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Human Umbilical Cord Wharton's Jelly Derived Mesenchymal Stromal Cells May Attenuate Sarcopenia in Aged Mice Induced by Hindlimb Suspension

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Data Interpretation D
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Background: Since the use of human umbilical cord Wharton's Jelly derived mesenchymal stromal cells (hWJ-MSCs) to treat sarcopenia has not been explored, we studied the effects of hWJ-MSCs in aged male C57BL/6J mice with sarcopenia induced by hindlimb suspension, and explored the potential mechanism.

Material/Methods: Hindlimb suspension was used to induce sarcopenia in 24-month-old C57BL/6J mice and green fluorescent protein-tagged hWJ-MSCs and controls were transplanted into mice via tail vein or local intramuscular injection. After hWJ-MSC transplantation, changes in whole body muscle strength and endurance, gastrocnemius muscle weight and myofiber cross-sectional area (CSA) were studied. Proliferation of skeletal muscle stem cell, apoptosis, and chronic inflammation were also investigated.

Results: We demonstrated that whole body muscle strength and endurance, gastrocnemius muscle mass, and CSA were significantly increased in hWJ-MSC-transplanted mice than in controls ($P<0.05$). In hWJ-MSC-transplanted mice, apoptotic myonuclei was reduced, and BrdU and Pax-7 expression indices of gastrocnemius muscles were increased ($P<0.05$). Tumor necrosis factor (TNF)-α and interleukin (IL)-6 were downregulated, and IL-4 and IL-10 were upregulated ($P<0.05$).

Conclusions: hWJ-MSCs may ameliorate sarcopenia in aged male C57BL/6J mice induced by hindlimb suspension, and this may be via activation of resident skeletal muscle satellite cells, reduction of apoptosis, and less chronic inflammation.

MeSH Keywords: Apoptosis • Hindlimb Suspension • Inflammation • Mesenchymal Stromal Cells • Sarcopenia

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Background

Sarcopenia is a syndrome characterized by age-dependent loss of muscle mass and function [1]. It can remarkably reduce quality of life in the elderly, especially those with limited mobility and long-term hospitalization. To date, its exact pathogenesis is unclear. There are likely several factors that contribute to sarcopenia. These include but may not be limited to: reduced satellite cell (SC) function, nuclear apoptosis, chronic inflammation, reduced protein synthesis, declines in neural function, hormonal deficits, oxidative stress, loss of mitochondrial function, and inappropriate signaling in muscle due at least in part to inadequate nutrition [2].

Unlike disuse amyotrophy, sarcopenia is slow and gradual muscular atrophy with age. Linear sarcopenic declines in muscle mass and strength are punctuated by transient periods of muscle disuse that can accelerate losses of muscle and strength [3]. Accumulating evidence suggests that many cellular events during sarcopenia are similar to those associated with prolonged muscle disuse, such as bed rest, casting, microgravity [4], and hindlimb suspension (HS). HS in murines has been recognized as a commonly used hypodynamic model of disuse-induced atrophy in order to study sarcopenia and muscle aging [5,6].

SCs are required for myogenesis and rejuvenation of senescent SCs is needed for recovery from sarcopenia. Studies indicate that muscle SCs function declines with advancing age, which impairs myofibers regenerative and/or growth response [7]. However, since it is unclear whether a decrease in their number or regenerative capacity is involved in sarcopenia [8,9], the use of SCs as therapeutic targets of sarcopenia is not well understood.

At this time, therapeutic regimens for sarcopenia have been unsatisfactory; exercise and nutritional supplementation are first-line treatments that only offer relatively minor benefit [10–12]. Mesenchymal stromal cells (MSCs) have been studied for their ability to improve regeneration of senescent muscles and studies suggest that MSCs may enhance muscular regeneration in animal and cellular models [13–19]. Furthermore, umbilical cord derived MSCs (UCMSCs) may hold promise as they have non-invasive collection procedures for autologous or allogeneic use, lower risk of infection and teratoma, multipotency, and low immunogenicity/good immunosuppressive ability [20]. Moreover, human umbilical cord Wharton's Jelly derived MSCs (hWJ-MSCs) may be superior to other UCMSCs, because their isolation is simple and easy to standardize. Additionally, hWJ-MSCs can be generated in large numbers with minimal manipulation, have fewer non-stem cell contaminants, are proliferative, and have broad and efficient differentiation potential [21]. Studies have shown that hWJ-MSCs differentiate into myofibers in muscle cell culture medium containing 5-azacytidine [22] and therapeutic effects have been demonstrated for muscular atrophy experimental models [15,16,19]. Since the use of hWJ-MSCs to treat sarcopenia has not been explored, we studied implanted hWJ-MSCs in a mouse model of sarcopenia to attempt to treat it. Using aged male C57BL/6J mice with sarcopenia induced by HS, we transplanted green fluorescent protein (GFP)-tagged hWJ-MSCs via tail vein or gastrocnemius muscle injection, and then studied how hWJ-MSCs may relieve or reverse sarcopenia.

Material and Methods

Cell preparation

GFP-tagged hWJ-MSCs (HUXUC-01101, passage #5) were purchased from Cyagen Biosciences Inc. (Guangzhou, China) and cultured in hWJ-MSCs complete medium (HUXUC-90011, Cyagen Biosciences Inc.) supplemented with 10% human UCMSCs specific FBS, 100 mg/mL streptomycin and 100 U/mL penicillin, and incubated at 37°C in a 5% CO_2 humidified incubator. After 72 hours, non-adherent cells were removed, and fresh medium was added; the medium was changed every 3 days. When adherent cells were 90% confluent, they were trypsinized (trypsin-EDTA solution; HUXUB-90011, Cyagen Biosciences Inc.) and seeded onto fresh plates (split 1: 3) until a homogenous population was obtained after 2 to 3 weeks of culture. hWJ-MSCs at passage 8–9 were used in all experiments.

Animals and experimental protocols

A total of 32 male C57BL/6J mice (Beijing HFK Bioscience, Beijing, China) aged 24 months were used in the study. All mice were housed in a temperature-controlled room on a 12-hour light/dark cycle with free access to food and water. A sarcopenia model was established by HS as previously described [6]. After 2 weeks of HS, mice were embedded subcutaneously in the posterior neck with 25 mg of BrdU (Innovative Research, Sarasota, FL, USA), after mice were anesthetized with 4% isoflurane and given freedom of movement. Then mice were randomized into one of 4 groups: tail vein transplant (TVT) group (n=8), muscular transplant (MT) group (n=8), TVT-control group (n=8), and MT-control group (n=8). The hWJ-MSC transplantation groups were treated directly in both hindlimb gastrocnemius muscles (0.5×10^6 cells in 50 µL Hank's buffered salt solution/limb)) or via the tail vein (1×10^6 cells in 100 µL Hank's buffered salt solution) weekly for 8 weeks. Efficacy was assessed at 8 weeks. Control groups were given an equivalent volume of Hank's buffered salt solution weekly for 8 weeks. Then animals were weighed and sacrificed after 8 weeks of cell transplantation for studies.

All procedures for animal care and treatment were performed in accordance with the Care and Use of Laboratory Animals Guidelines of the Affiliated Hospital of Jining Medical

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University, and all experimental procedures were approved by the Institutional Animal Use and Care Committee of the Affiliated Hospital of Jining Medical University.

Muscle strength and endurance analysis and tissue preparation

Whole body muscle strength and endurance was measured before HS and after HS and after 8 weeks of hWJ-MSCs transplant using a wire hang test, as previously done [23]. Latency to fall was analyzed from the average of the 2 best wire hang tests. After anesthetization, gastrocnemius muscles were removed from both hindlimbs and weighed. The muscles were bisected at the mid-belly, embedded in tragacanth gum, snap frozen in isopentane that was cooled by liquid nitrogen, and stored at -80°C. The remainder of the muscle was immediately frozen in liquid nitrogen and stored at -80°C for enzyme-linked immunosorbent assay (ELISA).

Histological and cross-sectional area (CSA) analysis of gastrocnemius muscles

Frozen cross-sections (12 μm) were stained with hematoxylin and eosin (H&E; Sigma-Aldrich), and then examined under a light microscope (CX31-LV320, Olympus, Japan) to measure cross-sectional area (CSA) of muscle fibers, as described previously [6]. CSA was determined by 100 myofibers randomly selected from each section and measured using ImageJ software.

Immunofluorescent staining

Gastrocnemius muscle sections (12 μm) were fixed in pre-cooled acetone at 4°C for 25 minutes and were permeabilized for 15 minutes in 0.1 M phosphate buffered saline (PBS) containing 0.2% Triton X-100. The sections were blocked by 5% normal goat serum for 1 hour and then incubated with anti-Pax-7 antibody (ab199010, Abcam, Cambridge, MA, USA, 1: 200) or anti-BrdU antibody (ab6326, Abcam, 1: 250) and anti-laminin antibody (ab11575, Abcam, 1: 500) overnight at 4°C in PBS with 5% normal goat serum, and then with Alexa Fluor 647-conjugeted goat anti-mouse IgG (ab150119, Abcam, 1: 300) or Alexa Fluor 647-conjugated donkey anti-rat IgG (ab150159, Abcam, 1: 300) and Alexa Fluor 488-conjugated donkey anti-rabbit IgG (ab150073, Abcam, 1: 300) for 1 hour at room temperature. Washed sections were mounted with 4',6-diamidino-2-phenylindole (DAPI) (Life Technologies, Japan) for nuclei identification. Immunofluorescent signals were captured using Observer Z1 confocal microscope (Zeiss, Jena, Germany).

TUNEL staining

Apoptosis was quantified with a terminal deoxynucleotidyl transferase (TdT)-mediated deoxy-UTP nick end labeling (TUNEL) kit (Roche Applied Science, Indianapolis, IN, USA), as described previously [6]. Briefly, tissue sections were fixed in pre-cooled acetone at 4°C for 25 minutes and were permeabilized for 15 minutes in 0.1 M PBS containing 0.2% Triton X-100. Then the slide was incubated at 4°C overnight with rabbit anti-laminin antibody (ab11575, Abcam, 1: 500). Thereafter, the cryosections were incubated with Alexa Fluor 647-conjugated donkey anti-rabbit IgG (ab150075, Abcam, 1: 300) and TUNEL reaction mixture for 1 hour at 37°C in the dark. Sections were mounted with DAPI (Life Technologies, Japan) to visualize nuclei. The glass slides were captured with Observer Z1 confocal microscope (Zeiss, Jena, Germany), then the apoptotic index was calculated according to the ratio of TUNEL-positive myonuclei and DAPI-positive myonuclei.

ELISA

Interleukin (IL)-6, tumor necrosis factor (TNF)-α, IL-4, and IL-10 in muscle homogenate were measured with ELISA according to the manufacturer's instructions from the eBioscience kits (IL-6, cat: ab100713; TNF-α, cat: ab100747; IL-4, cat: ab100710; IL-10, cat: ab108870; Abcam). Optical density was measured using a microtiter plate reader at 405 nm and 650 nm.

Statistical analysis

Statistical analyses were performed using SPSS 13.0 software (SPSS, Chicago, IL, USA). Data were shown as means ± standard error. Differences among means for transplant factors (TVT versus MT) and treatment (hWJ-MSCs versus Hank's buffered salt solution) were confirmed with 2-way ANOVA and Bonferroni post hoc analyses. When comparing muscle strengths before and after HS, and after hWJ-MSCs treatment, a repeated-measures ANOVA was used. $P<0.05$ was considered significant.

Results

hWJ-MSC transplantation improved whole body muscle strength and endurance

We first determined if the muscle functional decline that occurs in old mice after 2 weeks of HS. We assessed whole body muscle strength and endurance in old mice by using a hang test to measure latency-to-fall, which was significantly reduced in old mice after 2 weeks of HS ($P<0.001$; Figure 1). We also found that hWJ-MSC-transplanted mice have a longer latency to fall during a wire hang test compared to the corresponding control group or the same group before transplantation ($P<0.05$, n=8 per group; Figure 1), but it did not fully reverse the muscle functional decline induced by HS. This increment was more obvious in TVT than MT group, but there was no significant difference between the 2 groups ($P>0.05$; Figure 1).

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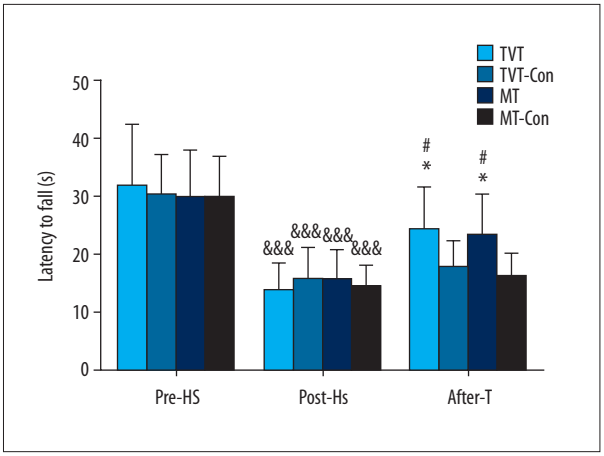

Figure 1. Effect of human umbilical cord Wharton's Jelly derived mesenchymal stromal cell (hWJ-MSC) transplantation on whole body muscle strength and endurance in sarcopenic mouse model. Quantification of whole body muscle strength and endurance using latency to fall during a wire hang test. Pre-hindlimb suspension (HS), before hindlimb suspension; post-HS, after 14 days of hindlimb suspension; after-T, after 8 weeks of hWJ-MSC transplantation. MT – muscular transplant group; MT-Con – MT-control group; TVT – tail vein transplant group; TVT-Con – TVT-control group. * $P<0.05$, compared to relative control in after-T group; # $P<0.05$, compared to relative post-HS group; &&& $P<0.001$, compared to relative pre-HS group.

hWJ-MSC transplantation increased muscle weight and CSA

Gastrocnemius wet mass in the hWJ-MSC-transplanted group was significantly increased compared with the control group ($P<0.001$), and increased gastrocnemius muscle mass was more prominent in the MT group compared to TVT group ($P<0.05$, n=8 per group; Figure 2). We found that hWJ-MSCs enhanced CSA of gastrocnemius myofibers in hWJ-MSC-transplanted mice compared to controls ($P<0.001$, n=8 per group; Figure 3). Gastrocnemius wet mass and CSA were normalized to body weight for comparisons. hWJ-MSCs enhanced normalized gastrocnemius muscle mass and CSA of myofibers in hWJ-MSCs-transplanted mice compared to controls ($P<0.05$; Figures 2, 3).

hWJ-MSC transplantation increased expression of BrdU and paired box 7 (Pax-7)

Paired box 7 (Pax-7) is a marker of the total number of quiescent and proliferating SCs, and BrdU is frequently used to indicate the proliferated SCs. We found hWJ-MSCs significantly increased BrdU and Pax-7 expression in the gastrocnemius muscle (MT: $P<0.001$, TVT: $P<0.01$, compared to relative control, n=8 per group; Figure 4). Increased Pax-7 was more prominent in MT group compared to TVT group ($P<0.01$; Figure 4), but expression of BrdU was not different between the 2 groups. We did not observe transplanted GFP-tagged hWJ-MSCs via tail vein or intramuscular injection in gastrocnemius muscle.

hWJ-MSC transplantation suppressed apoptosis

The apoptotic index as indicated by the ratio of TUNEL-positive myonuclei and DAPI-positive myonuclei, was significantly reduced after hWJ-MSC transplantation (MT: $P<0.001$, TVT: $P<0.01$, compared to relative control, n=8 per group; Figure 5). This reduction was more obvious in the MT group compared to the TVT group, but there was no significant difference between the 2 groups ($P>0.05$; Figure 5).

hWJ-MSC transplantation inhibited muscle chronic inflammation

hWJ-MSC transplantation significantly reduced expression of pro-inflammatory cytokines TNF-α and IL-6 and increased

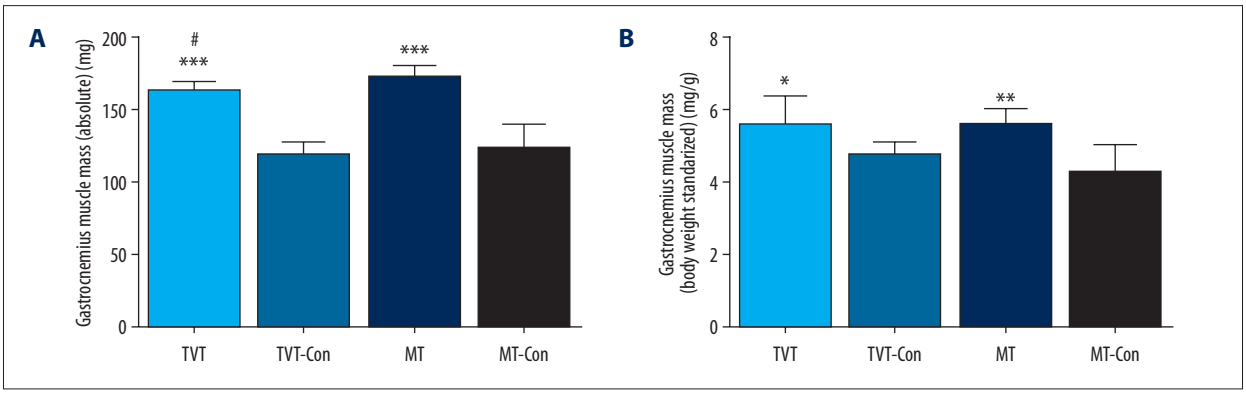

Figure 2. Effect of hWJ-MSC transplantation on muscle mass in sarcopenic mouse model. Absolute gastrocnemius mass (**A**), gastrocnemius mass (body weight standardized) (**B**). * $P<0.05$, ** $P<0.01$, *** $P<0.001$, compared to relative control; # $P<0.05$, compared to MT group. hWJ-MSC – human umbilical cord Wharton's Jelly derived mesenchymal stromal cell; MT – muscular transplant.

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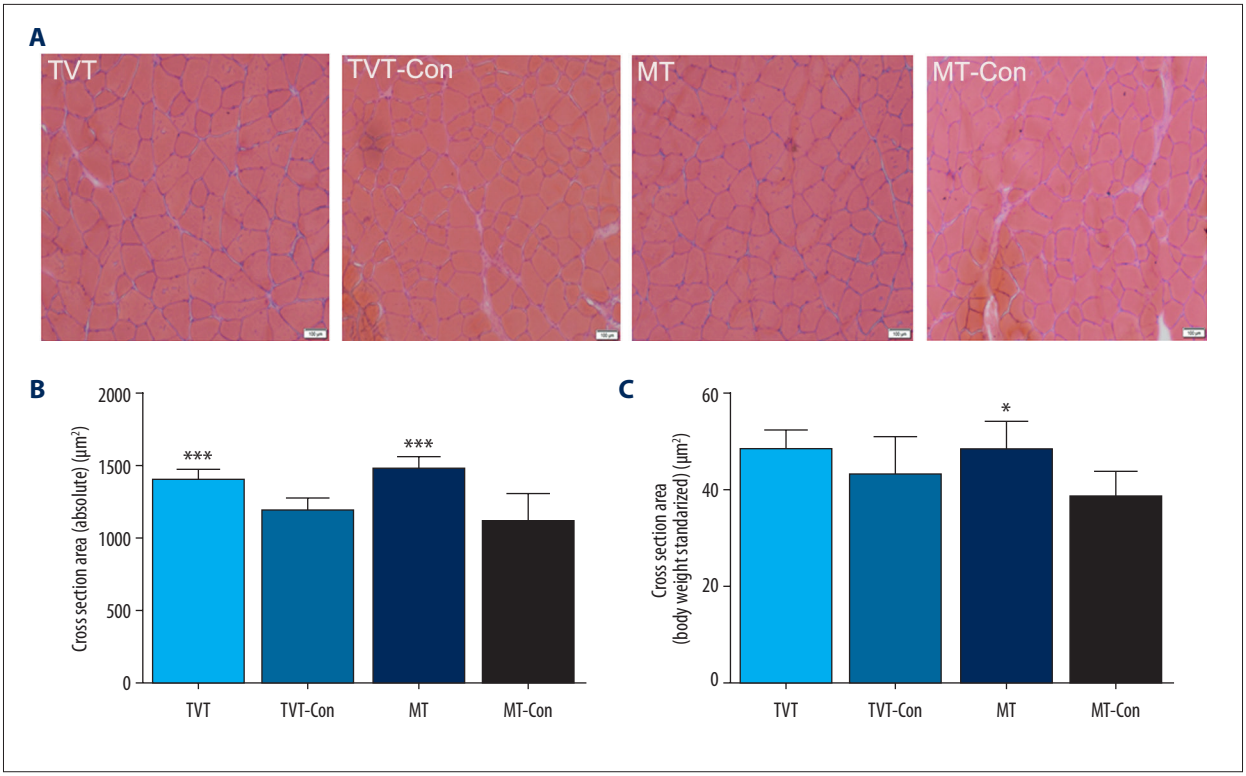

Figure 3. Histology of gastrocnemius muscle and measurements of the cross-sectional area (CSA). Hematoxylin and eosin (H&E) stain (**A**), absolute muscle fiber CSA (**B**), muscle fiber CSA (body weight standardized) (**C**). * $P<0.05$, *** $P<0.001$, compared to relative control.

expression of anti-inflammatory cytokines IL-4 and IL-10 in the gastrocnemius muscle of the TVT group and the MT group ($P<0.05$, n=8 per group; Figure 6). Reduced IL-6 was more prominent in the TVT group compared to the MT group ($P<0.05$; Figure 6), but expression of TNF-α, IL-4, and IL-10 were not different between the 2 groups. hWJ-MSC transplantation inhibited muscle chronic inflammation.

Discussion

hWJ-MSC transplantation was effective in attenuating sarcopenia in aged male C57BL/6J mice induced by HS. hWJ-MSCs increased whole body muscle strength and endurance, gastrocnemius wet weight and CSA of muscle fibers. How stem cell intervention occurs may be through activation of resident skeletal muscle SCs, inhibition of apoptosis, and reduction of chronic inflammation in myocytes of aged mice with sarcopenia.

Sarcopenia is accompanied by a loss of muscle mass and strength, most often arising from aging or disuse. For rodents, muscle atrophy, loss of muscle mass, and force is age-dependent [24]. Studies [5,25–28] show that HS attenuates muscle strength in aged rats, and we observed the parallel results in mice (Figure 1). And our results also showed that hWJ-MSC transplantation significantly improve whole body muscle strength and endurance in aged mice when compared to a control group in post-transplantation or the same group in pre-transplantation (Figure 1). Kim reported increased soleus mass and CSA after human MSCs (derived from bone marrow, adipose tissue, and umbilical cord) or human UCMSC-derived conditioned medium (hUCMSC-CM) injection in HS-induced amyotrophy in young (5-week-old) rats [15,16] and we noted similar results in older animals. Increased gastrocnemius muscle wet weight was more pronounced in the MT mice versus the TVT mice (Figure 2), indicating that treatment via intramuscular injection may be more effective. In addition, myofibers across groups did not differ significantly (Figure 3), and thus increases in gastrocnemius muscle weight may occur by increasing CSA of muscle fibers.

Nowadays, it remains controversial that whether a decrease in the number or regenerative capacity of SCs contributes to sarcopenia [8,9], but studies suggest depressed and blunted recovery of muscle after HS in aged rats [25,29] and SCs are indispensable for myogenesis and muscular recovery. We found that implanted hWJ-MSCs can increase expression of BrdU and Pax-7 in the MT mice and TVT mice compared to controls (Figure 4). Pax-7 is a marker of the total number of quiescent and proliferating SCs [30], and BrdU is frequently used

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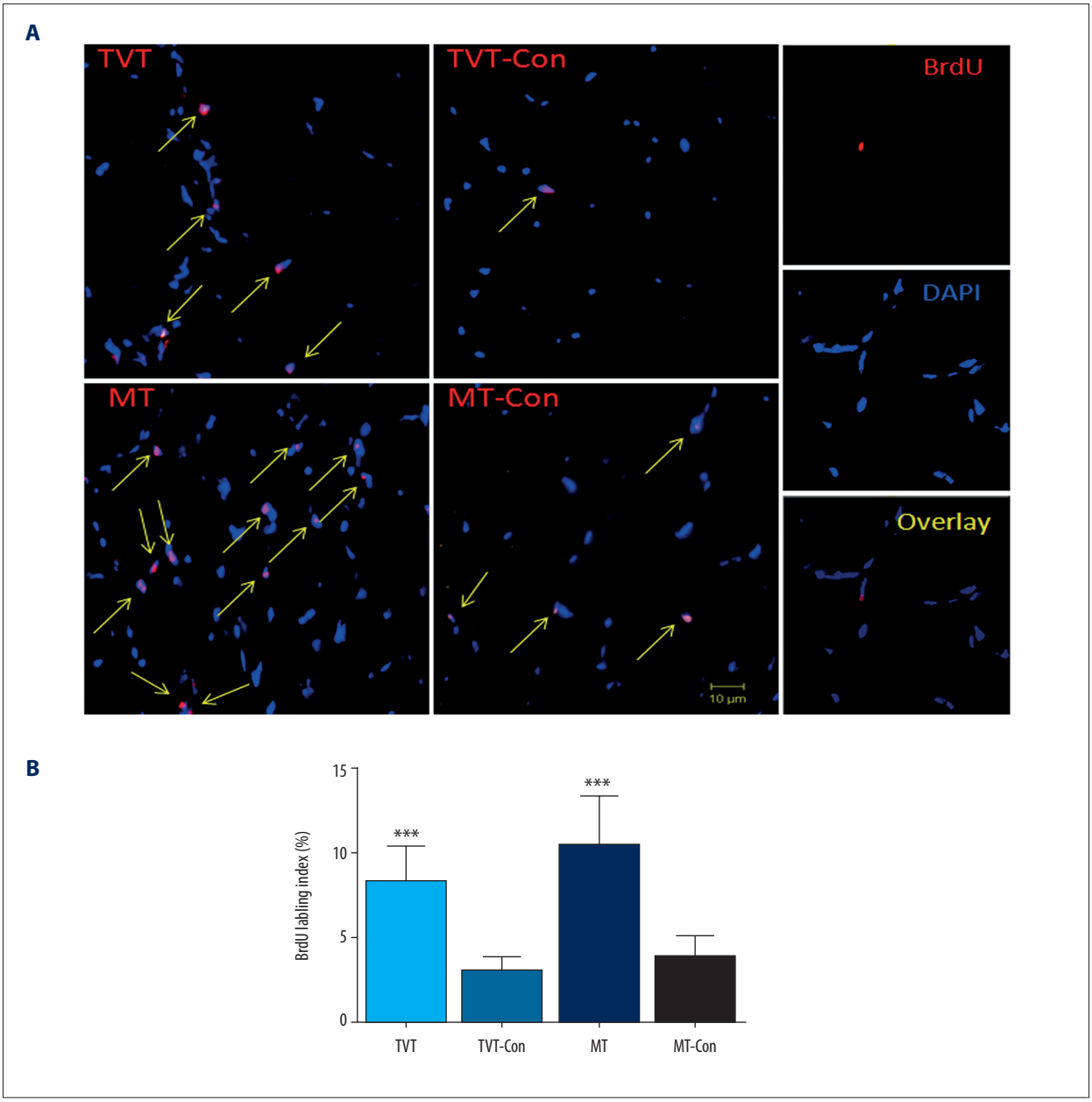
to indicate the proliferated SCs [31]. Hence, our results may suggest that hWJ-MSCs can promote proliferation of resident SCs to some extent, and then may cause differentiation and myogenesis during recovery after HS. Data by Li et al. suggest that SC (BrdU+/Pax-7+ subpopulations) proliferation was significantly greater after bone marrow derived MSC (BMMSC) treatment during immobilization-induced muscle atrophy of young (3-month-old) rats [18]. MSCs may activate resident SCs directly or indirectly to some degree. We did not observe that hWJ-MSCs via tail vein or intramuscular injection were directly differentiated and fused into the original muscle tissue, in other words, no transplanted GFP-tagged hWJ-MSCs were found in gastrocnemius muscle, but transplanted stem cells that can survive in muscle were expected. Therefore, our data agree with the literature [17,18,32,33]. Data show that the reparative effect of MSCs is largely via paracrine action rather than transdifferentiation and cell replacement [34–36]. Moreover, paracrine factors from MSCs may play a role via exosomes [37–39]. Thus, exosomes released by stem cells or other cells may be used to treat sarcopenia in the future.

Sarcopenia is associated with increased apoptosis [6,40,41] leading to muscle wasting. Muscle disuse in aging animals is accompanied by an increase in apoptotic signaling [6,42]. Oxidant stress is also a powerful initiator of mitochondrial damage leading to apoptosis and muscle wasting [43].



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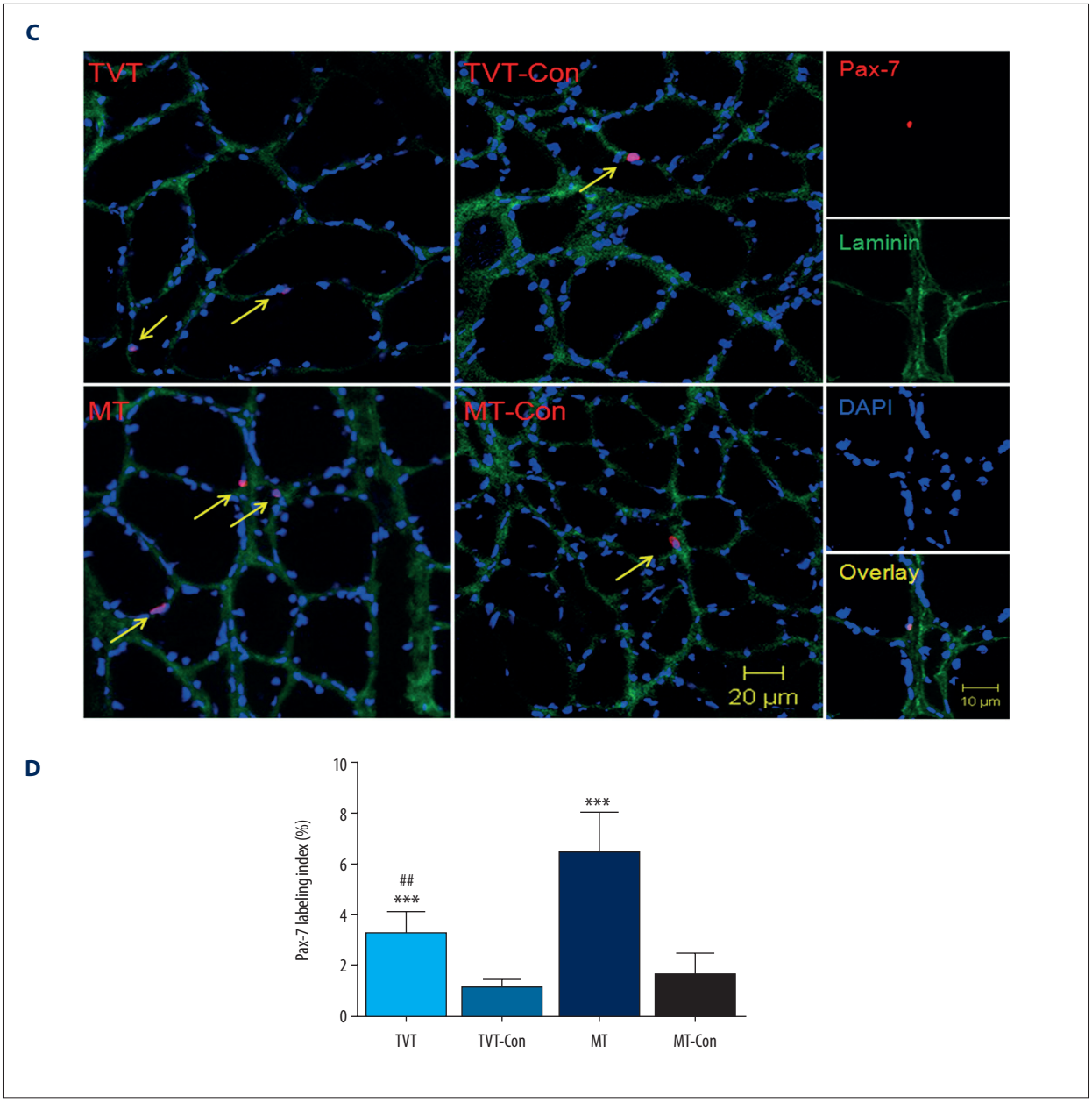

Figure 4. Changes in BrdU and Pax-7 expression in gastrocnemius muscle after hWJ-MSC transplantation. BrdU expression (**A, B**), Pax-7 expression (**C, D**). **A** and **C** show BrdU and Pax-7 immunofluorescent staining of gastrocnemius muscle cross section: Pax7 (red) and BrdU (red) expression, basal lamina (green) and nuclear (DAPI blue) identification. **B** and **D** show BrdU and Pax-7 positive cell index for each group. *** $P<0.001$, compared to relative control; ## $P<0.01$, compared to MT group. Pax-7 – paired box 7; hWJ-MSC – human umbilical cord Wharton's Jelly derived mesenchymal stromal cell; MT – muscular transplant.

Mitochondrial-regulated apoptosis provides a strong signaling network that contributes to sarcopenia [42]. We measured apoptosis with TUNEL staining to reveal DNA fragments. As expected, hWJ-MSC transplantation reduce apoptotic indices of myonuclei in MT and TVT groups compared to controls (Figure 5). This agrees with data from Li who showed that apoptotic myonucleus were reduced after BMMSC treatment in atrophied soleus muscles of young rodents [18]. Myofibers/myocytes are multinuclear cells, and during amyotrophy induced by aging and disuse, nuclei can be reduced by apoptosis to maintain a normal karyoplasmic ratio by reducing sarcoplasmic volume rather than inducing myofiber death, which will inevitably lead to amyotrophy [42]. Thus, apoptosis and amyotrophy may be partly alleviated by hWJ-MSCs in aging and atrophic muscles; however, we didn't conduct researches about the alterations of pro-apoptotic or anti-apoptotic proteins and genes

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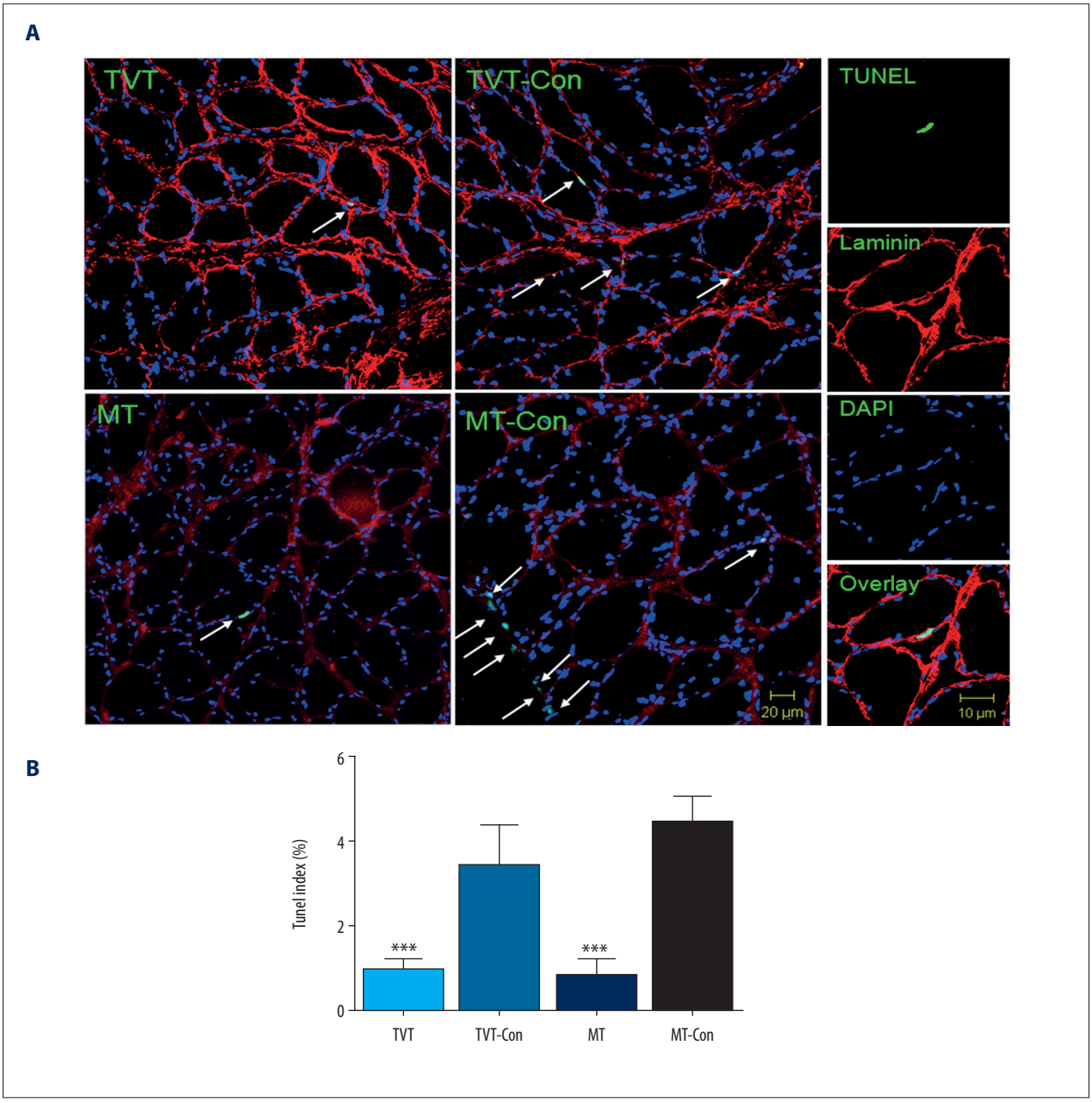

Figure 5. Changes of nuclear apoptotic indices after hWJ-MSC transplantation into gastrocnemius muscles of aged C57BL/6J mice. (**A**) TUNEL immunofluorescent staining of gastrocnemius muscle cross section: representative TUNEL labeled (green), basal lamina (red), and nuclear (DAPI blue) identification. (**B**) Nuclear apoptotic indices as indicated by the number of TUNEL-positive myonuclei to total myonuclei. *** $P<0.001$, compared with relative control. hWJ-MSC – human umbilical cord Wharton's Jelly derived mesenchymal stromal cell; TUNEL – terminal deoxynucleotidyl transferase (TdT)-mediated deoxy-UTP nick end labeling.

of apoptotic signaling pathways, and thus additional studies are required to elucidate the effects of hWJ-MSCs.

Immune cell secretion changes over a lifetime and TNF-α, IL-6, and IL-1 increase causing chronic low-grade inflammation or "inflammaging" [44], a reported contributor to age-related muscle wasting (sarcopenia) [45,46]. And elevated IL-6 and TNF-α have been described in the elderly, along with increased risk loss of skeletal muscle mass and strength [47,48]. The effect of inflammation on the homeostatic balance at the muscle level between protein synthesis and catabolism seems to provide an explanation for the relation between inflammation, muscle strength and muscle mass[49]. We report that transplanted hWJ-MSCs can significantly reduce expression of TNF-α and

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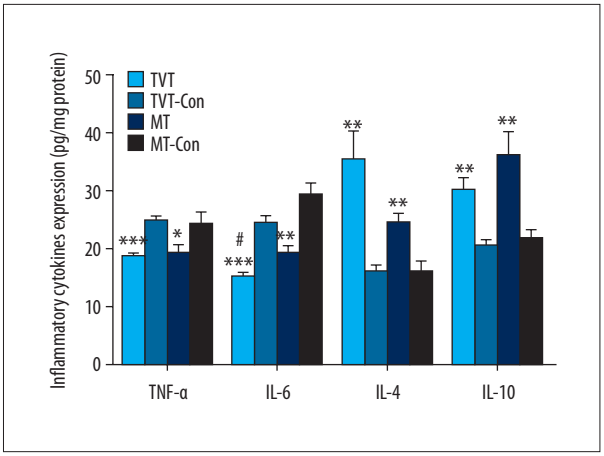

Figure 6. Cytokine expression after hWJ-MSC transplantation into gastrocnemius muscles of aged C57BL/6J mice. The expression levels of IL-4, IL-10, TNF-α, and IL-6 were detected by ELISA. * $P<0.05$, ** $P<0.01$, *** $P<0.001$, versus relative control. # $P<0.05$, compared to MT group. Data are means ± standard deviation and analyzed by a 2-way ANOVA. hWJ-MSC – human umbilical cord Wharton's Jelly derived mesenchymal stromal cell; IL – interleukin; TNF – tumor necrosis factor; ELISA – enzyme-linked immunosorbent assay; MT – muscular transplant.

IL-6 and increase expression of IL-4 and IL-10 in the gastrocnemius muscle of mice (Figure 6). Thus, hWJ-MSC therapy may inhibit chronic low-grade inflammation during rehabilitation in aging muscle after forced disuse. Recently, Pinheiro's group showed that adipose-derived MSCs treatment in mdx mice increased levels of IL-10 and IL-4 and reduced levels of TNF-α and IL-6 [50]. Although utilizing MSCs of different sources to treat 2 different disorders that sarcopenia and muscular dystrophy in rodent models both exists chronic inflammation possibly, we presented parallel data. Thus, our results imply that stem cell transplantation can ameliorate the inflammatory response caused by impaired mitochondria and mediate inflammation imbalance probably in gastrocnemius muscle tissue of aged C57BL/6J mice.

Conclusions

hWJ-MSCs may alleviate sarcopenia in aged male C57BL/6J mice induced by HS and this may occur via activation of resident SCs, reduction of apoptosis, and chronic inflammation in skeletal muscle. These findings provide a theoretical basis for therapy of sarcopenia with hWJ-MSC transplantation as well as a new method for therapy of amyotrophy mediated by long-term bed rest, zero gravity, and immobilization.

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