Research Article

Pharmacological Properties of a Traditional Korean Formula Bojungchiseup-tang on 3T3-L1 Preadipocytes and High-Fat Diet-Induced Obesity Mouse Model

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The global obesity epidemic has nearly doubled since 1980, and this increasing prevalence is threatening public health. It has been reported that natural products could contain potential functional ingredients that may assist in preventing obesity. Bojungchiseub-tang (BJT), mentioned in the Donguibogam as an herbal medication for the treatment of edema, a symptom of obesity, consists of eleven medicinal herbs. However, the pharmacological activity of BJT has not been investigated. The present study was designed to investigate the putative effect of BJT on the adipogenesis of 3T3-L1 cells and the weight gain of high-fat diet (HFD-) fed C57BL/6 mice. Oil Red O staining was conducted to examine the amount of lipids in 3T3-L1 adipocytes. Male C57BL/6 mice were divided into three groups: standard diet group (control, CON), 45% HFD group (HFD), and HFD supplemented with 10% of BJT (BJT). The expression levels of genes and proteins related to adipogenesis in cells, WAT, and liver were analyzed by quantitative real-time polymerase chain reaction (qRT-PCR) and western blot, respectively. We found that BJT treatment significantly decreased the protein and mRNA levels of peroxisome proliferator-activated receptor γ (PPARγ), CCAAT/enhancer-binding protein α (C/EBPα), and sterol regulatory element-binding protein 1 (SREBP1) in a dose-dependent manner in differentiated 3T3-L1 cells.

Similar to the results of the in vitro experiment, BJT suppressed HFD-induced weight gain in an obese mouse model. In addition, BJT effectively reduced the HFD-induced epididymal adipose tissue weight/body weight index. BJT also downregulated the mRNA levels of PPARγ, C/EBPα, and SREBP1 in the epididymal adipose and liver tissue of HFD-fed obese mice. These findings suggest that BJT induces weight loss by affecting adipogenic transcription factors.

1. Introduction

Obesity has been increasing in epidemic proportions in the United States and most of the Westernized world [1]. Obesity leads to various metabolic diseases, such as hypertension, nonalcoholic fatty liver disease, atherosclerosis, type 2 diabetes, and cardiovascular diseases [2]. Obesity is mainly caused by a chronic imbalance between energy intake and expenditure, resulting in increased adipose tissue mass [3]. White adipose tissue is a multifactorial organ conducting intricate metabolic functions under physiological conditions; however, during obesity, it may become severely dysfunctional and fail to appropriately expand and store the surplus energy [4]. Fat mass is locally determined via the function of white adipocytes and the modulation of white adipogenesis, the process of preadipocyte differentiation into adipocytes [5]. Increased adipocyte size is associated with increased systemic insulin resistance, while small adipocytes are correlated with decreased metabolic health and diabetes [6]. Additionally, obesity directly contributes to an increase in hepatic triglyceride accumulation, which is related to the progression of nonalcoholic fatty liver disease [7]. A number of genes have
been shown to be involved in the development of obesity, including peroxisome proliferator-activated receptor γ (PPARγ), CCAAT/enhancer-binding protein α (C/EBPα), and sterol regulatory element-binding protein 1c (SREBP1c) [8]. SREBP1c stimulates the expression of PPARγ, C/EBPα, and several other lipogenic enzyme products [9].

Natural products such as herbs, fruits, and vegetables have recently been shown to have inhibitory effects on adipocyte lipid accumulation through the induction of apoptosis, cell cycle arrest, and transcription factors. Consequently, they could be potential functional ingredients in preventing obesity, with high expectations regarding their efficacy, safety, and long-term effects [10]. Bojungchiseub-tang (BJT) is mentioned in the Donguibogam as an herbal medication that has been used to treat signs of edema, dampness-phlegm, and kidney failure [11]. Individuals with a phlegm-dampness constitution have a much higher risk of obesity than those with a balanced constitution [12]: edema is also a common finding in obesity [13]. BJT consists of 11 medicinal herbs, all with proven effects on health. Panax ginseng C. A. Meyer and Atractyloides macrocephala Koidzumi are known to prevent obesity and dyslipidemia in high-fat diet (HFD-) fed castrated mice [14, 15]. Poria cocos Wolf exerted a protective effect against hepatic steatosis in HFD obese mice [16]. The compound MDG-1 from Ophiopogon japonicas shows potent hypoglycemic and weight control effects in mice [17]. Decursin, present in Angelica gigas Nakai, has an antidiogenic effect in adipose-derived stem cells isolated from human visceral adipose tissue [18]. Akebia quinata extract also has antiobesity and hypolipidemic effects in HFD-fed mice [19]. Scutellaria baicalensis has favorable effects on hyperglycemia, glucose tolerance, hyperinsulinemia, and hypertriglyceridermia in mice [20]. In the present study, we investigated the putative effect of BJT on the adipogenesis of 3T3-L1 cells, as well as on the weight gain of HFD-fed C57BL/6 mice.

2. Materials and Methods

2.1. Chemicals and Reagents. 3-Isobutyl-1-methylxanthine (IBMX), dexamethasone (DEX), insulin, and Oil Red O powder were purchased from Sigma-Aldrich Co. LLC (St. Louis, MO, USA). Dulbecco’s modified Eagle’s medium (DMEM), bovine serum (BS), fetal bovine serum, and antibiotic-antimycotic (ABAM) were obtained from Life Technologies, C/EBPα, SREBP1c, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) oligonucleotide primers were purchased from Bioneer Corporation (Daejeon, Republic of Korea), and SYBR Premix Ex Taq was purchased from Takara Bio Inc. (Otsu, Japan). Antibodies against PPARγ (cat. No. sc-7273), C/EBPα (cat. No. sc-9314), SREBP1 (cat. No. sc-13551), and β-actin (cat. No. sc-81178) were purchased from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA). Horseradish peroxidase-conjugated secondary antibodies were obtained from Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA, USA).

2.2. Preparation of BJT. BJT contained Panax ginseng C. A. Meyer (4 g), Atractyloides macrocephala Koidzumi (4 g), Atractyloides chinensis Koidzumi (2.8 g), Citrus unshiu Markovich (2.8 g), Poria cocos Wolf (2.8 g), Liriope platyphylla Wang et Tang (2.8 g), Akebia quinata Decaisne (2.8 g), Angelica gigas Nakai (2.8 g), Scutellaria baicalensis Georgii (2 g), Magnolia obovata Thunberg (1.2 g), and Cimicifuga heracleifolia Komarov (1.2 g). All 11 herbs were acquired from Nanum Pharmaceutical Company (Seoul, Republic of Korea). The extraction yield of herbs was performed as previously described [21]. Herbs were extracted in the water at 99°C for 3 h. The extract was then freeze-dried, and the yield was calculated at 14%. The powder was dissolved in distilled water for subsequent experimentation, and the residual powder was stored at -20°C.

2.3. Cell Culture of 3T3-L1 Preadipocytes. 3T3-L1 preadipocytes were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA; CL-173) and were cultured in DMEM containing 10% BS, 1% ABAM, 1 g/l HEPES, and 1.5 g/l sodium bicarbonate. The 3T3-L1 adipocyte differentiation was performed as previously described [22]. To stimulate adipocyte differentiation, cells were seeded at a density of 2 × 10^6 per well into 6-well plates to confluence (day 0). Then, cells were differentiated with an MDI medium containing 0.5 mM IBMX, 1 μM DEX, and 1 μg/ml insulin in the culture medium (day 2). The cells were also differentiated in a culture medium containing 1 μg/ml insulin (day 4). The culture medium was changed every 2 days, until days 6–8.

2.4. Cell Viability Assay. Cell viability was performed was previously described [22]. 3T3-L1 preadipocytes (1 × 10^4 cells per well) were seeded on 96-well plates at density 1 × 10^3 cells per well. After 24 h, cells were treated with different concentrations of BJT for 48 h. The 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyl tetrazoliumbromide (MTT) solution (5 mg/ml) was treated and the cells were incubated at 37°C for 4 h. After discarding the supernatant, 100 μl of dimethyl sulfoxide was added to dissolve formazan crystals, and the MTT-formazan product was measured using an Epoch® microvolume spectrophotometer (Bio Tek Instruments Inc., Winooski, VT, USA) at 570 nm.

2.5. Oil Red O Staining of 3T3-L1 Adipocytes. The Oil Red O staining in 3T3-L1 adipocytes was performed as previously described [22]. 3T3-L1 preadipocytes were cultured with or without differentiation conditions in the presence or absence of indicated concentrations of BJT. After 8 days of differentiation, cells were washed three times with phosphate-buffered saline (PBS) and fixed with 10% formaldehyde in PBS at 25°C for 1 h. After fixation, cells were washed three times with distilled water and then stained with Oil Red O working solution (3 mg/ml ORO in 60% isopropanol) at 25°C for 2 h. Cells were rinsed three times with distilled water and photographed with an Olympus SZX10 microscope (Tokyo, Japan). The Oil Red O dye was dissolved by isopropanol and measured with an Epoch® microvolume spectrophotometer at 520 nm.
To the manufacturer

Zoletil 50 (20 mg/kg) by intraperitoneal injection according
At the end of the experiment, mice were anesthetized with

weight and food intake were measured weekly for 11 weeks.
all mice had ad libitum access to food and water. Mouse body

3T3-L1 preadipocytes. The results showed that concentra-

MTT assay

3. Results

3.1. BJT Suppressed Adipogenesis in 3T3-L1 Cells. MTT assay

water was provided). Animals were euthanized by cervical
dislocation. Liver and adipose tissue were excised, rinsed with
PBS, immersed into liquid nitrogen, and stored at −80°C
until further experimentation. All experiments were con-
ducted with approval from the Ethical Committee for Ani-
care and the Use of Laboratory Animal of Sangji University (reg.no. 2018-21).

2.6. Western Blot Analysis. Western blot analysis was per-
formed as previously described [23]. The liver tissue and
3T3-L1 cells were homogenized with PRO-PREP™ protein
 extraction solution (Intron Biotechnology, Seoul, Republic
of Korea). Equal amounts (15–30 μg) of protein samples were
separated on a sodium dodecyl sulfate-polyacrylamide gel
and then transferred onto a polyvinylidene fluoride mem-
brane. Membranes were incubated overnight with primary
antibody PPARγ, C/EBPα, and SREBP1 and incubated
with horseradish peroxidase-conjugated secondary antibody
for 2 h. The blots were again washed three times with tris
buffered saline with tween 20 and then visualized by
enhanced chemiluminescence using Amersham™ Imager
680 (GE Healthcare Bio-Sciences AB, Sweden).

2.7. Quantitative Reverse-Transcription Polymerase Chain
Reaction (qRT-PCR) Analysis. The qRT-PCR analysis was
performed as previously described [23]. In brief, the liver,
epididymal adipose tissue, and 3T3-L1 cells were homoge-

2.10. Histological Examination. Histological analyses of the
liver and adipose tissue were performed as previously
described [24]. Liver and epididymal adipose tissue from rep-
resentative mice were fixed in 10% formalin, embedded into
the paraffin, and cut into 5 μm sections. The sections were
then used for hematoxylin/eosin staining. The stained liver
sections were observed for the examination of lipid droplets.
The stained adipose tissue sections were used to measure the
size of adipocytes. All observations were performed using an
Olympus SZX10 microscope.

2.11. Statistical Analysis. Each result is represented as the
mean ± standard deviation of triplicate experiments. Statisti-
cal analysis was performed using SPSS version 19.0 (Interna-
tional Business Machines, Armonk, NY, USA). Statistical
significance was determined using analysis of variance and
Dunnnett’s post hoc test. P values of less than 0.05 were con-
sidered statistically significant amongst the experimented
groups.

2.9. Biochemical Analysis. Serum biochemical analysis was
performed as previously described [24]. Blood was collected
and immediately centrifuged (1000 × g for 20 min at 4°C)
to obtain the plasma. The levels of triglycerides (TG), total
cholesterol (TC), aspartate aminotransferase (AST), alanine
aminotransferase (ALT), and blood urea nitrogen (BUN) in
the plasma were measured using commercial kits (Asan
Pharmaceutical. Co. Ltd., Republic of Korea). The concentra-
tion of creatinine in the plasma was determined using a com-
mercial kit (BioAssay Systems, Hayward, CA, USA). All
biochemical assay was conducted according to the manufac-
turer’s instructions.

8-week-old male C57BL/6N mice (specific-pathogen-free (SPF) grade, 18–20 mg) were
obtained from Daehan Biolink (Daejeon, Republic of Korea) and maintained at modified conditions (22 ± 2°C and 55 ± 9%
humidity, 12 h light/dark cycle). Prior to the initiation of
the experiment, the mice were acclimatized to their environ-
ment for 1 week. Thereafter, mice were weighed and divided
into three groups (n = 6 per group) as follows: standard diet
group (control, CON), 45% HFD group (HFD), and HFD
supplemented with 10% of BJT (BJT). The animals were sub-
jected to experimentation for 11 weeks, during which period
all mice had ad libitum access to food and water. Mouse body
weight and food intake were measured weekly for 11 weeks.
At the end of the experiment, mice were anesthetized with
Zoletil 50 (20 mg/kg) by intraperitoneal injection according
to the manufacturer’s instructions after 12 h of fasting (only

| Gene      | Forward (5'-3')       | Reverse (5'-3')       |
|-----------|-----------------------|-----------------------|
| PPARγ     | ATCGAGTGCCCGAGTCTGTGG | GCAAGGCGACTTCAGAAACCG |
| SREBP1    | ATCGCAAACAGCTGACCTG   | AGATCCAGTGTGAGTGAGG   |
| C/EBPα    | GGAACCTTGAAGCCAATCGATC| TGGTTAGCATAGACCTGACCA | CACACCGACCTCACCATT TT |
| GAPDH     | GACGGCGGACATCTTCTTGT  |                       |
Figure 1: Continued.
intracellular lipid accumulation in a dose-dependent manner relative to that in the nondifferentiated cells (Figures 1(b) and 1(c)). As expected, the protein expression of PPARγ, C/EBPα, and SREBP1 increased relative to that in the nondifferentiation group. However, the BJT treatment markedly attenuated these increases in differentiated 3T3-L1 cells (Figure 1(d)). Consistently, the mRNA levels of PPARγ, C/EBPα, and SREBP1 increased in the differentiated cells compared to that in the nondifferentiated cells. BJT treatment significantly decreased the levels of PPARγ, C/EBPα, and SREBP1 in 3T3-L1 cells in a dose-dependent manner (Figures 1(e), 1(f), and 1(g)).

3.2. BJT Suppressed HFD-Induced Body Weight Gain in C57BL/6N Mice. After 1 week of acclimatization, mice were randomly sorted into three groups, CON, HFD, and BJT. The body weight of the HFD group was significantly more than that of the CON group, while the BJT group had significantly lower body weight than that in the CON group (Figure 1(d)). Consistently, the mRNA levels of PPARγ, C/EBPα, and SREBP1 were signiﬁcantly lower than those of the HFD group, while these levels of the BJT group were signiﬁcantly lower than those of the HFD group (Figure 1(e)).

3.3. BJT Suppressed HFD-Induced Lipid Accumulation in Epididymal Adipose Tissue of C57BL/6N Mice. The HFD group showed a significant increase in epididymal adipose tissue weight relative to that in the CON group, while BJT markedly attenuated the increase seen in the HFD group (Figure 1(f)). Additionally, the adiposity in the HFD group was more prominent than that in the CON group, while the BJT treatment appeared to block this change relative to that in the HFD group (Figure 2(b)). The food intake was comparable in all three groups (Figure 2(d)). Furthermore, the serum TG and TC levels of the HFD group were significantly higher than those of the CON group, while these levels of the BJT group were significantly lower than those of the HFD group (Table 2).
HFD group was larger than that in the CON group, and BJT dramatically inhibited the HFD-induced enlargement of epididymal adipocytes (Figures 3(c) and 3(d)). Furthermore, the adipogenic transcription markers PPARγ, SREBP1, and C/EBPα were all dramatically increased by HFD in the epididymal adipose tissue of mice. Importantly, BJT treatment effectively reversed the increases observed in the HFD group (Figures 3(e)–3(g)).

3.4. BJT Suppressed HFD-Induced Lipid Accumulation in the Liver Tissue of C57BL/6N Mice. To determine the effects of BJT on morphological and histological changes of liver tissue, hematoxylin/eosin staining was performed. The liver tissue in the HFD group was of a lighter color than that in the CON group, whereas BJT treatment reversed this color change. Consistently, HFD increased lipid accumulation compared to that in the CON group in liver tissue, while BJT treatment notably reduced HFD-induced enlargement of lipid droplets in the liver of obese mice (Figure 4(a)). Both the protein and mRNA levels of the adipogenic transcription markers PPARγ, SREBP1, and C/EBPα significantly increased in the liver tissue of the HFD group. In contrast, the BJT treatment markedly suppressed these increases in the HFD group (Figures 4(b)–4(e)).
expanding fat mass. BJT treatment significantly reduced the values of hepatic AST and ALT; BUN: blood urea nitrogen.

### Table 2: Effects of BJT administration on serum biochemical parameters in HFD-induced mice.

| Parameters groups | TG (mg/dl) | TC (mg/dl) | ALT (U/l) | AST (U/l) | Creatinine (mg/dl) | BUN (mg/dl) |
|-------------------|------------|------------|-----------|-----------|-------------------|-------------|
| CON               | 125.83 ± 10.62 | 100.39 ± 8.82 | 31.30 ± 4.96 | 98.52 ± 19.44 | 0.20 ± 0.07 | 21.94 ± 4.04 |
| HFD               | 179.79 ± 49.46** | 182.14 ± 31.32*** | 30.53 ± 5.38 | 93.66 ± 17.49 | 0.19 ± 0.07 | 20.16 ± 0.94 |
| BJT               | 116.95 ± 16.10*** | 153.57 ± 8.75* | 31.68 ± 6.72 | 97.34 ± 17.62 | 0.18 ± 0.07 | 19.41 ± 2.31 |

The values are represented as mean ± S.D (n = 6). Abbreviations: TG: triglyceride; TC: total cholesterol; ALT: alanine aminotransferase; AST: aspartate aminotransferase; BUN: blood urea nitrogen.

### 4. Discussion

BJT is an herbal medicine to treat symptoms and signs of edema and is widely used in Korean oriental medicine practitioners. Its effect in reducing adipocyte differentiation and adiopogenesis has been demonstrated in experiments with 3T3-L1 cells [25]. BJT is composed of eleven herbal plants; among them, five herbs are known to have antiobesity effects. Particularly, ginseng, the main herb of BJT, has been traditionally used in the treatment of most ageing-related diseases including obesity [26]. Ginseng and its active compounds ginsenosides inhibit obesity in several obese animal models [26–31]. In addition, Atractylodes macrocephala Koidzumi, another main herb of BJT, prevents diet-induced obesity and glucose intolerance in mice and inhibits adiopogenesis in 3T3-L1 cells [15, 32]. Despite its pharmacological studies for the compositional herbs of BJT in treating obesity, the antiobesity effect of BJT has not yet been elucidated. Therefore, we hypothesized that BJT would be effective in treating obesity, and we sought to validate this through in vivo model as well as in vitro model.

The global obesity epidemic has nearly doubled since 1980, and this increasing prevalence is a threat to public health [33]. Weight gain is the result of an imbalance between total energy intake and total energy expenditure, and it is thought that a substantial and sustained increase of total energy intake over the past 30 years has led to the increase in body weight across the global population [34]. In this study, mice were fed HFD to induce weight gain through an increase in energy intake. BJT has long been used to treat obesity [33], but the pharmacological activity of BJT has not yet been investigated. In the present study, we found that BJT suppressed HFD-induced weight gain in an obese mouse model. However, there were no significant changes in hepatic AST and ALT levels, and kidney BUN and creatinine levels in all three groups (Table 2), indicating that BJT reduced body weight without inducing liver and kidney toxicity.

Obesity is a multifactorial disorder, characterized by expanding fat mass. BJT treatment significantly reduced the weight of adipose tissue in HFD-induced obesity. Additionally, we examined the effect of BJT on the differentiation of 3T3-L1 adipocytes. 3T3-L1 cells are fibroblast-like preadipocytes, which differentiate into adipocytes, and are commonly used to study adipogenesis [35]. Master transcriptional regulators of adipogenesis, including PPARγ, C/EBPα, and SREBP1c, are necessary modulators of target gene expression that are involved in adipocyte differentiation at various stages [36]. C/EBPs consist of six different proteins, C/EBPα, C/EBPβ, C/EBPγ, C/EBPδ, C/EBPε, and C/EBPζ, that are widely expressed in numerous tissues and regulate many cellular processes, including cell cycle, inflammation, differentiation, metabolism, and cellular proliferation [37, 38]. Among them, C/EBPα is expressed in numerous tissues, including in the liver, adipose tissue, skeletal muscle, colon, and prostate, and is mainly observed in terminally differentiated cells [37]. Moreover, C/EBPα is expressed during adipocyte differentiation in culture cells at later stages and stays active in mature adipocytes [39]. Herein, BJT decreased the C/EBPα levels in 3T3-L1 adipocytes, epididymal adipose tissue, and liver, thus indicating that BJT can induce fat loss by reducing the adipocyte differentiation in the epididymal adipose tissue and liver. C/EBPα acts in concert with PPARγ to establish phenotypes of mature adipocytes [40]. The members of the PPAR family of nuclear receptors have vital roles in lipid metabolism [41]. In particular, PPARγ plays a key role in adipogenesis and has been implicated in the pathology of numerous diseases including obesity, type 2 diabetes, and atherosclerosis [42]. Adipocyte-specific deletion of PPARγ results in the complete absence of white adipose tissue in mice [41]. Several transcription factors are known to promote adipocyte development, but none are as important as PPARγ; therefore, this nuclear receptor family member is considered the major regulator of adipogenesis [35]. BJT also downregulated the PPARγ levels in 3T3-L1 adipocytes, epididymal adipose tissue, and liver, suggesting that BJT can block adipocyte development in adipose and liver tissue through the inhibition of PPARγ. The SREBP family of transcription factors modulate cholesterol and fatty acid metabolism; the family comprises of three members, SREBP1a, SREBP1c, and SREBP2 [43]. SREBP1a and SREBP2 are critical for the regulation of target genes involved in cholesterol metabolism, while SREBP1c is related to lipid synthesis [44]. Furthermore, the activation of SREBP1 leads to fatty liver through the stimulation of transcription of the network encompassing, at least in part, the synthesis of fatty acids and triglycerides [45]. Given that the expression of PPARγ, C/EBPα, and SREBP1 are the major gene targets for the suppression of adiopogenesis in differentiated cells and tissues, the hematoxylin/eosin staining showed that BJT decreased both the enlargement of adipocyte in epididymal adipose tissue and lipid accumulation in liver tissue, which is thought to be correlated with the inhibition of these adiopgenic markers in epididymal adipose and liver tissues (Figure 5). Overall, the suppressive effects of BJT on weight gain may be mediated by the
Figure 3: Effect of BJT on the lipid accumulation in epididymal adipose tissue of HFD-induced obese mice. At the end of the experimental period, the weight of epididymal adipose tissue was measured (a) and then divided by the body weight of mice (b). The hematoxylin/eosin staining images are shown at the magnification 100x (c). The average diameter of adipocytes in epididymal adipose tissue of each group (d). qRT-PCR was performed to determine the mRNA level of PPARγ (e), C/EBPα (f), and SREBP1 (g). The values are represented as mean ± S.D. *P < 0.05 and ***P < 0.001 vs. CON; **P < 0.05, ***P < 0.01, and ****P < 0.001 vs. HFD group; significances were determined using one-way ANOVA followed by a Dunnett’s post hoc test.
Figure 4: Continued.
downregulation of the expression of adipogenesis-related genes, and the present research provides a partial explanation for the antiadipogenic properties of BJT.

5. Conclusions

In the present study, BJT induced weight loss and alleviated lipid accumulation in epididymal adipose and liver tissues by affecting the adipogenic transcription factors: PPARγ, C/EBPα, and SREBP1. Further studies are needed to fully elucidate the antiadipogenic properties of BJT.

Abbreviations

BJT: Bojungchiseub-tang
HFD: High-fat diet
PPARγ: Peroxisome proliferator-activated receptor γ
C/EBPα: CCAAT/enhancer-binding protein α
SREBP1: Sterol regulatory element-binding protein 1.

Data Availability

The datasets used and/or analyzed in this study are available from the corresponding authors upon reasonable request.

Conflicts of Interest

The authors declare no competing interests with regard to the publication of this paper.

Authors’ Contributions

YJP, DWS, and HJA conceived and designed the experiments. YJP and DWS acquired the data and performed the experiments and wrote the manuscript. TYG and DCC conducted the animal experiments. LH and DSL provided technical support. IH contributed reagents, materials, and analysis tools. HJA substantially contributed to the analysis and interpretation of data and revised the manuscript. All authors read and approved the final manuscript.

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