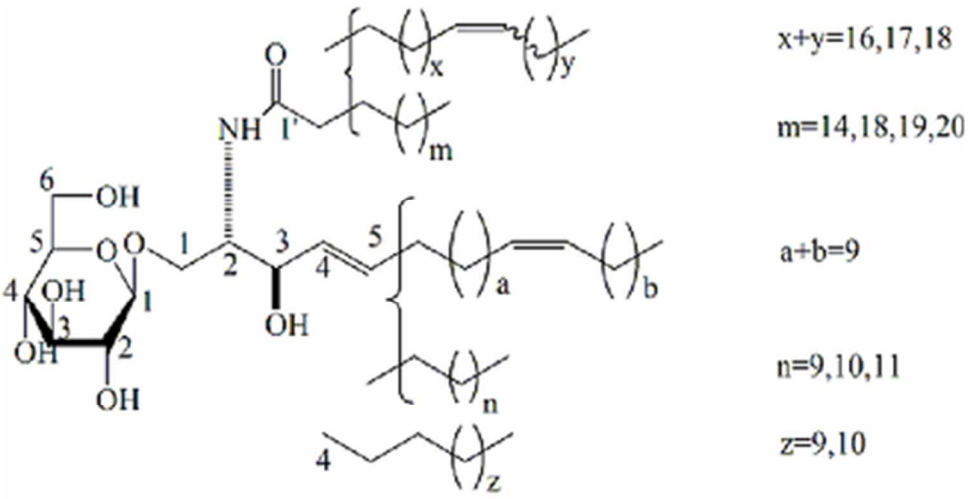

Fig. 1 Main chemical structure of CFC.
41x21mm (300 x 300 DPI)

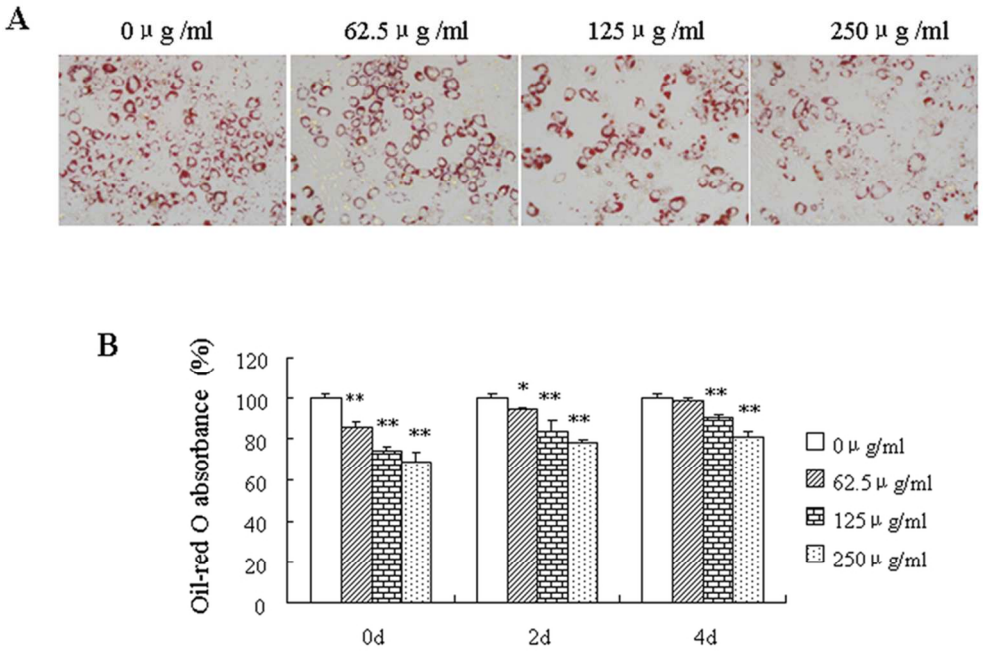

Fig. 2 Effects of CFC on adipocyte differentiation. 3T3-L1 cells were induced to mature adipocytes and stained with oil-red O at day 8. Data are represented as mean ± S.D. of three independent experiments. Multiple comparisons were done using one-way ANOVA analysis. *P < 0.05 versus control; **P < 0.01 versus control.
80x53mm (300 x 300 DPI)

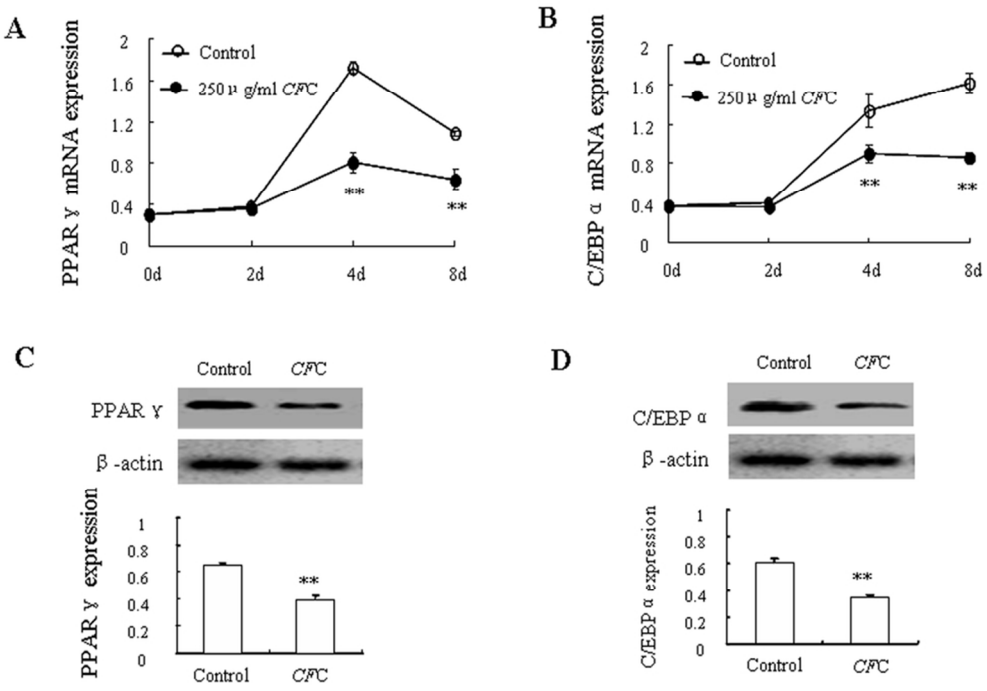

Fig. 3 Effects of CFC on C/EBPα and PPARγ expression. The mRNA expressions of C/EBPα and PPARγ were measured by RT-PCR and protein expression by western blotting. β-Actin was used as an internal control. Data are represented as mean ± S.D. of three independent experiments. Multiple comparisons were done using one-way ANOVA analysis. **P < 0.01 versus control.
83x57mm (300 x 300 DPI)

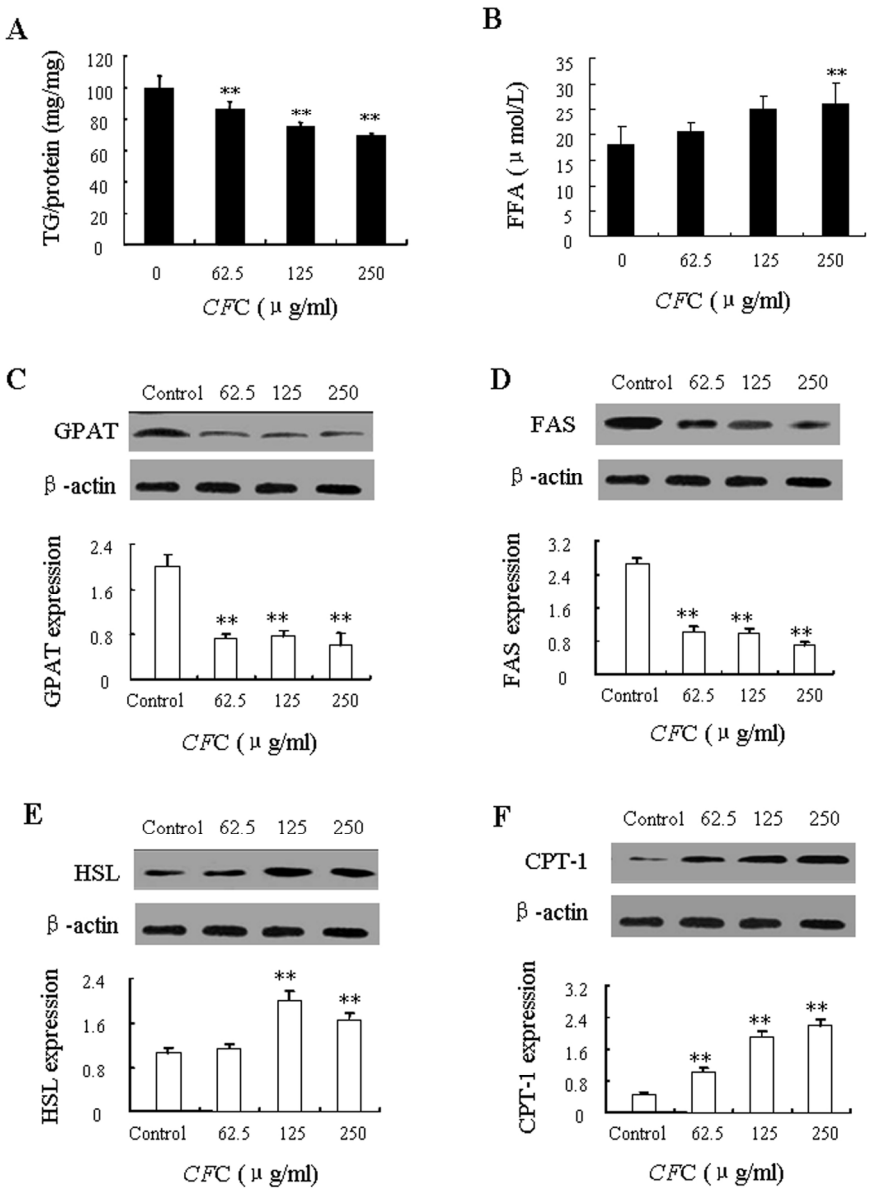

Fig. 4 Effect of CFC on the lipid metabolism in adipocytes. 3T3-L1 cells were induced to mature adipocytes and incubated with different concentrations of CFC for 24h. TG and FFA content were examined by the ELISA kits. Protein expression of GPAT, FAS, HAL and CPT-1 were measured by western blotting. β-Actin was used as an internal control. Data are represented as mean ± S.D. of three independent experiments. Multiple comparisons were done using one-way ANOVA analysis. **P < 0.01 versus control.
162x221mm (300 x 300 DPI)

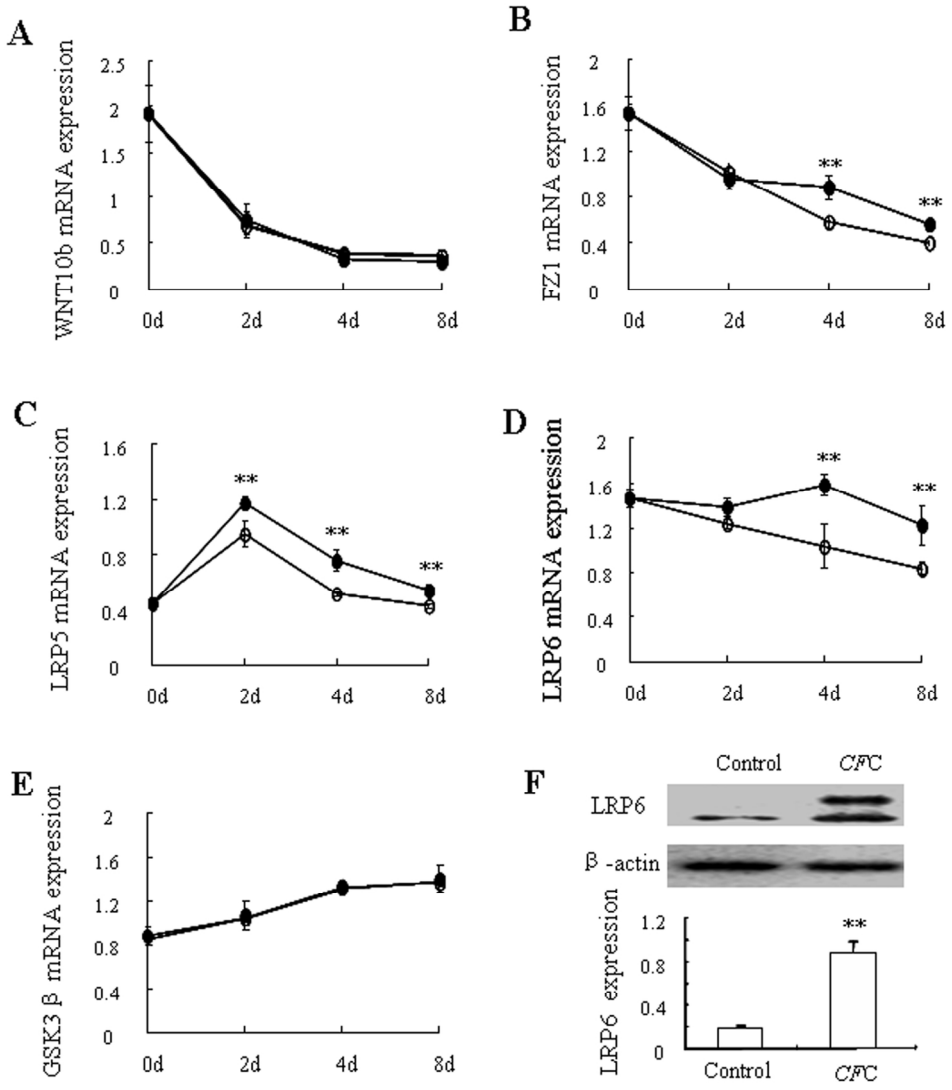

Fig. 5 Effects of CFC on the Wnt/β-catenin pathway. The mRNA expressions of Wnt10b, FZ1, LRP5, LRP6 and GSK3β were measured by RT-PCR and protein expression of LRP6 by western blotting. β-Actin was used as an internal control. Data are represented as mean ± S.D. of three independent experiments. Multiple comparisons were done using one-way ANOVA analysis. **P < 0.01 versus control.
132x146mm (300 x 300 DPI)

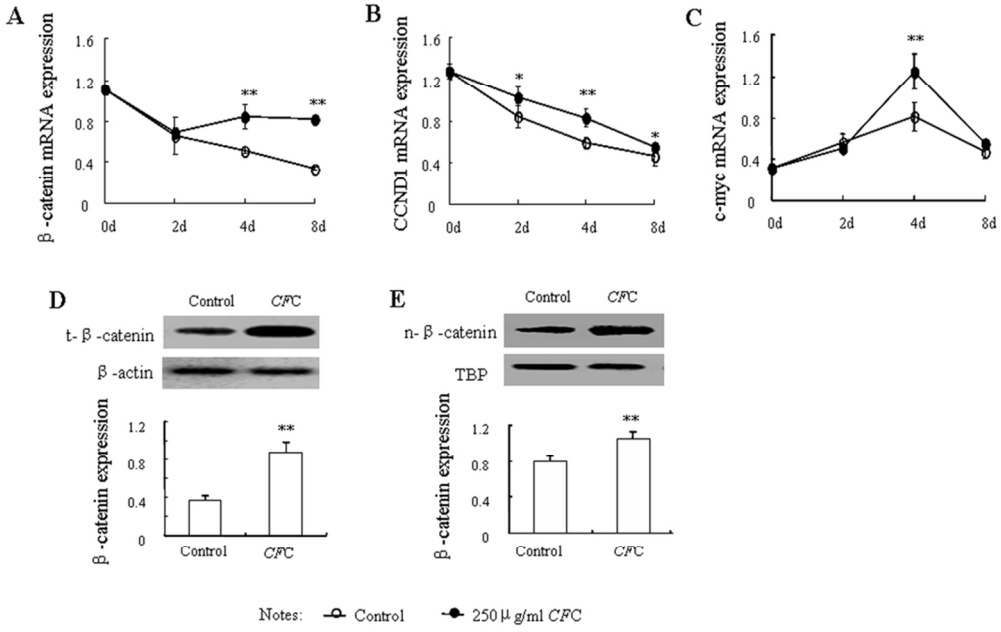

Fig. 6 Effects of CFC on β-catenin. The mRNA expressions of β-catenin, CCND1 and c-myc were measured by RT-PCR and protein expression of β-catenin by western blotting. β-Actin was used as an internal control for total protein test and TBP for nuclear protein test. Data are represented as mean ± S.D. of three independent experiments. Multiple comparisons were done using one-way ANOVA analysis. *P < 0.05 versus control; **P < 0.01 versus control.
90x58mm (300 x 300 DPI)

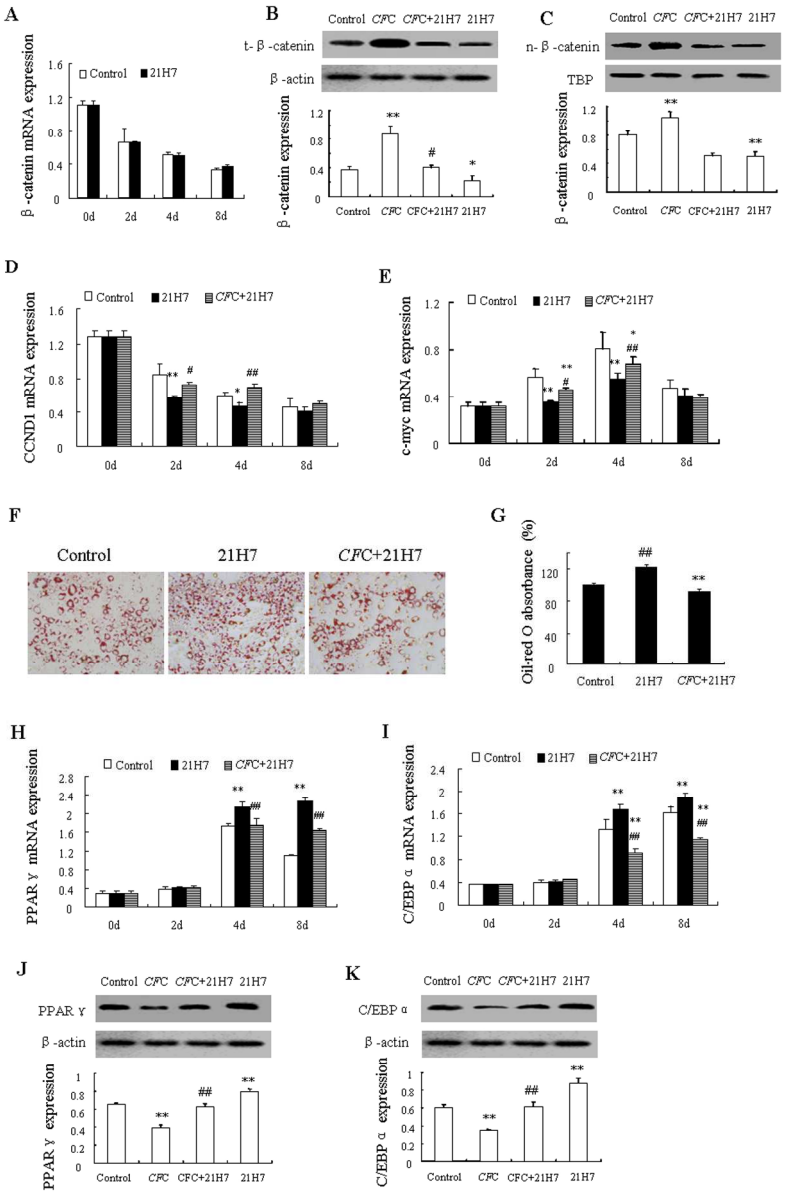

Fig. 7 Effects of CFC on adipogenesis under 21H7. 3T3-L1 cells were induced to mature adipocytes, incubated with 50μM 21H7 and stained with Oil-red O at day 8. The mRNA expressions of β-catenin, CCND1 and c-myc, C/EBPα and PPARγ were measured by RT-PCR and protein expression of β-catenin, C/EBPα and PPARγ by western blotting. β-Actin was used as an internal control for total protein test and TBP for nuclear protein test. Data are represented as mean ± S.D. of three independent experiments. Multiple comparisons were done using one-way ANOVA analysis. *P < 0.05 versus control; **P < 0.01 versus control; #P < 0.05 versus 21H7 group; ##P < 0.01 versus 21H7 group.
223x334mm (300 x 300 DPI)