***Astragalus* polysaccharides decrease muscle wasting through Akt/mTOR, ubiquitin proteasome and autophagy signalling in 5/6 nephrectomised rats**

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Abbreviations: AA, amino acid; Akt, thymoma viral proto-oncogene; APS, *Astragalus* polysaccharides; Atg, Autophagy associated gene; CKD, chronic kidney disease; CRP, C-reaction protein, ELISA, enzyme-linked immunosorbent assay; ERSD, end-stage renal disease; GC, gas chromatography; HPGFC, high performance gel filtration chromatography; IL, interleukin; LNAA, especial large neutral amino acid; MAFbx, Muscle atrophy F-box; MDA, malondialdehyde; MDC, monodansylcadaverine; mTOR, mammalian target of rapamycin; MuRF1, muscle ring finger 1; PCR, polymerase chain reaction; PEW, protein energy wasting; siRNA, small interfering RNA; SOD, superoxide dismutase; TA, Tibias anterior;

TNF, tumor necrosis factor; UPS, ubiquitin-proteasome dependent signaling.

Abstract:

Ethnopharmacological relevance: Existing evidences suggest that *Radix Astragali* and its polysaccharides composition (APS) can improve muscle mass, but the mechanisms need more research.

Aim of the study: In this study, we aimed to examine the effects of APS on muscle wasting at molecular level in 5/6 nephrectomised rats.

Materials and methods: We performed 5/6 nephrectomy or sham operation in 160 6-week-old Sprague–Dawley rats, and feed animals with or without 2% APS for 155 days. After treatment, we compared the change of weight, muscle fiber, protein metabolism, pro-inflammatory factors (TNF- α , IL-15, CRP) and oxidative factors (MDA, SOD) among each group. In addition, we detected the Akt/mTOR, ubiquitin proteasome, autophagy signaling and AA transporters *in vivo* and *in vitro*.

Results: Data *in vivo* show 2% APS could alleviate weight loss and improve protein metabolism in nephrectomised rats. The levels of serum pro-inflammatory factors and oxidative factors were restored by APS treatment. In molecular levels, APS restored Akt/mTOR, MAFbx, MuRF1, Atg7, LC3B-II/LC3B-I and SLC38A2 which changed in nephrectomised rats. Data *in vitro* show the optimal dose of APS is 0.2 mg/mL, and SLC38A2 siRNA attenuated the effects of 0.2 mg/mL APS on atrophy and autophagy.

Conclusions: Our results suggested APS could improve muscle wasting through Akt/mTOR, ubiquitin proteasome and autophagy signaling, and SLC38A2 may be one of potential targets.

1. Introduction

Muscle wasting always occurs in patients with chronic kidney disease (CKD), particularly those with end-stage renal disease (ERSD) (Wang and Mitch, 2014; Workeneh and Mitch, 2010). Mortality is independently related to loss of muscle mass in CKD patients (Rajan and Mitch, 2008). CKD-related muscle wasting causes malnutrition due to low protein intake and leads to protein energy wasting (PEW) (Buscher

et al., 2010; Stenvinkel et al., 2004), which has numerous causes, including persistent inflammation, acidosis, multiple endocrine disorders and dialysis (Wang and Mitch, 2014). This, simply altering the diet cannot fully correct the excessive morbidity and mortality in CKD patients (Stenvinkel et al., 2004). Due to the complex etiology, there is still no optimal treatment for muscle wasting in CKD. The imbalance between the anabolic and catabolic rates is responsible for the occurrence and progression of PEW. In CKD progression, protein catabolism is induced by proteolytic pathways, such as liposomal, ubiquitin-proteasome dependent signalling (UPS) and autophagy (de Palma et al., 2008; Galluzzi et al., 2014; Sala et al., 2014). The low anabolic rate is commonly attributed to attenuated mTOR signalling (Gordon et al., 2013; Wang et al., 2009). These aspects of protein metabolic signalling may be regarded as potential therapeutic targets for CKD-related muscle wasting.

Radix Astragali is the root of *Astragalus membranaceus* (Fisch.) Bunge, which is a popular medicinal and food plant in China, where it is known as Huang-qi. According to records of Chinese materia medica, Huang-qi was used to tonify Qi, and it has been used as therapy for “Wei Zheng”, a term for skeletal muscle fatigue and wasting (Gao et al., 2005; Zhou and Mei, 2014). The effect of *Radix Astragali* on muscle fatigue has been proven by a series of studies (Kuo et al., 2009; Yeh et al., 2014; Zhang et al., 2015a), but its medical use for treating muscle wasting still lacks experimental evidence. A previous study showed that *Radix Astragali* improves the protein levels of muscle in nephrotic patients (Li et al., 1995). However, the active ingredients and mechanisms remain unclear. The major bioactive constituents of *Radix Astragali* are astragloside, triterpene glycosides, polysaccharides, flavonoid, saponin, amino acid, alkaloid, organic acid, microelement etc (Liu et al., 2015b; Movafeghi et al., 2010). Previous studies reported some benefit effects of polysaccharides (APS), saponins (ASS), and flavonoids (ASF) fractions on muscle disorders such as insulin resistance and fatigue (Zhang et al., 2015b). APS are the polysaccharide components of the water extracted from *Astragalus* roots. They are made from the components rhamnose, arabinose and glucose (Fu et al., 2013). Previous studies have reported that APSs have beneficial bioactivities on some disorders,

including inflammation, oxidative stress and insulin resistance (Huang et al., 2013; Liu et al., 2015a; Liu et al., 2010; Liu et al., 2014; Mao et al., 2009), which are closely associated with the CKD-PEW process. Furthermore, our recent studies have shown that the polysaccharides in the water extract from *Astragalus* roots can reduce muscle atrophy *in vitro* by restoring the phosphorylation of mTOR and by suppressing myostatin (Liu et al., 2013; Lu et al., 2013a). Given this information, we speculate that APSs may be beneficial for patients with CKD-related muscle wasting.

In this study, we aimed to determine the effect of water-soluble polysaccharides (from *Astragalus mongholicus*) on muscle protein metabolism in a rat renal failure model. We also explored possible mechanisms for these effects. This research may help in the search for effective treatments for CKD-related muscle wasting.

2. Materials and methods

2.1. Animals

Five-week-old male Sprague–Dawley rats (obtained from the Experimental Animal Center of Southern Medical University, China, certification no. SCXK (Yue) 2011-0015) weighing 145-155 g were housed at a constant temperature with a 12/12-h light-dark cycle and less than 60 dB of noise. The rats were kept according to the guidelines for Care and Use of Laboratory Animals formulated by the Ministry of Science and Technology of China, and all experimental procedures were approved by the Ethics Committee of Southern Medical University. Animals were allowed to adapt to their surroundings for 1 week with normal feeding based on semi-purified diet AIN 93-G before starting the experiments. Male rats were randomly assigned to either the 5/6 nephrectomised group or the sham-operated group. Each animal in the nephrectomised group underwent a 5/6 nephrectomy, consisting of ablation of two thirds of the mass of the left kidneys, followed by right unilateral nephrectomy after 1 week. In the sham-operated rats, a sham operation was performed according to previous methods (Wang et al., 2014c).

2.2. APS preparation

Astragalus membranaceus (Fisch) Bunge was purchased from the Guangzhou Qingping Medicine Market (Guangzhou, China) and identified by the Department of Authentication of Chinese Medicine, Chinese Traditional Medicine (Shanghai, China) by comparison to the specimens deposited under the voucher number SHCTM-817.

APSs were extracted and purified with optimized techniques using direct water decoction according to previous methods (Mu et al., 2009). The APSs were purified using the Sevage method and column chromatography with a Sephadex G-200. The polysaccharide content was 95.25%, as determined by the phenol–sulphuric acid method. The weight average molecular mass of the APSs was measured by high-performance gel filtration chromatography (HPGFC); the results are shown in Supplementary Figure 1. The individual monosaccharides from the polysaccharide fractions were identified by gas chromatography (GC); the results are shown in Supplementary Figure 2 and Table 1.

2.3. Diet preparation and experimental design

Two dietary treatments were used in this study. The control diet was amended based on an AIN-93G diet. The control diet was fed to the sham-operated group (control group) and the 5/6 nephrectomised group (model group). The treatment diet consisted of the control diet with 2% APS substituted for maize starch, and this diet was fed to 40 of the 5/6 nephrectomised rats (M-APS group) and 40 of the sham-operated rats (C-APS group). To guarantee the comparability of each group, we ensured that all diets contained similar protein contents. The gross energy of each diet was greater than 4.4 kcal/g. The ratio of caloric density in the APS diets to other diets was kept to at least 98% by adjusting the carbohydrate-fat ratio of the AIN-93G diet. The ingredients and chemical composition of the diets are listed in Table 1. All rats were allowed to drink tap water ad libitum. All diets were prepared by the Guangdong Provincial Medical Laboratory Animal Center (Foshan, China). The duration of the study was 155 days. Food intake was determined daily. We define weight loss as an end event if weight gain did not reach 20% of the average weight gain of the control group for two consecutive weeks. (This standard was based on a receiver operating characteristic curve

comparing weight loss and muscle pathology in a pre-experiment). Body weights, weight loss events and death were recorded weekly.

2.4. Myofibre cross-sectional area measurements and gradient screening of APSs

Myofibre cross-sectional area and C2C12 fibre size measurements were performed according previous experiments(Lu et al., 2013c; Wang et al., 2014a). Tibias anterior (TA) muscle samples were fixed in paraformaldehyde and embedded in paraffin. The muscle samples were sectioned and stained with H&E according to previously described methods. Muscle fibre cross-sectional area was measured in a “blinded” fashion on coded pictures; the investigator analysing the image was unaware of the group. In each muscle, six sections of fifty contiguous myofibres were demarcated so that the averages of 200 fibres were obtained for fibre area measurement in each group. Using the Image Pro-Plus software (Media Cybernetics, Bethesda, MD), the borders were delineated with a calibrated pen by circling each fibre. Each fibre was further traced with a handheld mouse to measure the area in pixels and mm².

To investigate the effect of extract on C2C12 myotubes, fibre size was also determined by staining the cells with H&E and treating with TNF- α (80 ng/mL) and/or a concentration gradient of APSs (0.05, 0.1, 0.2, 0.5, 1 or 2 mg/mL) in C2C12 myoblasts/myotubes. Then, C2C12 myoblasts/myotubes were incubated in an optimum concentration of APSs for different amounts of time (8, 12, 24 or 48 h) to determine the optimum incubation time. We selected fibres containing two or more nuclei to measure the short axis through the nucleus in each group. Three hundred myotubes were used for fibre area measurement in each group.

2.5. Dry weight and protein synthesis/degradation

TA muscle samples were dried in incubator at 65°C for at least 24 h until the weight remained stable. Then, the weight of every sample was measured. Protein synthesis/degradation rates were measured using our previous protocol (Wang et al., 2014c). Protein synthesis was measured *in vitro* in the soleus muscle using the incorporation of [¹⁴C] phenylalanine as described previously. [¹⁴C] Phenylalanine (0.05 mCi/ml) was obtained from the China Institute of Atomic Energy (Beijing, China). Protein synthesis was calculated

by dividing the protein-bound radioactivity by the specific activity of free phenyl-alanine in the incubation medium, and it is expressed as pmol phenylalanine incorporated/mg protein per 2 h. Protein degradation was measured using tyrosine release from the isolated muscle. The strips of soleus muscle were pre-incubated for 30 min in Krebs Ringer buffer (1.2 mmol/L NaCl, 4.8 mmol/L KCl, 25 mmol/L NaHCO₃, 2.5 mmol/L CaCl₂, 1.2 mmol/L KH₂PO₄ and 1.2 mmol/L MgSO₄; pH 7.4), supplemented with glucose (5.5 mmol/L), bovine serum albumin (1.0 g/L), insulin (5 U/mL) and cyclohexamide (5 mmol/L), saturated with a gas mixture of 95% O₂/5% CO₂. The strips were then transferred into fresh medium with the same composition and incubated for 2 h. At the end of the incubation, samples of the incubation medium were used to measure tyrosine based on the procedure of Waalkes and Udenfriend (Galban et al., 2001).

2.6. Measurement of pro-inflammatory and oxidative factors

As pro-inflammatory and oxidative factors can cause muscle wasting directly by enhancing catabolism, we detected representative selections that reported in previous studies (Argiles et al., 2009b; Roth et al., 2006). Blood samples were collected at weeks 3, 7, 14 and 23 by tail incision and were stored at -80°C. Serum CRP, serum interleukin-15 (IL-15) and serum TNF- α levels were measured using enzyme-linked immunosorbent assay (ELISA) kits. CRP levels were determined using an ELISA kit (Bogoo Inc., Shanghai, China) with a detection limit of 0.5 ng/mL. IL-15 levels were determined using an ELISA kit (Bogoo Inc., Shanghai, China) with a detection limit of 0.2 ng/mL. TNF- α levels were determined using an ELISA kit (Biocheck Inc., Foster City, CA, USA) with a detection limit of 0.2 pg/mL.

Aliquots of gastrocnemius muscle tissue were homogenized in ice-cold buffer (0.25 M sucrose, 10 mM Tris-HCl, and 0.25 mM phenylmethylsulfonyl fluoride; pH 7.4), and a portion of the homogenate was measured immediately to determine the malondialdehyde (MDA) level. Another portion of the homogenate was centrifuged at 15,000 g for 30 min at 4°C. The supernatant was decanted and assayed for superoxide dismutase (SOD) activity. The SOD activities and MDA levels were determined according to the recommended procedures provided by the commercial kits (Jiancheng Bioengineering Institute, Nanjing,

China).

2.7. Cell culture and treatment

Cell culture were performed according our previous protocol(Shi et al., 2014). C2C12 myoblasts were purchased from the Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China). Cells were cultured in high-glucose DMEM containing 10% FBS, 2 mM glutamine, 100 unit/mL penicillin, and 100 g/mL streptomycin. Cells were maintained at 37°C under 5% CO₂ (v/v) in a humidified incubator. Differentiation of myoblasts into myotubes was induced when the cells had achieved 80% confluence by replacing the media with DMEM containing 2% horse serum. Five to seven days later, the differentiated myotubes (80% differentiated) were used for the experiments.

2.8. Transfection of siRNA

Transfection of siRNA were performed according our previous protocol(Wang et al., 2014e). Validated SLC38A2 siRNA (5'- CUUUCCGGAUACAUAGGAUUU -3') and negative-control siRNA (5'- CUUUCCCGGGAAAGUUAUUU -3') were purchased from Shanghai GenePharma Co. (Shanghai, China). C2C12 myoblasts or myotubes were seeded in 6-well plastic culture plates and cultured in medium B for 48 h at 37°C. A total of 30 nmol of validated siRNA or negative control siRNA were transfected into the cells using lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions.

2.9. Autophagy detection *in vivo*

Autophagosome were marked by LC3B, and could be stained by Monodansylcadaverine (MDC) (Vazquez and Colombo, 2009). So MDC and immunofluorescence of LC3B were used to detect autophagy in C2C12 myoblast/myotubes. After treatment, MDC (50 nM) was added and incubated for an additional 30 min, after which the samples were examined. Other samples were fixed for 15 min at room temperature in 4% paraformaldehyde, permeabilized using 1% Triton X-100 for 15 min at 4°C, and blocked for 2 h at room temperature using 1% normal serum. The prepared sections were probed overnight using antibodies to

LC3B (1:200) and GAPDH (1:400). Secondary antibodies were incubated for 45 min. Because C2C12 myotubes were unable to adhere in confocal dishes, C2C12 myotubes were cultured in 96-well plates and measured in a microplate reader (Bio-Rad). The C2C12 myoblasts were cultured in confocal dishes and examined by confocal laser scanning microscopy (Olympus, Tokyo, Japan).

2.10. Western blotting

Western blotting were performed according our previous protocol (Yin et al., 2015). Aliquots of gastrocnemius muscle tissue (40 mg) were snap frozen and ground in a mortar, thawed and homogenized in lysis buffer supplemented with 8 mL of protease inhibitor cocktail (Calbiochem) and phosphatase inhibitor cocktail (Sigma-Aldrich). C2C12 myotubes were also lysed in the lysis buffer. After being quantified by BCA analysis, protein samples were separated by SDS-PAGE and transferred onto PVDF membranes. After being placed in blocking buffer, the membranes were incubated with the following primary antibodies: Anti-Akt (1:1,000 dilution), anti-p-AKT (1:1,000 dilution), anti-mTOR (1:1,000 dilution), anti-p-mTOR (1:1,000 dilution), anti-MuRF1 (1:1,000 dilution), anti-MUFbx (1:1,000 dilution), anti-Atg 7 (1:1,000 dilution), anti-LC3B (1:1,000 dilution) and anti-GAPDH (1:5,000 dilution). They were then treated with HRP-conjugated secondary antibodies (1:10,000 dilution). The protein bands of LC3B were exposed by developing the film in a darkroom, and the remaining protein bands were captured and documented through a CCD system (imagerstation 2000 MM, Kodak, NY, USA). The intensities of the protein bands were analysed by Molecular Imaging Software Version 4.0, which was provided with the Kodak 2000 MM System. The GAPDH protein was used as an internal control.

2.11. Real-time PCR

Three-step PCR method were used to detected mRNA levels of some amino acid carriers (Wang et al., 2014a). One-milligram aliquots of gastrocnemius muscle tissue and C2C12 myotubes were used for real-time PCR analysis. Total RNA was extracted with Trizol reagent (Invitrogen, Carlsbad, CA, USA) and dissolved in RNase-free water. Total RNA was reverse transcribed to cDNA using 50 ng of total RNA in a

20 µL reaction mixture containing 200 U of Revert Aid M-MuLV reverse transcriptase. Quantitative PCR was performed using the SYBR green method in a total reaction volume of 20 µL containing 0.2 U of JumpStart Taq polymerase (Sigma-Aldrich), 10 pmol of each forward and reverse primer, and 1 µL of cDNA in a MyiQ real-time PCR detection system (Bio-Rad) in triplicate. The sequences of specific primers are as follows: SLC38A2 (F 5'-TAATCTGAGCAATGCGATTGTGG-3', R 5'-GATGGACGGAGTATAGCGAAAA-3'); SLC7A5 (F 5'-ATATCACGCTGCTCAACGGTG-3', R 5'-CTCCAGCATGTAGGCGTAGTC-3'); SLC36A1 (F 5'-GACTACAGTTCCACAGACGTG-3', R 5'-CCATGTCATGCTACTGCTCTCT-3'); Atf4 (F 5'-AAGGAGGAAGACACTCCCTCT-3', R 5'-CAGGTGGGTCATAAGGTTTGG-3') and GAPDH. The detected levels of target mRNAs were calculated using the $2^{-\Delta\Delta C_t}$ method and normalized to GAPDH in arbitrary units.

2.12. Statistical analysis

Statistical significance was estimated using one-way analysis of variance, and Chi-squared tests were used for categorical data. The weight events were determined using the Kaplan–Meier method. P values < 0.05 were considered significant. Results are expressed as means ± S.E.M. Statistical analyses were performed using SPSS 13.0 (SPSS, Chicago, IL, USA)

3. Results

3.1. Effects of APSs on body weight

Figure 1A shows the changes in body weight that occurred during the experiment. The body weights of rats in the model group showed a significant decrease compared with rats in the control group from weeks 4 to 23 ($P < 0.05$). Compared with the body weights in the model group, rats fed APSs showed significant increases in body weight at weeks 13 and 15–23 ($P = 0.0377$ – 0.002). Body weight did not significantly differ between the C-APS group and control group ($P = 0.081$ – 0.599). Figure 1B shows the incidence of body weight events. A total of 49 body weight events (Control: 4; Model: 24; M-APS: 16; C-APS: 5) and 39 death events (Control: 2; Model: 20; M-APS: 14; C-APS: 3) occurred during the experiment. In the analysis,

rats in the model group were more likely to experience body weight events than those in the control group ($P<0.0001$). Compared with the model group, rats in the M-APS groups appeared to experience fewer body weight events ($P=0.0572$). There was no significant difference between the C-APS group and control group ($P=0.7197$).

3.2. Effects of APSs on muscle protein metabolism *in vivo*

We used the cross-sectional area of muscle fibres to evaluate muscle atrophy as previously described, and a total of 800 muscle fibres were analysed. Representative views are shown in Figure 2A. The mean cross-sectional area of TA muscle in the model group was significantly lower than in the control group ($P<0.001$). Compared with the model group, fibre atrophy was attenuated in the M-APS group ($P=0.003$, Figure 2B). The dry weight and protein synthesis rate of TA muscle were significantly decreased (Dry weight: $P<0.001$; protein synthesis rate: $P<0.001$), and the protein degradation rate was significantly increased ($P<0.001$) in the model group compared with the control group (Figure 2B). In the M-APS group, supplementation with APSs increased the dry weight ($P=0.0065$, Figure 2C) and the protein synthesis rate ($P=0.0139$, Figure 2D), and it decreased the protein degradation rate ($P=0.043$, Figure 2E). Notably, the dry weight was decreased in the C-APS group compared with the control group ($P=0.021$).

3.3. Effects of APSs on Akt/mTOR, MuRF1/MuFbx and autophagy signalling *in vivo*

To determine the potential mechanism of APSs, we examined the signalling pathways involved in protein anabolism (Akt/mTOR) and catabolism (MuRF1/MuFbx and autophagy). All of these pathways were found to be involved in the mechanism. As shown in Figure 3, the levels of phosphorylated Akt and mTOR were reduced in the model group (p-Akt/t-Akt: $P=0.0001$; p-mTOR/t-mTOR: $P=0.006$) and were restored in the M-APS group (p-Akt/t-Akt: $P=0.039$; p-mTOR/t-mTOR: $P=0.001$). The levels of MuRF1, MuFbx, and Atg 7 and the ratio of LC3B-II to LC3B-I were increased in the model group (MuRF1/GAPDH: $P<0.0001$; MuFbx/GAPDH: $P=0.008$; Atg 7/GAPDH: $P=0.017$; LC3B-II/LC3B-I/GAPDH: $P=0.006$) and were down-regulated when APS was added (MuRF1/GAPDH: $P=0.004$; MuFbx/GAPDH: $P=0.018$; Atg

7/GAPDH: $P=0.032$; LC3B-II/LC3B-I/GAPDH: $P=0.007$).

3.4. Effects of APSs on amino acid carriers *in vivo*

Endochylema amino acid (AA) levels were closely associated with protein metabolism, and we detected three AA carriers (SLC38A2, SLC7A5 and SLC36A1) and a molecular chaperone (ATF4). Our results show that the mRNA levels of SLC38A2, SLC36A1 and ATF4 were significantly decreased in the model group compared with the control group (SLC38A2: $P<0.001$; SLC36A1: $P=0.009$; ATF4: $P<0.001$, Figures 4A, C and D). The down-regulated SLC38A2 and ATF4 mRNAs were restored in the M-APS group (SLC38A2: $P=0.042$; ATF4: $P=0.013$). The expression level of SLC7A5 did not differ significantly between groups ($P=0.493$ - 0.767 , Figure 4B).

3.5. Effects of APSs on inflammatory and oxidative factors *in vivo*

As shown in Figure 5, compared with the control group, the levels of TNF- α , IL-15 and CRP were significantly increased in the model group at all time points ($P<0.001$). The levels of inflammatory factors were lower in the M-APS group at certain time points, as shown in Figure 5A-C. At the end of the experiment, the levels of MDA were significantly decreased ($P<0.0001$) and SOD was increased ($P<0.0001$) in the muscles of rats in the model group compared with those in the control group. The levels of MDA and SOD were partially restored in the M-APS group (MDA: $P<0.0001$; SOD: $P=0.0003$).

3.6. Gradient screening of the effects of APSs on the C2C12 myotube diameter

We performed gradient screening to determine the optimum concentration of APSs and the optimum incubation time *in vitro*. As shown in Figure 6, the C2C12 myotube diameter decreased when 80 ng/mL TNF- α was used ($P=0.005$). APSs significantly restored the myotube diameter in the concentration range of 0.1 to 2 mg/mL, and the effect was most notable with 0.2 mg/mL APS ($P<0.001$). In the gradient screening of incubation time, 80 ng/mL TNF- α significantly decreased the C2C12 myotube diameter at all time points ($P<0.001$), and 0.2 mg/mL APS increased the diameter at 12 and 24 h. Impaired myotubes showed the greatest recovery after 24 h of treatment ($P<0.001$). Thus, we selected 0.2 mg/mL APS for the *in vitro*

experiments.

3.7. Effects of APSs on muscle cell protein metabolism *in vitro*

As shown in Figures 7A and 7B, C2C12 myotube diameter decreased when 80 ng/mL TNF- α was used ($P=0.001$), and the diameter increased with 0.2 mg/mL APS treatment ($P=0.006$). The effect of APSs was inhibited by SLC38A2 siRNA ($P<0.001$). The fluorescence intensity of MDC increased after TNF- α treatment ($P=0.007$, Figure 7C). APSs could reduce the optical intensity in negative siRNA transfected myotubes ($P=0.006$), but this was not true in the SLC38A2 siRNA transfected group ($P=0.022$). The fluorescence intensity of LC3B increased with TNF- α treatment and decreased with APS treatment. However, no quantitative analysis was performed to determine the autophagosome quantity and fluorescence intensity.

3.8. Effects of APSs with RNA interference on autophagy signalling *in vitro*

To confirm the association between SLC38A2 and autophagy signalling, we measured mTOR and LC3B levels in C2C12 myotubes with or without SLC38A2 siRNA. As shown in Figure 8, the ratio of phosphorylated mTOR to total mTOR was decreased after TNF- α treatment ($P=0.006$), whereas the ratio of LC3B-II to LC3B-I increased after TNF- α treatment ($P=0.008$). APSs restored the phosphorylation of mTOR and increased the ratio of LC3B-II to LC3B-I (p-mTOR/t-mTOR: $P=0.023$; LC3B-II/LC3B-I/GAPDH: $P=0.001$). The improved effect of APSs was blocked by SLC38A2 siRNA (p-mTOR/t-mTOR: $P=0.015$; LC3B-II/LC3B-I/GAPDH: $P=0.001$).

4. Discussion

In this study, we tested the anti-wasting effect of APSs and explored potential mechanisms for this effect. In CKD patients, muscle wasting is closely linked with morbidity and mortality. Maintaining the protein content in muscles may improve these patients' clinical outcomes. Early research attributed wasting to malnutrition and insufficient protein intake, and researchers focused on appetite-regulating pathways, such as neuropeptide signalling (Lu et al., 2015; Morley and Farr, 2008). However, epidemiological studies

have concluded that the excessive morbidity and mortality of CKD patients is rarely attributable to malnutrition (Fouque et al., 2008; Stenvinkel et al., 2004). Ikizler et al. found that although increasing the intake of protein and calories improved protein turnover, it did not fully correct the endpoint of CKD (Carrero et al., 2013; Ikizler et al., 2002). This suggests that the PEW of cellular protein catabolism may play a more important role in CKD-related muscle wasting progression. Some uremia disorders, such as persistent inflammation, acidosis and insulin resistance, lead to PEW. Experimentally, carbohydrate polymers can increase colonic microbial activity and improve lipid profiles. Few studies have focused on the relationship between functional polysaccharides and protein metabolism. Our results show that if adequate amounts of nitrogen and energy are available, APSs can improve muscle mass and protein metabolism in 5/6 nephrectomised rats, in agreement with previous studies (Liu et al., 2013; Lu et al., 2013a). By measuring skeletal muscle dry weight and evaluating muscle pathology, we found that APSs may have a weight-loss effect in sham-operated rats. This result is not consistent with data on protein metabolism. The reason for this discrepancy is not clear, but some polysaccharides may affect how other nutrients and chemicals are absorbed through bulking and viscosity. To obtain more evidence of an anti-wasting effect, we measured representative inflammatory and oxidative factors that have already been linked with CKD-related PEW. Previous studies have shown that some pro-inflammatory cytokines, such as TNF- α and IL-15, can cause muscle wasting directly by enhancing catabolism. Anti-cytokine treatments may be effective in treating cachexia (Argiles et al., 2009a; Dogra et al., 2007; Quinn et al., 2002; Steffen et al., 2008). Oxidative stress has also been proven to induce PEW through stress signalling (Buck and Chojkier, 1996; McClung et al., 2010). Our experiment shows that APSs can lower pro-inflammatory cytokine levels and improve oxidative stress to varying degrees. This may represent an important underlying mechanism of the anti-wasting effect.

To determine the potential mechanism of the anti-wasting effect, we studied relevant signalling pathways. The imbalance between protein anabolic and catabolic rates is responsible for muscle wasting. Protein catabolism is primarily induced by two major pathways: ubiquitin-proteasome-dependent proteolysis

and autophagy. The E3 Ub-ligases play important roles in the attachment of ubiquitin to protein substrates. MuRF1 and MAFbx are two E3 Ub-ligases that are significantly up-regulated during CKD-related muscle wasting (de Palma et al., 2008; Du et al., 2005; Mitch, 2007). In addition, autophagy dominantly regulates muscle mass through targeted protein binding and protein degradation. Some cases of sarcopenia have been linked to excessive autophagy (Sala et al., 2014; Teixeira Vde et al., 2012). However, there have been few reports regarding the role of autophagy in CKD-related muscle wasting. In contrast, protein anabolic factors, such as IGF-1, can promote protein synthesis via the Akt/mTOR signalling pathway. The phosphorylation of mTOR activates the P70 ribosomal protein s6 kinase (P70s6k), which activates at least three regulators: eIF4b, rpS6 and (eEF)2. These regulatory factors accelerate protein translation (Brooks and Myburgh, 2014; Ham et al., 2014). Akt/mTOR signalling has rarely been considered in CKD-related muscle wasting. However, recent research found that Akt1-mediated muscle growth reduces renal damage in a model of obstructive kidney disease (Hanatani et al., 2014). Our results show that in addition to the E3 Ub-ligase pathway, Akt/mTOR signalling and autophagy signalling may also be involved in the CKD-related muscle wasting process. APSs could decrease Ub-ligases levels and autophagy signalling and could restore the phosphorylation of Akt and mTOR. Because there is a complex network of interactions between these signalling proteins, some factors (e.g., FoxO3a and FoxO1) are linked to both pathways (Milan et al., 2015; Stitt et al., 2004). Further investigation will be needed to find the target of APSs.

Although CKD-related muscle wasting is marked by insufficient protein content, it does not correspond to AA deficiency. However, AA profiles change due to wasting. The levels of several AAs, including leucine, valine and isoleucine, were reduced in CKD patients with PEW (Yang et al., 2001). However, these AAs (particularly large neutral AAs, or LNAAs) play important roles in protein metabolism. The increased availability of certain AAs (e.g., glutamine and leucine) is associated with the up-regulation of AA transporters (e.g., SLC38A2 and SLC7A5) in human skeletal muscles (Tsien et al., 2015). A series of AA transporters act as upstream triggers of mTOR and can regulate protein metabolism (Nicklin et al., 2009;

Ogmundsdottir et al., 2012). Although AA profile changes may indicate that AA transporters are affected during CKD, there have been no studies of AA transporter profiles in muscle during the CKD process. A previous animal study found that APSs can enhance the ileal digestibility and serum concentrations of AAs (Yin et al., 2009). Despite the lack of direct evidence, we speculate that the function of APSs may involve ileal AA transporters. Our study found that SLC38A2, a Na⁺-dependent neutral AA transporter, was expressed at lower levels in the model group, and its expression was restored by APSs. A previous study showed that SLC38A2 acts directly as the initiating sensor for mTOR signalling (Pinilla et al., 2011). The phosphorylation of mTOR inhibits the dephosphorylation of Atg13, causing autophagosome levels to decrease. Thus, SLC38A2 may be a target of APSs that connects it to mTOR signalling and autophagy signalling. To test this hypothesis, we used RNA interference in an *in vitro* experiment. Our results show that APSs can alleviate problems with catabolism and autophagy in model rats, and this effect can be blocked by SLC38A2 siRNA. Moreover, results from Western blots indicated that the inhibition of SLC38A2 attenuates the phosphorylation of mTOR and increases the LC3B-II/LC3B-I ratio, which should be restored by APSs. Our findings suggest that APSs can restore Akt/mTOR and autophagy signalling, and this result is consistent with our *in vivo* experiments. Some AA transporters, such as SLC38A2, may be targets of APSs. However, our findings suggest that AA transporter changes may cause CKD-related muscle wasting. Thus, we have begun to identify the changes in AA transporters that occur in CKD patients by using high-throughput screening.

In conclusion, our results suggest that 2% APSs (or 0.2mg/mL *in vitro*) alleviate muscle wasting by acting on the Akt/mTOR, ubiquitin proteasome and autophagy signalling pathways in 5/6 nephrectomised rats. Specific AA transporters, such as SLC38A2, may be involved in disease progression. These findings may lead to the development of therapeutic natural products that can be used to manage CKD-related muscle wasting.

Author Contribution:

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Table 1. Ingredient of experimental diets to rats (g/kg diets).

Components	Control	Model	M-APS	C-APS
Casein	220	220	220	220
Dextrin	379	379	379	379
Maize starch	140.5	140.5	120.5	120.5
APS	-	-	20	20
Sucrose	100	100	100	100
Soyabean oil	90	90	90	90
Mineral mix	35	35	35	35
Inherent fibre	20	20	20	20
Vitamin mix	10	10	10	10
L-Cystine	3	3	3	3
Choline hydrochloride	2.5	2.5	2.5	2.5
Gross energy, kcal/g	4.50	4.50	4.42	4.42

Note: APS, *Astragalus* polysaccharide

Caption of Figures

Figure 1. Change in body weight over 155 days of 2% APS intervention (A) and Kaplan–Meier curve of weight events in each group (B). Weight loss is defined as an end event if weight gain does not reach 20% of average weight gain of Control group for two consecutive weeks (This standard were based on results of receiver operating characteristic curve between weight loss and muscle pathology in pre-experiment).

Figure 2. Change of muscle protein metabolism in each group. (A) Cross-sections of tibialis anterior (TA) muscle stained with H&E. Scale bar 50mm. (B) Quantitation of cross-sections area were determined. (C) Dry weight of TA muscle in each group. (D) Protein synthesis rate of TA muscle in each group. (E) Protein degradation rate of TA muscle in each group. * $P < 0.05$, Control group vs. Model group. # $P < 0.05$, Model group vs. M-APS group. & $P < 0.05$, Control group vs. C-APS group.

Figure 3. Effects of APS on Akt/mTOR, MuRF1/MAFbx and Autophagy signaling *in vivo*. (A) Relative levels of p-Akt / total Akt and p-mTOR/total mTOR . Quantitation was determined by densitometry of the blots. (B) Relative levels of MuRF1/GAPDH and MAFbx/ GAPDH. Quantitation was determined by densitometry of the blots. (C) Relative levels of Atg7/GAPDH and LC3BII/LC3BI/ GAPDH. Quantitation was determined by densitometry of the blots. * $P < 0.05$, Control group vs. Model group. # $P < 0.05$, Model group vs. M-APS group.

Figure 4. Effects of APS on amino acid carriers *in vivo*. (A) SLC38A2 mRNA levels were determined by real-time PCR. (A) SLC7A5 mRNA levels were determined by real-time PCR. (A) SLC36A1 mRNA levels were determined by real-time PCR. (A) ATF4 mRNA levels were determined by real-time PCR. * $P < 0.05$, Control group vs. Model group. # $P < 0.05$, Model group vs. M-APS group.

Figure 5. Effects of APS on pro-inflammatory and oxidative factors *in vivo*. (A) Change of serum TNF- α levels during the experience. (B) Change of serum IL-15 levels during the experience. (C) Change of serum CRP levels during the experience. (D) Levels of MDA in each group after treatment. (E) Levels of SOD in each group after treatment. * $P < 0.05$, Control group vs. Model group. # $P < 0.05$, Model group vs. M-APS group. & $P < 0.05$, Control group vs. C-APS group.

Figure 6. Effects of APS on C2C12 myotubes diameter. (A) C2C12 myotubes incubated in 80 ng/mL TNF- α with different concentrations of APS for 24 h; (B) C2C12 myotubes incubated in 80 ng/mL TNF- α with 0.2mg/mL APS for the indicated times. * $P < 0.05$, Control vs. TNF- α intervene. # $P < 0.05$, TNF- α intervene vs. APS treatment.

Figure 7. Effects of APS on protein metabolism of C2C12 myoblasts or myotubes. (A) The images listed successively as follows: Images of C2C12 myotubes with H&E stain and captured by optical microscope ($\times 200$), Images of C2C12 myoblasts with Monodansylcadaverine (MDC) treatment and captured by confocal microscope ($\times 9000$), Images of C2C12 myoblasts with immunofluorescence captured by confocal microscope ($\times 1000$). (B) Quantitation of C2C12 myotubes diameter changes. (C) luorescence intensity of MDC in C2C12 myotubes measured by microplate reader. * $P < 0.05$, APS negative control vs. TNF- α intervene. # $P < 0.05$, TNF- α intervene vs. APS treatment. & $P < 0.05$, APS treatment vs. APS treatment add siRNA

Figure 8. Relative levels of p-mTOR/total mTOR and LC3B-II/LC3B-I/ GAPDH. Quantitation was determined by densitometry of the blots. * $P < 0.05$, APS negative control vs. TNF- α intervene. # $P < 0.05$, TNF- α intervene vs. APS treatment. & $P < 0.05$, APS treatment vs. APS treatment add siRNA

Astragalus membranaceus Bunge

Polysaccharide
extraction

Identification and
component analysis

SD rats with 5/6 nephrectomy
or sham operation

APS

C2C12 myoblasts or
myotubes

Separate skeletal muscle

Functional and
pathological
examination

protein

mRNA

siRNA

Identification

Identification