

Effects of a traditional Chinese medicine formula and its extraction on muscle fiber characteristics in finishing pigs, porcine cell proliferation and isoforms of myosin heavy chain gene expression in myocytes

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Objective: This study evaluated the effects of a traditional Chinese medicine formula (TCMF) on muscle fiber characteristics in finishing pigs and the effects of the formula's extract (distilled water, ethyl acetate and petroleum ether extraction) on porcine cell proliferation and isoforms of myosin heavy chain (*MyHC*) gene expression in myocytes.

Methods: In a completely randomized design, ninety pigs were assigned to three diets with five replications per treatment and six pigs per pen. The diets included the basal diet (control group), TCMF1 (basal diet+2.5 g/kg TCMF) and TCMF2 (basal diet+5 g/kg TCMF). The psoas major muscle was obtained from pigs at the end of the experiment. Muscle fiber characteristics in the psoas major muscle were analyzed using myosin ATPase staining. Cell proliferation was measured using 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) dye and cytometry. Isoforms of *MyHC* gene expression were detected by real-time quantitative polymerase chain reaction.

Results: The final body weight and carcass weight of finishing pigs were increased by TCMF1 ($p < 0.05$), while the psoas major muscle cross-sectional area was increased by TCMF ($p < 0.05$). The cross-sectional area and diameter of psoas major muscle fiber I, IIA, and IIB were increased by TCMF2 ($p < 0.05$). The cross-sectional area and fiber diameter of psoas major muscle fiber IIA and IIB were increased by diet supplementation with TCMF1 ($p < 0.05$). Psoas major muscle fiber IIA and IIB fiber density from the pigs fed the TCMF1 diet and the type IIB fiber density from the pigs fed the TCMF2 diet were lower compared to pigs fed the control diet ($p < 0.05$). Pigs fed TCMF2 had a higher composition of type I fiber and a lower percentage of type IIB fiber in the psoas major muscle ($p < 0.05$). The expression levels of MyHC I, MyHC IIa, and MyHC IIx mRNA increased and the amount of MyHC IIb mRNA decreased in the psoas major muscle from TCMF2, whereas MyHC I and MyHC IIx mRNA increased in the psoas major muscle from TCMF1 ($p < 0.05$). Peroxisome proliferator-activated receptor γ coactivator-1 α and CaN mRNA expression in the psoas major muscle were up-regulated by TCMF ($p < 0.05$). Porcine skeletal muscle satellite cell proliferation was promoted by 4 $\mu\text{g/mL}$ and 20 $\mu\text{g/mL}$ TCMF water extraction ($p < 0.05$). Both 1 $\mu\text{g/mL}$ and 5 $\mu\text{g/mL}$ of TCMF water extraction increased MyHC IIa, MyHC IIb, and MyHC IIx mRNA expression in porcine myocytes ($p < 0.05$), while MyHC I mRNA expression in porcine myocytes was decreased by 5 $\mu\text{g/mL}$ TCMF water extraction ($p < 0.05$). Porcine myocyte MyHC I and MyHC IIx mRNA expression were increased, and MyHC IIa and MyHC IIb mRNA expression were down-regulated by 5 $\mu\text{g/mL}$ TCMF ethyl acetate extraction ($p < 0.05$). MyHC I and MyHC IIa mRNA expression in porcine myocytes were increased, and the MyHC IIb mRNA expression was decreased by 1 $\mu\text{g/mL}$ TCMF ethyl acetate extraction ($p < 0.05$). Four isoforms of MyHC mRNA expression in porcine myocytes were reduced by 5 $\mu\text{g/mL}$ TCMF petroleum ether extraction ($p < 0.05$). MyHC IIa mRNA expression in porcine myocytes increased and MyHC IIb mRNA expression decreased by 1 $\mu\text{g/mL}$ in a TCMF petroleum ether extraction ($p < 0.05$).

Conclusion: These results indicated that TCMF amplified the psoas major muscle cross-sectional area through changing muscle fiber characteristics in finishing pigs. This effect was confirmed as TCMF extraction promoted porcine cell proliferation and affected isoforms of *MyHC* gene expression in myocytes.

Keywords: Muscle Fiber; Cell Proliferation; Traditional Chinese Medicine Formula; Pigs

INTRODUCTION

Muscle fibers are the basic units of muscular tissue. Muscle fiber characteristics include the number

of fibers, cross-sectional area, diameter, density and types of muscle fiber. In general, the smaller the muscle fiber diameter is, the smaller the fiber cross-sectional area is, and the larger the fiber density is [1]. According to the isoforms for myosin heavy chain (MyHC) structure, metabolic patterns and contraction speed, muscle fibers are classified into four categories: slow oxidation type (type I), fast oxidation type (type IIA), fast glycolysis type (type IIB) and intermediate type (type IIC) [2].

The number of postnatal myofibril and muscle fibers do not change, but the diameter, cross-sectional area, density, type and composition are not constant [3]. Many factors, such as breed, nutrition, age, hormone, and exercise, can affect muscle fiber diameter, type and its composition. Additionally, feed additives, such as antioxidants (e.g., ethoxyquin, butylated hydroxytoluene, butylated hydroxyanisole, tertiary butylhydroquinone, vitamins E and C), have been applied to improve the oxidative ability, meat quality and muscle fiber characteristics [4,5]. However, it is widely reported that using these chemical synthetic additives in animal feedstuff leads to cytotoxicity [6]. Recently, certain traditional Chinese medicines have been used as feed additives for livestock, and this practice has attracted attention from scientific and technical workers because of the functional performance of the bioactive components in the medicine with either less or no toxicity and residue [7,8]. *Radix Astragali*, *Radix Glycyrrhizae*, and *Fructus Ligustri Lucidi* have beneficial dietary effects on growth performance, immunity and antioxidant properties [9,10]. The traditional Chinese medicine formula (TCMF) that contains screened herbs at suitable doses and rationale according to the principles of an herbal combination and a long history of successful clinical practice [11] is expected to provide effects through its combined activities. However, researchers have not evaluated the effects of TCMFs on muscle fiber characteristics in pigs.

Muscle fiber types are translated from different isoforms of the *MyHC* gene [2]. Additionally, muscle fiber types are modulated by a network of multiple interconnected genes in signal transduction pathways. Peroxisome proliferator-activated receptor γ coactivator-1 α (PGC-1 α) is known to be a key transcriptional regulator of mitochondrial biogenesis, energy substrate utilization and oxidative capacity in skeletal muscle fiber type formation [12,13]. PGC-1 α is preferentially expressed in type I fiber enriched muscle [14]. Overexpression of PGC-1 α up-regulated genes related to mitochondrial oxidative metabolism (nuclear respiratory factor-2 [NRF-2] and cytochrome c oxidase subunit IV [COX IV]) and increased the redness of type II fiber enriched muscles [15]. A shift from muscle fiber type I and IIA toward IIB and IIC, as well as a reduction in endurance capacity, were observed in specific-skeletal muscle PGC-1 α knock-out mice [16]. Calcineurin (CaN), which is also known as calcium/calmodulin-dependent protein phosphatase 2B, co-precipitated with the nuclear factor of activated T cells (NFATc1), which is required for muscle fiber type remodeling [17]. Loss-of-function of endogenous NFAT isoforms using siRNA acting downstream of

CaN has been used to mediate slow muscle fibers in adult muscle [18]. The MyHC I expression increased and the expression of MyHC IIa and MyHC IIx decreased after constitutively activating CaN in the soleus [19]. Herb extract, exercises and reactive oxygen species promoted or initiated PGC-1 α expression [20-23]. CaN/NFAT was activated in response to mechanical stretching [24].

In our study, a diet for pigs supplemented with a TCMF was developed to investigate its effects on muscle fiber characteristics in the psoas major muscle. We also analyzed the expression level of the psoas major muscle isoforms of *MyHC* genes and key genes in the regulation pathways of muscle fibers. To further verify the combined properties of this formula, we divided this formula into three active ingredient sections, including distilled water, ethyl acetate and petroleum ether extraction (from polarity to non-polarity) according to solubility parameter. Next, we investigated the effects of a medicine extraction on porcine skeletal muscle satellite cells (SCs) proliferation and isoforms of *MyHC* gene expression levels in myocytes.

MATERIALS AND METHODS

Traditional Chinese medicine formula and its extraction

The TCMF powders were provided by Nong Zhi Dao Co., Ltd., Guangzhou, Guangdong, China. The formula contains 25% *Radix Astragali* (root), 20% *Radix Glycyrrhizae* (root), 15% *Cortex Eucommia* Leaf (bark and leaf), 10% *Rhizoma Atractylodis Macrocephalae* (rootstalk), 10% *Rhizoma Zingiberis* (rootstalk), 10% of *Fructus Crataegi* (fruit), 5% *Fructus Rosae Laevigatae* (fruit), 3% *Fructus Ligustri Lucidi* (fruit), and 2% *Pericarpium Citri Reticulatae* (pericarp). Effective ingredients of the TCMF are listed in Table 1. The herbs in the formula were screened through a 60 mesh sieve after drying and crushing. The TCMF was shown to be safe by an acute toxicity test using male Sprague-Dawley rats (Guangdong Medical Experimental Animal Center, Guangzhou, Guangdong, China) that were fed the diet (basal diet+100 g/kg TCMF, up to 40 times the dosage used in swine) with no deaths [25]. The formula was extracted using distilled water (Spring and Omni, Ewell, Guang-

Table 1. Effective ingredients of traditional Chinese medicine formula

Effective ingredients	Content (mg/kg)
Calycosin	216.20
Liquiritin	187.06
Eucommiol	181.66
6-Gingerol	164.03
Ursolic Acid	107.63
Atractylósíde	75.44
Glycyrrhízic acid	56.40
Chlorogenic acid	55.93
Salídrosíde	53.35
Nóbíletín	48.18
Specnuezheníde	23.97

zhou, Guangdong, China), ethyl acetate (Fuyu, Tianjin, China) and petroleum ether (Fuyu, Tianjin, China) at 60°C for 2 h with 3 repetitions, respectively, and then the filtrate was dried before it was used.

Ethics approval of animal experiments

The experimental protocol was undertaken according to procedural guidelines (SCAU-AEC-2010-0416) approved by the Institutional Animal Care and Use Committee (IACUC) of South China Agricultural University for the care and use of animals. The animals were reared and slaughtered strictly according to the Welfare and Ethics of Laboratory Animals Regulations (WELAR) established by the Chinese Association for Laboratory Animal Sciences (CAFLAS).

Experimental design, animals, diets

In a completely randomized design, ninety 146-day-old castrated Pietrain×Duroc×Landrace×Yorkshire (PDLY) finishing swine with an average initial body weight (BW) of 84.05±0.86 kg were divided into groups fed the following three dietary treatments: CON (the control group, basal diet), TCMF1 (basal diet+2.5 g/kg the TCMF) and TCMF2 (basal diet+5 g/kg the TCMF). There were five replications per treatment and six pigs per pen. Feed and water were supplied to the pigs *ad libitum* during the 35-day experiment. The experimental protocol was approved by the Animal Care and Use Committee of South China Agricultural University (ACUC) (certificate number: SCXK [Guangdong] 2013-0002). The experimental diets (Table 2) were formulated based on the NRC [26] requirement for all nutrients, and all calculated values are in line with NRC [26] tabular values.

Determination of muscle fiber characteristics

At the end of the experiment, one pig with a BW close to the average BW from each replicate was slaughtered using electrical stunning and was later exsanguinated through the jugular vein. The final BW, carcass weight and the psoas major muscle cross-sectional area were calculated. The psoas major muscle was resected from the left flesh carcass for experimental analysis. Muscle fiber characteristic analysis in the psoas major muscle was performed as described in the methods of Brook and Kaiser and Lind and Kernell [27,28]. Briefly, serial 10-µm thick cross-sections were prepared with a cryostat microtome (Leica CM 1850, Leica Microsystems Nussloch GmbH, Wetzlar, Hessen, Germany) and stained using myosin ATPase. The sections were pre-incubated at pH 4.7 and then incubated at pH 9.4. Sections of the psoas major muscle were photographed using a microscope (100×) (Motic BA310, Motic Medical Diagnostic System Co., Ltd, Xiamen, Fujian, China) and were measured using Motic Images Advanced 3.2 software (Motic Medical Diagnostic System Co., Ltd, China) for calculating muscle fiber characteristics. Muscle fibers were classified into types I, IIA, and IIB at the same location on the different samples. Approximately 600 fibers of each sample were

Table 2. Ingredients and nutrition levels of the experimental diets

Items	CON ¹⁾	TCMF1	TCMF2
Ingredients (%)			
Corn (8% CP)	69.64	69.79	70.14
Soybean meal (43% CP)	16.20	16.50	16.70
Wheat bran (15% CP)	5.90	5.20	4.40
Wheat flour (13% CP)	5.00	5.00	5.00
Limestone	0.82	0.82	0.82
Dicalcium phosphate	0.60	0.60	0.60
Phytase	0.60	0.60	0.60
TCMF powder	0	0.25	0.50
Choline chloride (50%)	0.10	0.10	0.10
Premix ²⁾	0.20	0.20	0.20
Salt	0.17	0.17	0.17
L-lysine HCL (70%)	0.54	0.54	0.54
L-threonine (98%)	0.13	0.13	0.13
DL-methionine (98%)	0.10	0.10	0.10
L-tryptophan (98%)	0.006	0.006	0.006
Nutrient level ³⁾			
Dry matter (%)	86.83	86.83	86.83
Digestible energy (MJ/kg)	13.73	13.73	13.73
Net energy (MJ/kg)	10.05	10.05	10.05
CP (%)	14.49	14.49	14.49
Calcium (%)	0.51	0.51	0.51
Total phosphate (%)	0.46	0.46	0.46
Non-phytate phosphorus (%)	0.23	0.23	0.23
Na (%)	0.17	0.17	0.17
Lys (%)	0.86	0.86	0.86
Met (%)	0.31	0.31	0.31
Met+Cys (%)	0.83	0.83	0.83
Thr (%)	0.56	0.56	0.56
Trp (%)	0.14	0.14	0.14

CP, crude protein.

¹⁾ TCMF, traditional Chinese medicine formula; CON, Control group, basal diet; TCMF1, basal diet+2.5 g/kg TCMF; TCMF2, basal diet+5 g/kg TCMF.

²⁾ Premix that provided the following vitamin and minerals per kg diet: vitamin A, 3,500 IU; vitamin D₃, 600 IU; vitamin E, 140 IU; vitamin K, 1.5 mg; vitamin B₁, 1 mg; vitamin B₂, 4.8 mg; vitamin B₆, 1.5 mg; pantothenic acid, 12 mg; niacin, 30.4 mg; biotin, 0.05 mg; folacin, 0.3 mg; vitamin B₁₂, 18 µg; Fe (ferrous sulfate heptahydrate, 20.09% Fe), 150 mg; Cu (copper sulfate pentahydrate, 25.45% Cu), 15.99 mg; Mn (manganese oxide, 77.45% Mn), 2.58 mg; Zn (zinc oxide, 80.34% Zn), 73.44 mg; Co (cobaltous sulfate monohydrate, 32% Co), 0.40 mg; Se (sodium selenite, 45.66% Se), 0.42 mg; and I (potassium iodate, 59.06% I), 0.40 mg.

³⁾ Calculated from NRC [26] tabular values.

calculated. Cross-sectional area and the diameter of muscle fibers were evaluated. Muscle fiber composition was calculated based on the relative area. Fiber density was calculated from the mean number of muscle fibers per mm².

Real-time quantitative polymerase chain reaction for genes

The total RNA was extracted from the psoas major muscle using the Total RNA Kit I reagent (Omega Bio-Tek, Norcross, GA, USA) based on the manufacturer's protocol. RNA purity was identified by determining the absorbance of the RNA at 260 and 280 nm [29]. Two micrograms were reverse transcribed to cDNA using

the OligdT18 primer and M-MuLV Reverse Transcriptase (Promega, Madison, WI, USA). An Applied Biosystems 7500 (Foster City, CA, USA) real-time quantitative polymerase chain reaction (real-time qPCR) system performed quantitative real-time qPCR using a SYBR Premix Ex Taq Kit (Promega, Madison, WI, USA). The method to select primer sequences for isoforms of the *MyHC* gene was based on the study by Peuker and Pette [30]. PCR primer sequences are shown in Table 3. Statistical analyses were displayed by the $2^{-\Delta\Delta Ct}$ method in which ΔCt is the difference between the target genes and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) [31].

Isolation, culture and identification procedures of the porcine skeletal satellite cells

The cells were isolated from the skeletal muscle from a large White Landrace-Duroc pig at 1 day of age according to protocols approved by the Institutional Animal Ethics Committee (IAEC) and the Animal Care and Use Committee (ACUC) of South China Agricultural University. Skeletal muscle was cut into tiny pieces and then digested with 0.2% collagenase Type II (Sigma, St. Louis, MO, USA) at 37°C for 2 h. Subsequently, the cells and suspension were filtered through 200- and 400-mesh sieves. After differential adhesion, skeletal muscle SCs were cultured in Dulbecco's modified Eagle's medium F-12 (DMEM/F-12) (containing 15% fetal bovine serum (FBS) [Gibco, New York, NY, USA], 100 U/mL penicillin [Gibco, USA], and 0.1 mg/mL streptomycin [Gibco, USA]) at 37°C and 5% CO₂ in a cell culture incubator (BB15, Thermo, Waltham, MA, USA). To identify the porcine SCs, we used the Desmin immunohistochemistry method. The cells were washed with 5% bovine serum albumin (BSA) blocking buffer (SA1021, Boster, Wuhan, China) and then incubated with mouse anti-Desmin (MS376, Thermo Scientific, Waltham, MA, USA) and the secondary antibodies of goat anti-mouse immunoglobulin G (IgG) (SA1021, Boster, Wuhan, China) at 37°C for 20 min.

Next, streptavidin biotin complex (SA1021, Boster, Wuhan, China) was added to incubate the cells. The skeletal SCs were stained with diaminobenzidine (002114, Thermo Scientific, USA) solution.

Cell proliferation and the traditional Chinese medicine formula extraction treatment in myocytes

Cell proliferation was analyzed using 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) dye (M2128, Sigma-Aldrich, St. Louis, MO, USA) and cytometry [32,33]. The absorbance of MTT stained cells at 490 nm against the appropriate blank was determined using a spectrophotometer (imark680, Bio-Rad, Hercules, CA, USA). To investigate the effects of a water extraction of TCMF on the proliferation of porcine skeletal muscle SCs, 0, 0.0064, 0.32, 0.16, 0.8, 4, and 20 µg/mL of water extraction were added to the cell culture for 1 d, 3 d, and 5 d. After 80% confluence was reached, porcine SCs were induced to differentiation in myocytes by 2% horse serum (Gibco, USA). After 4 days of induced differentiation, the myocytes were subjected to three kinds of TCMF extraction (water, ethyl acetate and petroleum ether extraction) with a level of 0, 1, and 5 µg/mL with DMEM/F-12 (Gibco, USA) supplemented in 10% FBS (Gibco, USA), respectively. After 48 h of cultivation, the myocytes were collected for muscle fiber type related isoforms of *MyHC* gene expression analysis.

Statistical analysis

The data were analyzed using a One-way analysis of variance procedure in SPSS 19.0 (SPSS Inc.233 South Wacker Drive, Chicago, IL, USA). A general linear model analyzed the interaction effects between the time and dose of water extraction of the TCMF on SCs proliferation. Less than 0.05 p value was considered to be a significant difference between the means of the groups. The results were presented as the means and standard error of the mean.

Table 3. Primers for gene RT-qPCR

GenBank accession No.	Genes	Primer sequence (5'-3')	Annealing temperature (°C)
NM_001206359.1	<i>GAPDH</i>	F: GAAGGTCGTCGGAGTGAACGGAT R: CATGGGTAGAATCATACTGGAACA	58
NM_213855.1	<i>MyHC I</i>	F: GAGAAGGGCAAAGGCAAGG R: ACGAAGTGGGGATGTGTGG	63
NM_214136.1	<i>MyHC IIa</i>	F: GCACCGTGGACTACAACATT R: CCAGGGGTTTTCTGTCTCA	60
NM_00123141.1	<i>MyHC IIb</i>	F: GTCACCGTCAACCCCTACAAGT R: CGGCGATTGTGGCAAAGTA	61
NM_001104951.1	<i>MyHC IIx</i>	F: GCACCGTGGACTACAACATT R: CGCCAGTGAAGAGGAAAGC	56
NM_213963.2	<i>PGC-1α</i>	F: GCAGAAGAGCCGTCTACTTAAGA R: CGATTGAGGGCATTATCAGAACA	58
NM_001318324.1	<i>CaN</i>	F: GGATGTTCTTGCCTCTGATGGT R: GGCATACCCCAAAGAGGTGT	58

RT-qPCR, real-time quantitative polymerase chain reaction; *GAPDH*, glyceraldehyde-3-phosphate dehydrogenase; *MyHC*, myosin heavy chain; *PGC-1α*, peroxisome proliferator-activated receptor γ coactivator-1 α ; *CaN*, calcineurin.

RESULTS

Muscle fiber characteristics in the psoas major muscle

The final BW and carcass weight of finishing pigs were increased by TCMF1 ($p < 0.05$), whereas the psoas major muscle cross-sectional area was increased by the TCMF added to the diet ($p < 0.05$) (Figure 1A, B, C). TCMF2 increased the final BW of finishing pigs ($p = 0.08$) (Figure 1A). The cross-sectional area and fiber diameter of type I, IIA, and IIB in the psoas major muscle were increased by diet supplementation with TCMF2 ($p < 0.05$) (Figure 2A, 2B), and the cross-sectional area and fiber diameter of type IIA and IIB in the psoas major muscle were increased by diet supplementation with TCMF1 ($p < 0.05$) (Figure 2A, 2B). Type IIA and IIB fiber density in the psoas major muscle from the pigs fed the TCMF1 diet, and type IIB fiber density in the psoas major muscle from the pigs fed TCMF2 diet were lower compared to pigs fed the control diet ($p < 0.05$) (Figure 2C). Pigs that consumed diet supplementation with TCMF2 had a higher percentage of muscle fiber type I and a lower ratio of muscle fiber type IIB in the psoas major muscle ($p < 0.05$), compared to the control group (Figure 2D). Muscle fiber typing by enzyme histochemical staining of the psoas major muscle from the CON, TCMF1, and TCMF2 groups (100 \times) are shown in Figure 2E to 2I, respectively.

Expression level of isoforms of the myosin heavy chain

gene and the key genes in the regulation pathways of muscle fibers in the psoas major muscle

The psoas major muscle MyHC I mRNA and MyHC IIx mRNA expression level from TCMF1, as well as the MyHC I mRNA, MyHC IIa mRNA, and MyHC IIx mRNA expression level from TCMF2, increased compared to the control group ($p < 0.05$) (Figure 3A). MyHC IIb mRNA expression level in the psoas major muscle was decreased by diet supplementation with TCMF2 ($p < 0.05$) (Figure 3A). PGC-1 α mRNA and CaN mRNA expression levels in the psoas major muscle were increased by diet supplementation with TCMF ($p < 0.05$) (Figure 3B).

Culture and identification procedures of porcine satellite cells

To obtain high-purity porcine skeletal muscle SCs, we used a differential adhesion method to separate fibroblasts and SCs after 2 h of inoculation (Figure 4A). SCs began to adhere at 24 h, to proliferate at 48 h and then to fuse at 120 h (Figure 4B to 4D). Compared to the fibroblasts, SCs were stained yellowish-brown using the Desmin immunocytochemistry method (Figure 4E, 4F). These results indicated that the isolated SCs could be used for the next experiments. To further determine the cellular activity, a cell proliferation curve was performed in our study. Both MTT (3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide) and cell counting methods showed the logarithmic phase lasted from 2 d to 6 d after cell inoculation. The results suggested

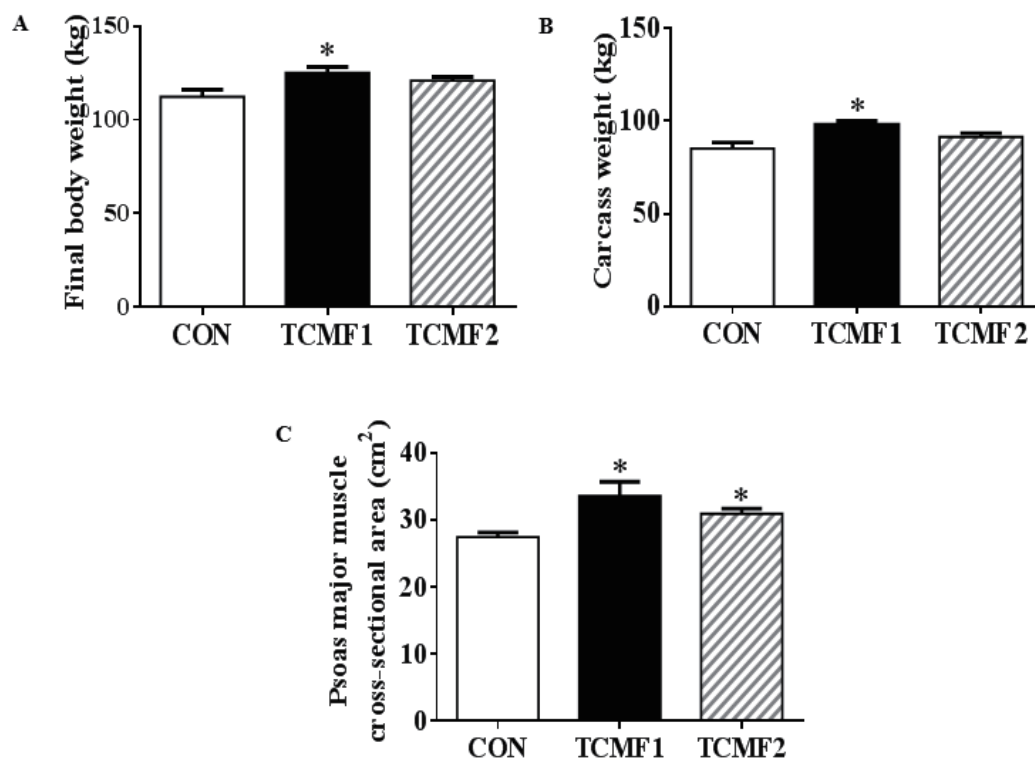


Figure 1. Effects of the traditional Chinese medicine formula (TCMF) on finishing pig carcass traits. (A) Effects of TCMF on the final body weight of finishing pigs. (B) Effects of TCMF on the carcass weight of finishing pigs. (C) Effects of TCMF on the psoas major muscle cross-sectional area of finishing pigs. CON, Control group, basal diet; TCMF1, basal diet+2.5 g/kg TCMF; TCMF2, basal diet+5 g/kg TCMF. Compared to CON, * represents $p < 0.05$. $n = 5$.

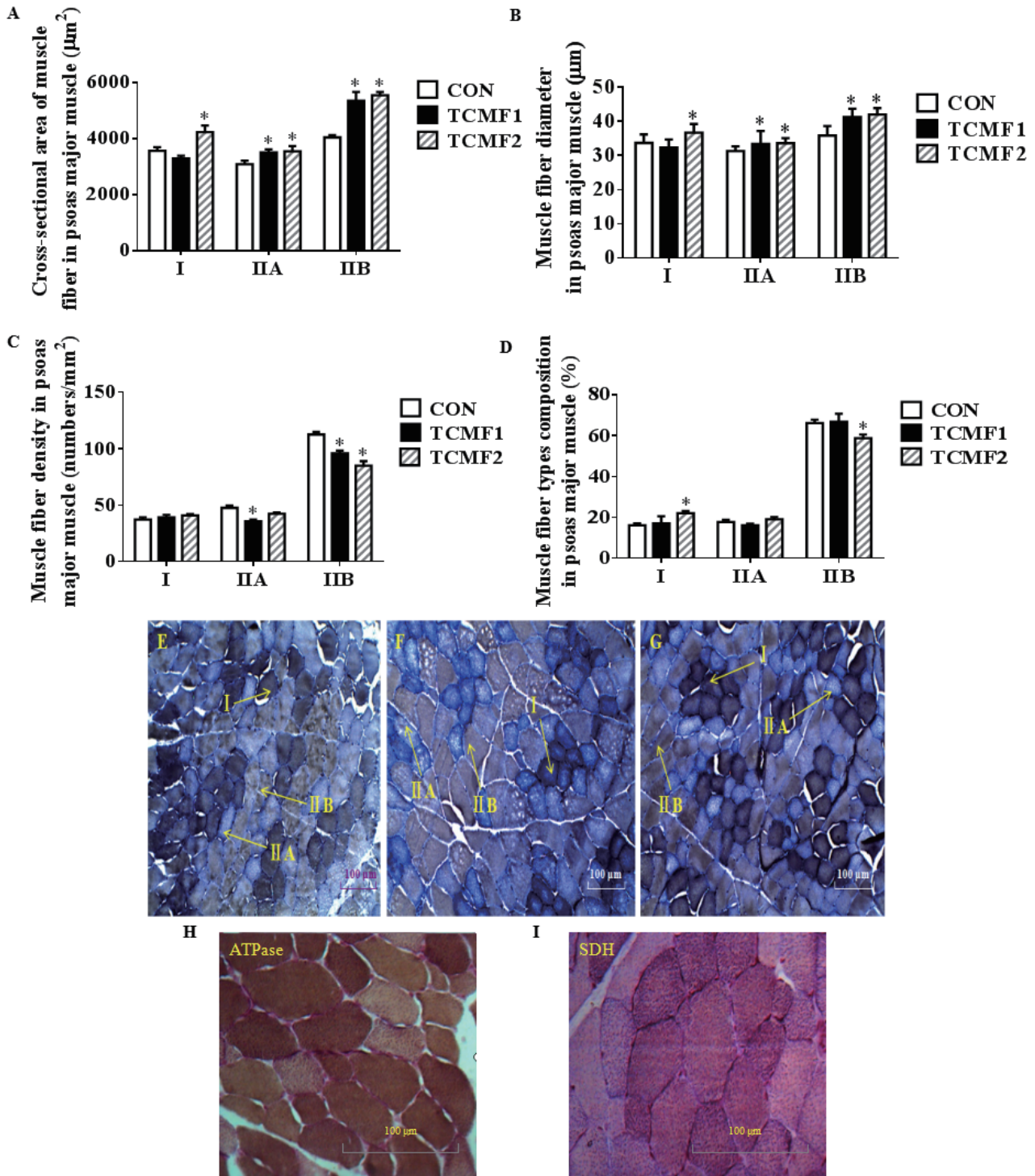


Figure 2. Effects of the traditional Chinese medicine formula (TCMF) on muscle fiber type characteristics in the psoas major muscle in finishing pigs. (A) Effects of TCMF on the cross-sectional area of the muscle fiber in the psoas major muscle of finishing pigs. (B) Effects of TCMF on muscle fiber diameter in the psoas major muscle of finishing pigs. (C) Effects of TCMF on muscle fiber density in the psoas major muscle of finishing pigs. (D) Effects of TCMF on muscle fiber types in the psoas major muscle of finishing pigs. (E, F, G) Enzyme histochemical staining of muscle fiber types in the psoas major muscle from pigs fed CON, TCMF1, and TCMF2 (100 \times), respectively. (H, I) Separate sections stained for mATPase and SDH, respectively. CON, control group, basal diet; TCMF1, basal diet+2.5 g/kg TCMF; TCMF2, basal diet+5 g/kg TCMF. Compared to CON, * represents $p < 0.05$. $n = 5$. Bar = 100 μm .

that the isolated SCs had high activity and could be used to induce cell differentiation (Figure 5A, 5B). SCs were gradually differen-

tiated from parallel arrangement of myocytes after 24 h to 96 h induction of 2% horse serum (Figure 6A to 6D). Therefore, we

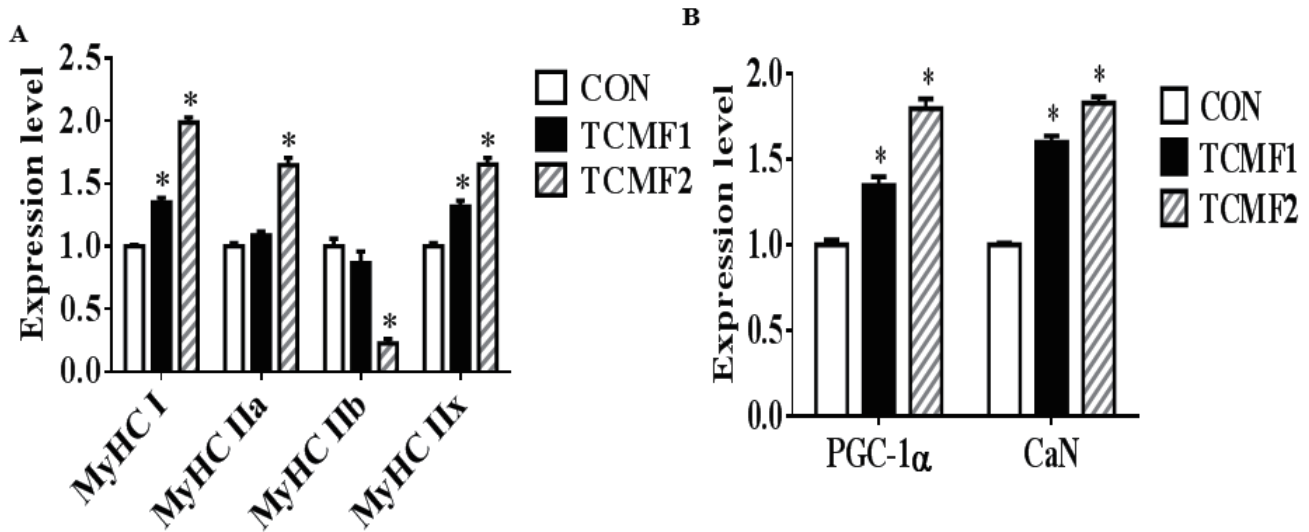


Figure 3. Effects of traditional Chinese medicine formula (TCMF) on gene expression levels in the psoas major muscle in finishing pigs. (A) Effects of TCMF on the four isoforms of the myosin heavy chain (*MyHC*) gene expression level in the psoas major muscle in finishing pigs. (B) Effects of TCMF on expression levels of key genes (*PGC-1α* and *CaN*) in the regulation pathways of muscle fiber type in the psoas major muscle of finishing pigs. CON, control group, basal diet; TCMF1, basal diet+2.5 g/kg TCMF; TCMF2, basal diet+5 g/kg TCMF. Compared to CON, * represents $p < 0.05$. $n = 5$. *PGC-1α*, peroxisome proliferator-activated receptor γ coactivator-1 α ; *CaN*, calcineurin.

treated the myocytes with the TCMF extractions after 96 h of induction with 2% horse serum.

Cell proliferation and the traditional Chinese medicine formula extraction treatment in myocytes

With the increase in time (d 1 to d 5), porcine skeletal muscle

SCs proliferation was increased by treating cells with 0, 4, and 20 $\mu\text{g/mL}$ water extraction of TCMF ($p < 0.05$) (Figure 7A). The SCs proliferation was repressed by treating cells with 0.0064, 0.032, 0.16, and 0.8 $\mu\text{g/mL}$ water extraction of the TCMF ($p < 0.05$) (Figure 7A). Among these cells, 20 $\mu\text{g/mL}$ water extraction of the TCMF had the best effect on SCs proliferation (Figure 7A). With

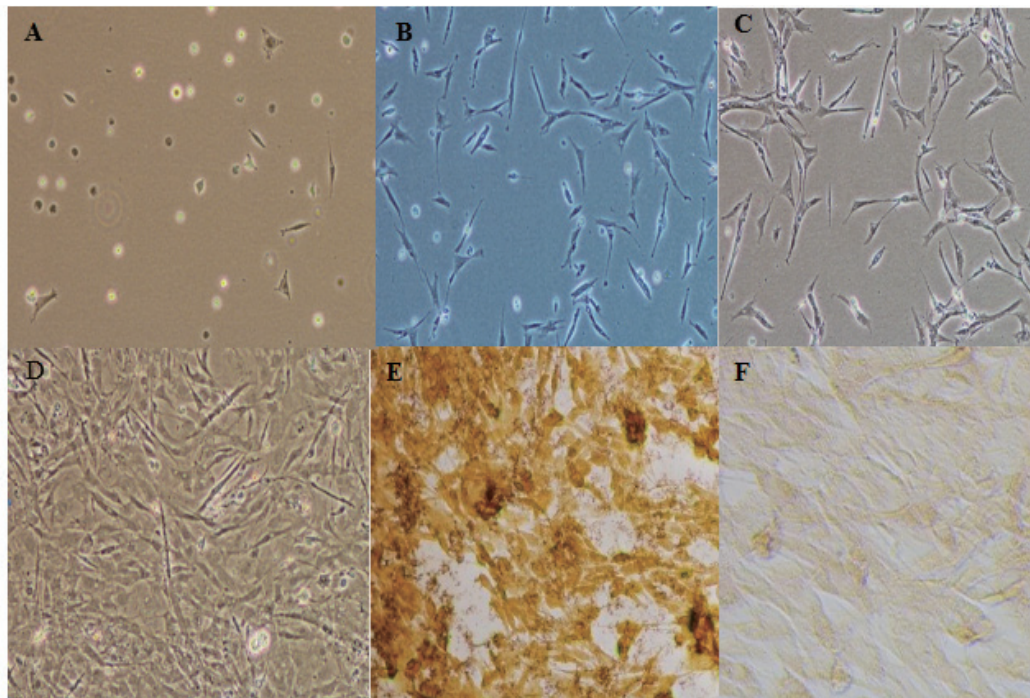


Figure 4. Morphology and identification of porcine skeletal muscle satellite cells (SCs) (100 \times). (A) Morphology of SCs after incubation for 2 h. (B) Morphology of SCs after incubation for 24 h. (C) Morphology of SCs after incubation for 48 h. (D) Morphology of SCs after incubation for 120 h. (E) Identification of SCs using Desmin immunocytochemistry. (F) Identification of fibroblasts with Desmin immunocytochemistry. Fibroblasts were used as a negative control for the identification of SCs.

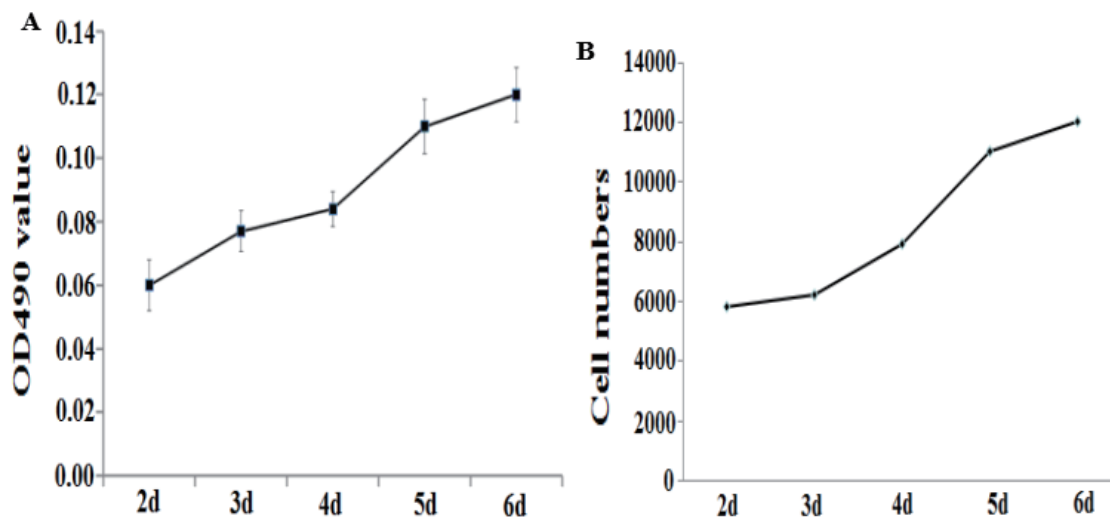


Figure 5. Porcine skeletal muscle satellite cells (SCs) proliferation curve from day 2 to day 6. (A) Porcine SCs proliferation curve was determined by the MTT method. $n = 6$. (B) Porcine SCs proliferation curve was measured by cell counting.

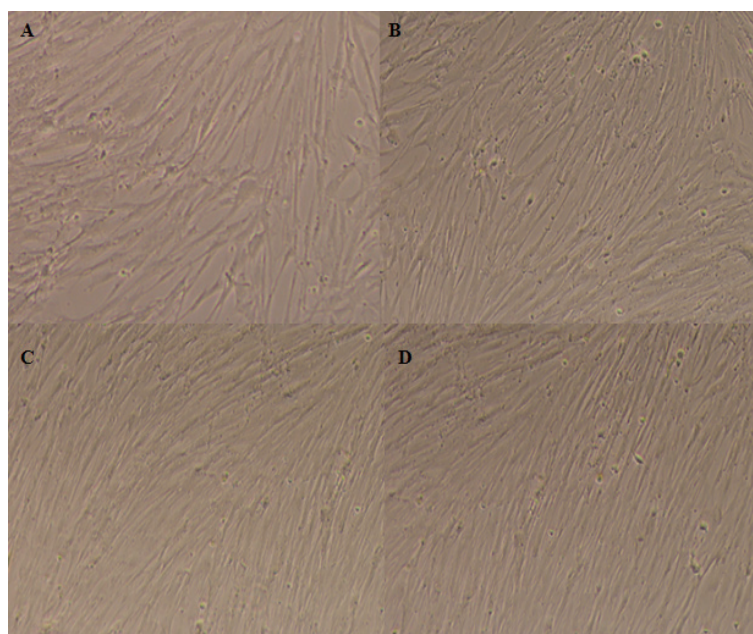


Figure 6. Induction of porcine skeletal muscle satellite cells (SCs) (100 \times). (A) Morphology of SCs after 2% horse serum induction for 24 h. (B) Morphology of SCs after 2% horse serum induction for 48 h. (C) Morphology of SCs after 2% horse serum induction for 72 h. (D) Morphology of SCs after 2% horse serum induction for 96 h.

the increase in the concentration of the water extraction of the TCMF, SCs proliferation went down initially at 0 $\mu\text{g}/\text{mL}$ and then up at 0.8 $\mu\text{g}/\text{mL}$ on days 1, 3, and 5 (Figure 7B). There was no interaction effect between time and dose for the TCMF water extraction on SCs proliferation (Table 4).

MyHC I expression level in porcine myocytes was increased by 1 and 5 $\mu\text{g}/\text{mL}$ ethyl acetate extraction of the TCMF ($p < 0.05$), whereas the MyHC I expression level in myocytes was decreased by the 5 $\mu\text{g}/\text{mL}$ water extraction and 5 $\mu\text{g}/\text{mL}$ petroleum ether extraction of the TCMF ($p < 0.05$) (Figure 8A). The MyHC I

expression level in myocytes was increased by treating cells with 1 and 5 $\mu\text{g}/\text{mL}$ water extraction, 1 $\mu\text{g}/\text{mL}$ ethyl acetate extraction and 1 $\mu\text{g}/\text{mL}$ petroleum ether extraction of the TCMF ($p < 0.05$). However, the MyHC IIa expression level in myocytes was decreased by treating cells with the 5 $\mu\text{g}/\text{mL}$ ethyl acetate extraction and 5 $\mu\text{g}/\text{mL}$ petroleum ether extraction of the TCMF ($p < 0.05$) (Figure 8B). The MyHC IIb expression level in myocytes was increased by 1 and 5 $\mu\text{g}/\text{mL}$ water extraction of the TCMF ($p < 0.05$), while the MyHC IIb expression level in myocytes was decreased by 1 and 5 $\mu\text{g}/\text{mL}$ of ethyl acetate extraction and 1 and

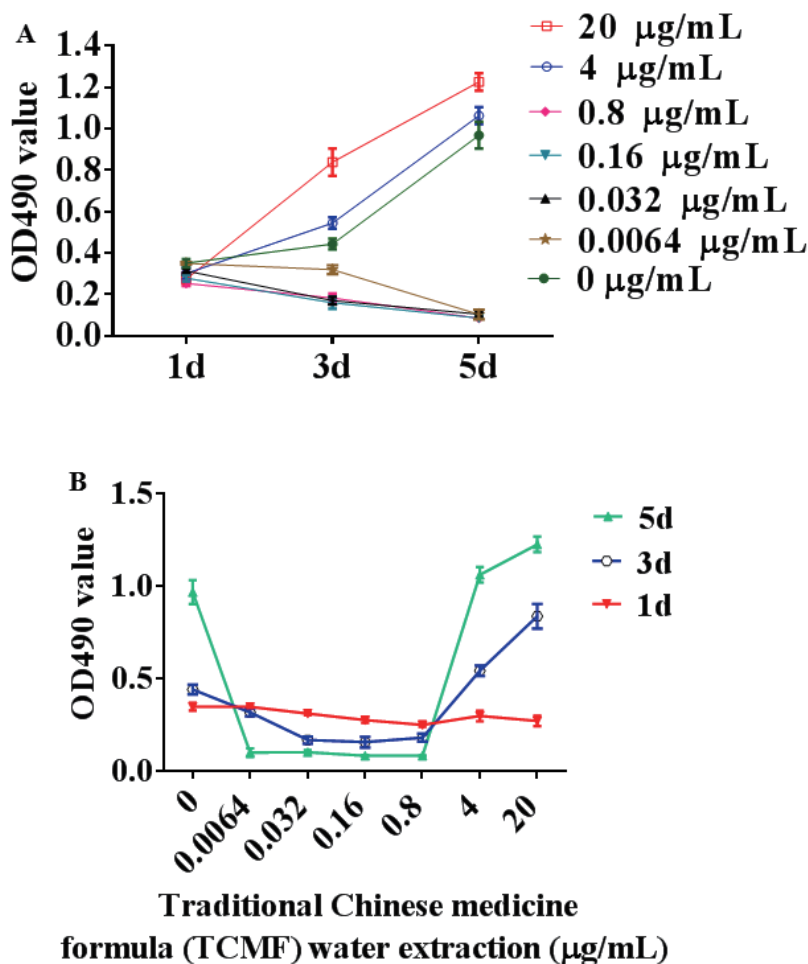


Figure 7. Effects of traditional Chinese medicine formula (TCMF) water extraction on porcine skeletal muscle satellite cells (SCs) proliferation. (A) Effects of different levels (0, 0.0064, 0.032, 0.16, 0.8, 4, and 20 µg/mL) of TCMF water extraction on porcine SCs proliferation from day 1 to day 5. n = 6. (B) Comparison of the effects of different levels of TCMF water extraction on porcine SCs proliferation on the same day.

5 µg/mL of petroleum ether extraction of the TCMF ($p < 0.05$) (Figure 8C). MyHC IIX expression level in myocytes was increased by treating cells with 1 and 5 µg/mL water extraction and 5 µg/mL ethyl acetate extraction of the TCMF ($p < 0.05$), but MyHC IIX expression in myocyte levels was decreased by treating cells with a

5 µg/mL petroleum ether extraction of the TCMF ($p < 0.05$) (Figure 8D).

Table 4. Interaction effects between the dose of the traditional Chinese medicine formula water extraction and its treatment time on skeletal muscle satellite cells (SCs) proliferation

Items	N	p-value ¹⁾
Dose ²⁾	7	0.83
Time ³⁾	3	0.49
Dose × time	21	0.23

¹⁾ $p < 0.05$ means that there was an interaction effect between the dose of the TCMF water extraction and its treatment time on skeletal muscle satellite cells (SCs) proliferation.

²⁾ 0; 0.0064; 0.032; 0.16; 0.8; 4; 20 µg/mL traditional Chinese medicine formula (TCMF) water extraction.

³⁾ SCs were treated with different doses of TCMF water extraction for 1, 3, or 5 days.

DISCUSSION

In our study, the final BW and carcass weight were increased by TCMF1, while the psoas major muscle cross-sectional area was increased by TCMF. Additionally, TCMF2 increased the final BW of pigs. The results indicated that diet supplementation with TCMF2 increased the cross section area and fiber diameter of type I, IIA, and IIB in psoas major muscle, and TCMF1 increased the cross-sectional area and fiber diameter of type IIA and IIB in the psoas major muscle. Pigs fed TCMF1 had lower type IIA and IIB fiber density in the psoas major muscle, and pigs fed TCMF2 had lower type IIB fiber density in the psoas major muscle compared to pigs fed the control diet. The TCMF consists of calycosin, liquiritin, eucommiol, 6-gingerol, ursolic acid,

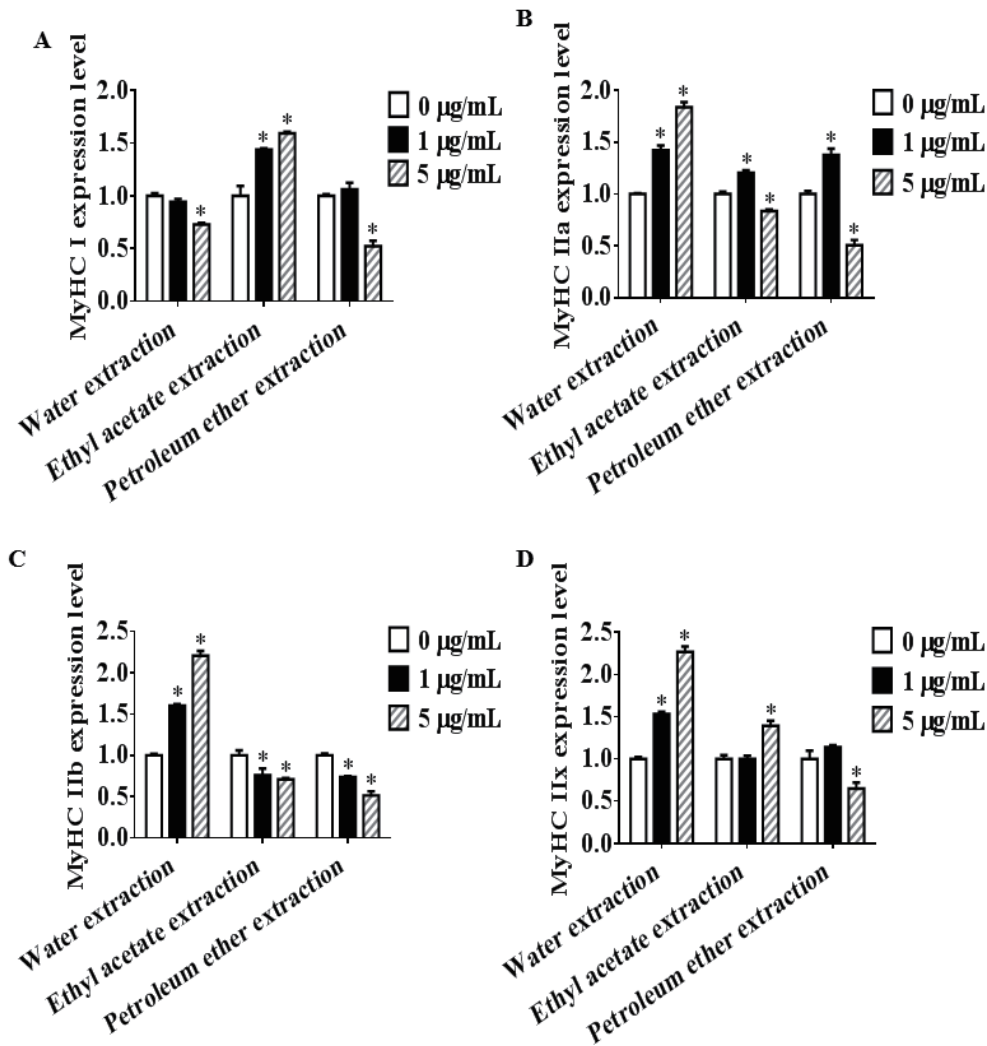


Figure 8. Effects of different levels of traditional Chinese medicine formula (TCMF) extractions (water extraction, ethyl acetate extraction and petroleum ether extraction) on four isoforms of the myosin heavy chain (*MyHC*) gene expression in porcine myocytes. (A) Effects of different levels (0, 1, and 5 µg/mL) of TCMF extractions (water extraction, ethyl acetate extraction, and petroleum ether extraction) on the MyHC I expression level. (B) Effects of different levels (0, 1, and 5 µg/mL) of TCMF extractions (water extraction, ethyl acetate extraction and petroleum ether extraction) on MyHC IIa expression level. (C) Effects of different level (0, 1, and 5 µg/mL) of TCMF extractions (water extraction, ethyl acetate extraction and petroleum ether extraction) on the MyHC IIb expression level. (D) Effects of different levels (0, 1, and 5 µg/mL) of TCMF extractions (water extraction, ethyl acetate extraction and petroleum ether extraction) on the MyHC IIx expression level. Compared to 0 µg/mL, * represents $p < 0.05$. $n = 6$.

atractyloside, glycyrrhizic acid, chlorogenic acid, salidroside, nobiletin, and specnuezhenide effective ingredients. It was reported that ginger extract containing 6-gingerol increased the number of type I muscle fibers [34]. Ursolic acid has been reported to encourage glycolytic muscle fibers to transform to IIA and slow muscle fibers [35]. There has been no research about the effects of calycosin, liquiritin, eucommiol, atractyloside, glycyrrhizic acid, chlorogenic acid, salidroside, nobiletin, and specnuezhenide on muscle fiber characteristics until now. PGC-1 α , which is a coactivator of various nuclear receptors and other transcription factors, has been reported to up-regulate the expression of genes associated with slow muscle fibers, mitochondrial function and fatty acid oxidation [36,37]. Zhang et al [38] observed that pigs with a higher PGC-1 α mRNA expression level showed a greater

expression level of MyHC I, MyHC IIa, and MyHC IIx mRNA, as well as a lower expression level of MyHC IIb mRNA. CaN acts as a modulatory agent in the regulation of MyHC I, MyHC IIa, and MyHC IIx transcription [39]. Active CaN specifically elevated MyHC I [40] and MyHC IIa mRNA expression, as well as repressing MyHC IIb and MyHC IIx mRNA expression [41]. The up-regulation of PGC-1 α and CaN mRNA expression in the psoas major muscle could be attributed to the increase in MyHC I and MyHC IIa mRNA expression levels, as well as the higher composition of muscle fiber I in the psoas major muscle were induced by TCMF2 in the present study. The different compositions of muscle fiber types in the psoas major muscle were caused by the differential expression of the isoforms of the *MyHC* gene family, which encodes proteins for the four types of muscle fibers [42].

The decreased percentage in type IIB fibers in the psoas major muscle was directly related to the comprehensive effectiveness of the decline of the MyHC IIB mRNA expression level and the increase in the MyHC IIX mRNA expression level. Furthermore, the decline in the proportion of the type IIB fiber indicated that some fibers of type IIB were transformed to type IIA and some fibers of type IIA were converted to type I. This muscle fiber type transformation was consistent with a regular pattern as follows: $I \leftrightarrow IIA \leftrightarrow IIX \leftrightarrow IIB$ [43]. Therefore, our results suggested that the muscle fiber characteristics in the psoas major muscle can be altered by dietary supplementation with TCMF.

To ensure that the cells we obtained from porcine muscle were skeletal muscle SCs, we cultivated the cells from a low density stage to high density stage for 5 d. Morphological characteristics of the isolated porcine cells were confirmed to identify SCs using the methods of Li et al [44]. The isolated cells were further verified by Desmin antibodies, which have been putatively shown to identify SCs derived from distinct species [45]. Furthermore, an SCs proliferation curve assessment showed the raw cell growth was characterized by an 'S' shaped [46] curve and the cells showed high proliferative capacity. Taken together, these results showed that highly pure SCs can be used as a cell model for the next experiment. As shown in the experiment, porcine myocytes were identified by proliferation and differentiation within 5 days with a nearly 80% differentiation fusion index manifested in an abundance of discrete MyHC [47]. We treated the myocytes with the TCMF extractions after 96 h of induction with 2% horse serum.

In our study, the high dosage of the water extraction of the TCMF promoted SCs proliferation. In contrast, the low concentration of this water extraction suppressed SCs proliferation. These results indicated that the TCMF water extraction had dosage-dependent effects on SCs proliferation. Among them, 20 $\mu\text{g}/\text{mL}$ water extraction of this formula exerted the best promotion effects on SCs proliferation. With the increase in the concentration of water extraction, SCs proliferation increased at first and decreased subsequently. Our results indicated that there was no interaction effect of time and dose on SCs proliferation. Although little was known of the effects of TCMF extraction on cells until now, licorice (one component of the TCMF) extracts have been reported to inhibit cultured hepatic carcinoma cell (Hep-3B) proliferation [48]. In addition, *Astragali Radix*, another ingredient of the TCME, was effective at promoting human fibroblast (Hs27) proliferation [49]. The dose used to investigate the effects of the TCMF extractions on isoforms of *MyHC* gene expression in myocytes was based on our previous studies and the research of Paduch et al [50]. The water extraction and ethyl acetate extraction of the TCMF mainly elicited positive effects on the four isoforms of the *MyHC* gene in porcine myocytes in our experimental dose, except that 5 $\mu\text{g}/\text{mL}$ water extraction of the TCMF had a negative effect on MyHC I expression level and 5 $\mu\text{g}/\text{mL}$ ethyl acetate extraction of the TCMF had a negative effect on the MyHC IIA expression level. Additionally, the 1 $\mu\text{g}/\text{mL}$ and 5 $\mu\text{g}/\text{mL}$ ethyl

acetate extraction of the TCMF had negative effects on the MyHC IIB expression level. The petroleum ether extraction primarily had negative effects on the four isoforms of the *MyHC* gene in myocytes in our experimental dose, except that 1 $\mu\text{g}/\text{mL}$ petroleum ether extraction of the TCMF had a positive effect on the MyHC IIA expression level. However, there were no significant effects of 1 $\mu\text{g}/\text{mL}$ TCMF water extraction and 1 $\mu\text{g}/\text{mL}$ TCMF petroleum ether extraction on MyHC I expression in porcine myocytes. The MyHC IIX expression was not significantly affected by 1 $\mu\text{g}/\text{mL}$ TCMF ethyl acetate extraction and 1 $\mu\text{g}/\text{mL}$ TCMF petroleum ether extraction in myocytes. A fundamental principle of the TCMF is combined therapy, which maximizes the pros and minimizes the cons of different substances [51]. The TCMF has been considered to be an empirical system of multiple components with antagonistic effects and synergistic properties in biological multilayer networks [11]. The interaction effects of the three extractions on the four isoforms of the *MyHC* gene in myocytes reflected the TCMF effects on the muscle fiber type in the psoas major muscle. Therefore, our results suggested that overall TCMF performed the integrated and combined effects on muscle fiber characteristics in porcine myocytes.

CONCLUSION

The enlarged psoas major muscle cross-sectional area in finishing pigs from diet supplementation with a TCMF led to an increase in cross-sectional area and the diameter of muscle fibers IIA and IIB. Including 5 g/kg TCMF in the diets of pigs elevated the composition and isoforms of *MyHC* gene expression for slow muscle fibers by up-regulating the expression of PGC-1 α and CaN. *In vitro*, the proliferation of porcine skeletal muscle SCs was promoted by 4 $\mu\text{g}/\text{mL}$ and 20 $\mu\text{g}/\text{mL}$ TCMF water extraction. The TCMF extraction affected the *MyHC* gene expression in porcine myocytes, including up-regulation of MyHC IIA, MyHC IIB, and MyHC IIX by TCMF water extraction, up-regulation of MyHC I and down-regulation of MyHC IIB by TCMF ethyl acetate extraction as well as repression of MyHC IIB with a TCMF petroleum ether extraction.

AUTHOR CONTRIBUTIONS

QPY, DYF, and JJZ conceived the concept and design. QPY and XJH conducted muscle fiber characteristic assays in this study. XJH and YHL performed cell culture, cell identification and cell proliferation. MHX and TD carried out the real-time quantitative polymerase chain reaction experiment and statistical analysis. QPY drafted the manuscript and created Table 1 to 4. FW and QPY generated Figure 1 to 8. HZT, SGZ, TZ, and XHO participated in design and coordination of the study. JJZ revised the manuscript. All authors read and approved the final manuscript.

CONFLICT OF INTEREST

We certify that there is no conflict of interest with any financial organization regarding the material discussed in the manuscript.

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