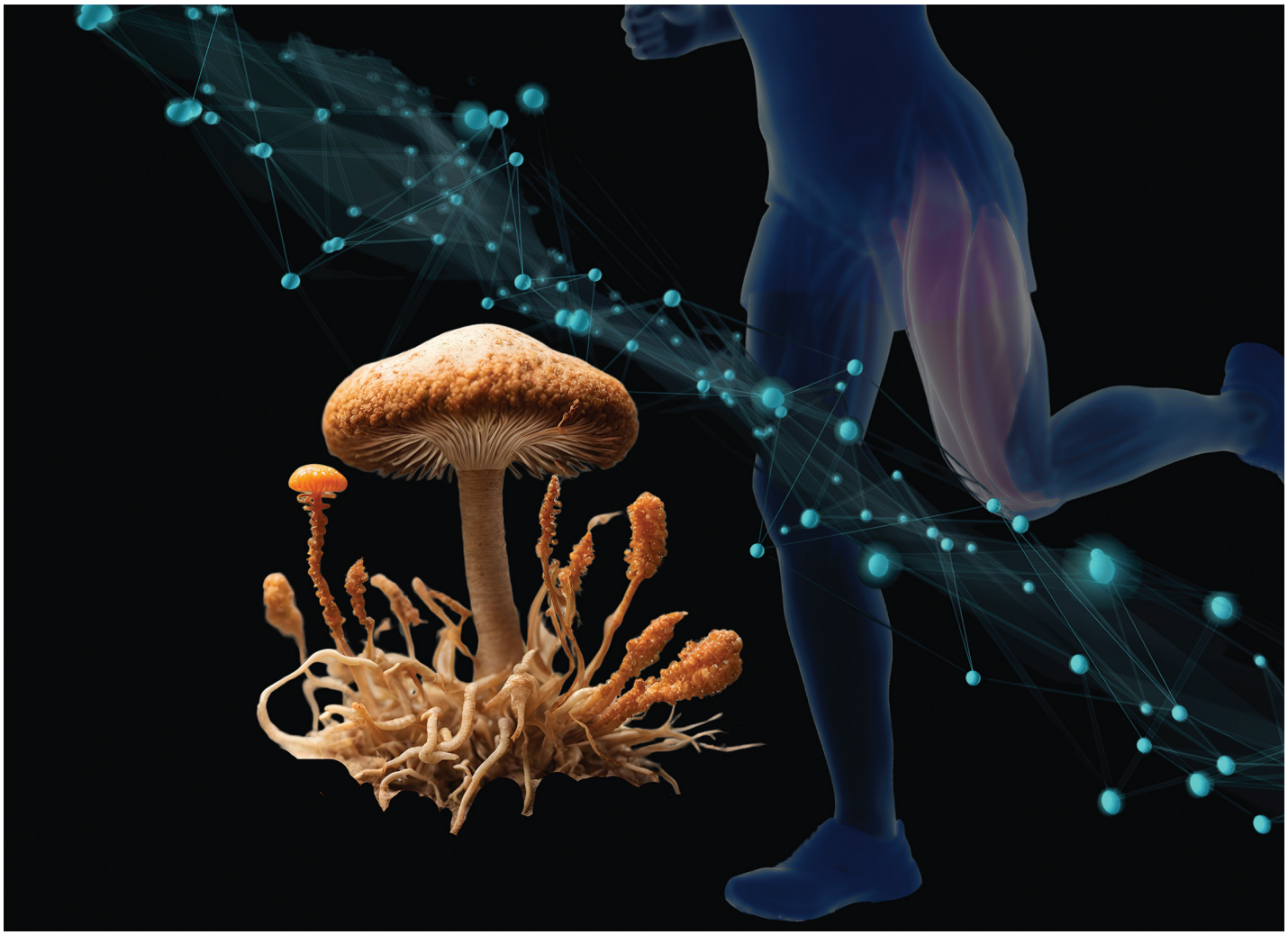

Showcasing research from Professor Chia-Hua Kuo's laboratory, Laboratory of Exercise Biochemistry, University of Taipei, Taipei, Taiwan.

Cordyceps sinensis accelerates stem cell recruitment to human skeletal muscle after exercise

Cordyceps sinensis is a parasitic fungus lethal to some insects and is known to induce a mild immune response in mammals. Nevertheless, immunity is essential for wound healing. In this study, we observed an earlier recovery from the exercise-induced muscle damage when men were pre-conditioned with oral Cordyceps supplementation before high-intensity cycling. This effect is associated with accelerated recruitment of $CD34^{+}/Pax7^{+}$ stem cells into the damaged sites for muscle regeneration. These findings highlight a novel concept of potentiating immunity to expedite exercise recovery using the fungus.

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Cordyceps sinensis accelerates stem cell recruitment to human skeletal muscle after exercise†

Luthfia Dewi, ‡[a,i] Yu-Chieh Liao,‡[a] Wei-Horng Jean,[b] Kuo-Chin Huang,[c] Chih-Yang Huang, [d,e,f,m] Liang-Kung Chen,[g,h,l] Andrew Nicholls,[a] Li-Fan Lai[a] and Chia-Hua Kuo *[a,j,k]

Cordyceps sinensis is a parasitic fungus known to induce immune responses. The impact of *Cordyceps* supplementation on stem cell homing and expansion to human skeletal muscle after exercise remains unexplored. In this study, we examined how pre-exercise *Cordyceps* supplementation influences cell infiltration, $CD34^+$ cell recruitment, and $Pax7^+$ cell expansion in human skeletal muscle after high-intensity interval exercise (HIIE) on a cycloergometer. A randomized, double-blind, placebo-controlled crossover study was conducted with 14 young adults (age: 24 ± 0.8 years). A placebo (1 g cornstarch) and *Cordyceps* (1 g *Cordyceps sinensis*) were administered before exercise (at 120% maximal aerobic power). Multiple biopsies were taken from the vastus lateralis for muscle tissue analysis before and after HIIE. This exercise regimen doubled the VEGF mRNA in the muscle at 3 h post-exercise ($P = 0.006$). A significant necrotic cell infiltration (+284%, $P = 0.05$) was observed 3 h after HIIE and resolved within 24 h. This response was substantially attenuated by *Cordyceps* supplementation. Moreover, we observed increases in $CD34^+$ cells at 24 h post-exercise, notably accelerated by *Cordyceps* supplementation to 3 h (+51%, $P = 0.002$). This earlier response contributed to a four-fold expansion in $Pax7^+$ cell count, as demonstrated by immunofluorescence double staining ($CD34^+/Pax7^+$) ($P = 0.01$). In conclusion, our results provide the first human evidence demonstrating the accelerated resolution of exercise-induced muscle damage by *Cordyceps* supplementation. This effect is associated with earlier stem cell recruitment into the damaged sites for muscle regeneration.

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[a]*Laboratory of Exercise Biochemistry, University of Taipei, Tianmu Campus, Taipei, Taiwan. E-mail: kuochiahua@gmail.com, kch@utaipei.edu.tw*
[b]*Department of Anaesthesiology, Far East Memorial Hospital, No. 21, Sec. 2, Nanya S. Rd, Banciao Dist., New Taipei, Taiwan*
[c]*Department of Family Medicine, College of Medicine, National Taiwan University, Taipei, Taiwan*
[d]*Cardiovascular and Mitochondrial Related Disease Research Center, Hualien Tzu Chi Hospital, Buddhist Tzu Chi Medical Foundation, Hualien 970, Taiwan*
[e]*Center of General Education, Buddhist Tzu Chi Medical Foundation, Tzu Chi University of Science and Technology, Hualien 970, Taiwan*
[f]*Department of Medical Research, China Medical University Hospital, China Medical University, Taichung, Taiwan*
[g]*Center for Healthy Longevity and Aging Sciences, National Yang Ming Chiao Tung University, Taipei, Taiwan*
[h]*Center for Geriatrics and Gerontology, Taipei Veterans General Hospital, Taipei, Taiwan*
[i]*Department of Nutrition, Universitas Muhammadiyah Semarang, Semarang, Indonesia*
[j]*School of Physical Education and Sports Science, Soochow University, Suzhou, China*
[k]*Department of Kinesiology and Health, College of William and Mary, Williamsburg VA, USA*
[l]*Taipei Municipal Gan-Dau Hospital (Managed by Taipei Veterans General Hospital), Taipei, Taiwan*
[m]*Graduate Institute of Biomedical Sciences, China Medical University, Taichung, Taiwan*

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‡These authors contributed equally to this work.

Introduction

Cordyceps sinensis is a parasitic fungus that can survive at high altitudes and is known to induce pro-inflammatory response *in vivo*[1] and enhanced phagocytosis.[2] However, *Cordyceps sinensis* supplementation extends longevity in mice by ~10%.[3] Pro-inflammatory preconditioning is a classic approach to enhance muscle adaptation in humans.[4] *Cordyceps* has been widely consumed by athletes and sport enthusiasts.[5,6] It remains unknown whether pre-exercise *Cordyceps* supplementation influences recovery from muscle damage induced by high intensity exercise.

Muscle regeneration after injury requires inflammation.[7] This wound healing program requires bone marrow stem cells to develop hematopoietic immune cells and specialized progenitors. Both types of cells work in concert to clear unhealthy cells and repopulate the damaged/senescent tissues into a relatively younger cell population,[8] resulting in enhanced fitness of challenged tissues. $CD34^+$ cells are a well-characterized population of bone marrow-derived stem cells that have traditionally been used for cell regeneration in clinical practice.[9,10]

In response to exercise, circulating $CD34^+$ cells transiently increase and then quickly decrease.[11] A significant increase in

$CD34^+$ cells in human skeletal muscle is observed 24 h after resistance exercise, suggesting bone marrow stem cell homing and expansion in challenged tissues.[12] In mice, $CD34^+$ bone marrow stem cell transplantation increased $Pax7^+$ satellite cells in skeletal muscle, demonstrating the hematopoietic contribution to myogenesis.[13] Currently, no study has directly validated the contribution of $CD34^+$ cells to $Pax7^+$ cells in injured human skeletal muscle. In this study, we hypothesized that $CD34^+$ cells contribute to $Pax7^+$ satellite cell expansion in damaged muscle tissues following high intensity exercise.

$Pax7^+$ satellite cells are located between the sarcolemma and basal lamina for nucleus donation to regenerating myofibers of muscle tissues.[14] In this study, we examined dynamical changes of $CD34^+$/$Pax7^+$ cells in human skeletal muscle during a 24 h recovery period following HIIE. Furthermore, the effect of *Cordyceps* supplementation on this exercise response was also examined.

Materials and methods

Cordyceps supplementation

In the study, *Cordyceps sinensis* mycelium (*Paecilomyces hepiali*, TCM777, LivPhcD, TCM Biotech International Corp., Taipei, Taiwan), produced by a GMP manufacturer, was used for pre-exercise consumption. The dosage comprised a total of 3.5 mg of adenosine and 40 mg of polysaccharide. The adenosine content, a recognized active ingredient, may vary depending on the cultured environment. Therefore, the product is standardized based on the adenosine content using HPLC. Polysaccharides, specifically 1,3 : 1,6-β-glucan, present in yeast and mushrooms, are indicators for mushroom and yeast products.

Participants

We recruited sedentary young men (aged 23–30 years) with the body mass index (BMI) ranging from 18–25 kg m^{-2}. A total of 20 candidates were initially enrolled. Six participants dropped out due to time conflict. The remaining 14 participants completed the entire counter-balanced crossover trials. Inclusion criteria were sedentary young men (exercise < 2 times a week) aged between 20 and 30 years. Exclusion criteria were smoking, inflammatory conditions, metabolic conditions, and exercise training. Participants were informed to avoid exercise or receive medical/nutraceutical supplements before HIIE. All participants provided written informed consent before study participation. The experimental protocol was conducted in compliance with the guidelines of the Declaration of Helsinki. Institutional ethical approval was obtained from the Institutional Review Board of University of Taipei with the number IRB-2020-050.

Study design

To assess the effect of pre-exercise *Cordyceps sinensis* supplementation on muscle recovery after HIIE, a randomized placebo-controlled counterbalanced crossover trial was performed. The participants were required to visit the laboratory on seven occasions. A familiarization to the VO_{2max} test and the experimental protocol in the lab was performed on the first visit. The body composition was measured by dual-energy X-ray absorptiometry (DXA) (Lunar iDXA; GE Medical Systems, Madison, Wisconsin, USA) using Encore software V.13.60.033 (Encore, Madison, Wisconsin, USA) to obtain the baseline characteristics of participants (Table 1).

During the second visit, the maximal aerobic power (cycling power at VO_{2max}) of all participants was assessed 2 days after the familiarization. A baseline muscle biopsy was conducted at rest two weeks after the second visit. Participants ingested either *Cordyceps* or placebo capsules (1 g in total), 11 h (0.5 g) and 1 h (0.5 g) before HIIE. Washout period of the crossover trial was 3 weeks. To avoid the potential nutritional effect on the assessed outcomes, a 250 kcal isocaloric standard diet (Ensure; Abbott 58 Nutrition, Taipei, Taiwan) was provided 12 h and 2 h prior to the challenge and 12 h before the last muscle biopsy (24 h post-HIIE). Participants were asked to maintain their normal diet and were allowed drinking water *ad libitum* for the rest of the time. The daily energy intake of participants was 1760 kcal (carbohydrate 44%, fat 39%, protein 17%) during the placebo trial and 1714 kcal (carbohydrate 45%, fat 38%, protein 17%) during the *Cordyceps* trial, according to a 3-day dietary recall survey by a registered dietitian. Muscle samples from vastus lateralis were collected by needle biopsy 3 h on one leg and 24 h on contra-lateral leg following HIIE. The collected biopsied muscles were used for quantitative PCR analysis (VEGF and CD34 mRNA) and immunofluorescence double staining.

Maximal aerobic power (W_{max})

An incremental exercise protocol was performed in the daytime on a cycle ergometer (Monark LC6, Stockholm, Sweden). To obtain reliable W_{max} at VO_{2max}, participants were familiarized with the cycle ergometer, the Borg scale 0–10, and the technical details of the testing. The seat height was adjusted to align with the participants' preferred knee joint flexion when the pedals were in the bottom position. Additionally, the handlebar height was adjusted to accommodate the trunk and arms. The individual setup recorded during the familiarization phase was replicated in the experimental session to ensure consistency in positioning. The Cortex gas analyser system (Metalyzer, Leipzig, Germany) was

Table 1 Subject characteristics

$N = 14$	Mean ± SE
Age (y)	24 ± 0.8
Height (cm)	172 ± 1.8
Weight (kg)	68 ± 1.7
BMI (kg m^{-2})	23 ± 0.6
VO_{2max} (mL min^{-1} kg^{-1})	42 ± 1.1
Bone (kg)	2.7 ± 0.07
Bone to weight ratio (%)	4.0 ± 0.09
Muscle mass (kg)	51 ± 1.3

connected to a mask to collect and analyse the participant's expired air. Gas values (O_2 and CO_2) were recorded each second by using the gas analyser system using MetaSoft Studio software. Heart rate signals were transferred wirelessly using Polar H10 (Polar, Kuala Lumpur, Malaysia) synchronized with the gas analyser. The gas analyser was calibrated daily during the exercise session. The maximal aerobic power (W_{max}) at VO_{2max} was established before conducting HIIE at 120% W_{max}. The VO_{2max} protocol was started with warming up for 3 min at 50 watts, followed by resting for 3 min. The incremental exercise protocol commenced at 125 W and was increased by 25 W every 2.5-minute interval until the participant reached exhaustion. Participants were instructed to maintain a pedal cadence of 60 to 70 rpm. To determine the VO_{2max} value, participants needed to meet at least two of the following four criteria: (1) respiratory exchange ratio ≥ 1.2, (2) heart rate ≥ 95% of the age-estimated maximal heart rate for >30 seconds, (3) rated perceived exertion (RPE) on the Borg scale reaching 9–10, and (4) inability to sustain a cadence of 60 rpm for more than 10 seconds. The VO_{2max} was determined by calculating the mean value of the final 30 seconds of oxygen uptake, while the W_{max} was the maximum work rate achieved in the final stage.[15]

High-intensity interval exercise (HIIE)

Participants performed HIIE in the morning (around 10:30 am). The HIIE protocol utilized a cycling exercise procedure based on a previously established method.[8] This involved exercising at an intensity of 120% of W_{max}, maintaining a pedal cadence of 90 rpm, for 20 s, followed by a 20 s rest interval. This pattern was repeated for a total of 15 sets. The HIIE protocol was programmed using the Monark Test Software (Ver. 1.1.9.0) and conducted on the same Monark LC6 cycloergometer for VO_{2max} determination. Prior to the HIIE session, each participant commenced with a 4 min warm-up phase at a comfortable load of 50 W, followed by a 3 min rest period. During the HIIE, participants were instructed to increase their cadence within 3–5 s to reach a target of 90 rpm at the conclusion of each rest phase. The total work achieved during the exercise from both the placebo and *Cordyceps* trials (placebo: 19 779 kJ; *Cordyceps*: 19 624 kJ) was similar across all participants.

Muscle biopsy

The muscle biopsy procedure followed the methodology outlined in a previous study.[8] A qualified physician performed the muscle biopsy using an 18-G Temno disposable cutting needle (Cardinal Health, McGaw Park, Illinois, USA) under the administration of local anaesthesia (2% lidocaine). Muscle tissue samples were obtained from the vastus lateralis, located at a depth of 3 cm and 20 cm proximal to the kneecap. Three weeks following the baseline muscle biopsy, two consecutive muscle biopsies for tissue collection were performed 3 h and 24 h following HIIE from the contralateral leg at the same position during each trial. Muscle samples were immediately placed into a microtube containing 4% of paraformaldehyde (PFA) solution for immunofluorescence and hematoxylin and eosin (HE) staining.

Hematoxylin and eosin (HE) staining

Muscle necrosis and centrally nucleated myofibers of muscle cross-section were assessed by HE stains according to the previous protocol.[8] A histological procedure was conducted by professional pathologists from Toson Technology Corporation (Zhubei City, Hsinchu, Taiwan). Formalin-fixed paraffin embedded muscle tissues were sliced 2–3 μm thick, deparaffinized in xylene followed by rehydrating in a graded alcohol series using ethanol (99.9%, 95%, 85% and 75%) each for 2 min, and stained with hematoxylin (for nuclei) for 3 min (BioTnA Inc., TA01HE, Kaohsiung, Taiwan). The tissue sections were stained using eosin (for the cytoplasm) for 15 s and dehydrated in ethanol after being washed in distilled water.

Immunofluorescence staining for $CD34^+$ and $Pax7^+$cells

Double staining was performed to localize $CD34^+$ cells and $Pax7^+$ cells on a muscle cross-section for each participant. Glass slides were first incubated with primary antibodies against Pax7 and CD34 protein antigens. The primary antibody against CD34 was rabbit anti-CD34 (human) (EP373Y) (ab81289, 1 : 200, Abcam, Cambridge, UK). The secondary antibody used was IgG (H + L)-488 goat anti-rabbit (TAFB01-F, Biotna, Kaohsiung, Taiwan), which reflects bright green fluorescence. The primary antibody against Pax7 was mouse anti-Pax7 (human) (clone 2F12H4) (MABD20, 1 : 200, Merck, Darmstadt, Germany). The secondary antibody was IgG (H + L)-594 goat anti-mouse (TAAB01-T, Biotna, Kaohsiung, Taiwan) which reflects red fluorescence. Nuclei were labelled with DAPI (4′,6-diamindino-2-phenylindole) (TA01DP, Biotna, Kaohsiung, Taiwan) after incubation (5–10 min) in the dark at room temperature, followed by 4 times rinsing in PBS buffer.

Imaging and quantification

To obtain unbiased results, HE staining and immunofluorescence staining of muscle tissues were analysed by two independent assessors according to the same counting criteria. Results were accepted only when values from both assessors achieved the intraclass correlation coefficient (ICC) > 0.7.

HE staining was analysed using Motic Digital Slide Assisstant System Lite 1.0 (Motic Hong Kong Limited, Hong Kong, China) at ×40. The criterion to identify the necrotic area included shrinking myofibers (disintegrated membrane) associated with cell aggregation > 7 nuclei in the wide spaces between the myofibers of muscle cross-sections. The nuclei located in the central myofiber (centronucleation) was a marker for damage-induced myofiber regeneration. For immunofluorescence, representative images were captured at ×20 magnification using an OLYMPUS OlyVIA 3.21. (OLYMPUS, Tokyo, Japan) with a scale bar ranging 200–500 μm. To avoid the effect of colouring background, the brightest slide was manually set. The captured images were analyzed using ImageJ (National Institute of Health, USA) to determine the positive area. The positive markers of $CD34^+$

(green), $Pax7^+$ (red), and $CD34^+/Pax7^+$ cells (yellow) were set at a minimal size of 0.8 μm^2 based on the smallest diameter of detectable $Pax7^+$ cells (1 μm).[16]

Real-time polymerase chain reaction (RT-PCR)

The current study used the RNeasy® Fibrous Tissue Mini Kit (QIAGEN, Hilden, North Rhine-Westphalia, Germany) to extract total RNA from the biopsied muscle. Approximately, 5 mg of muscle tissue was homogenized using 300 μl of buffer RLT with a POLYTRON® system (PT 3100 D, KINEMATICA AG, Malters, Switzerland) on ice. The buffer RLT contained guanidine thiocyanate, which inhibited the activity of endogenous ribonucleases. After incubating the lysate with RNase-free water (590 μl) and proteinase K solution (10 μl) at 55 °C for 10 min to digest proteins and release nucleic acids, RNA was isolated by binding to the silica membrane of the RNeasy Mini spin column. The addition of 450 μl of 99.5% ethanol and centrifugation facilitated RNA separation. DNase treatment at room temperature for 15 min on the silica membrane eliminated DNA contaminants. The DNase and contaminants were removed by using RPE buffer, and RNA was eluted by collecting in the tubes with 30 μl of RNase-free water.

The extracted RNA was reverse transcribed into cDNA using the iScript™ cDNA Synthesis Kit (Bio-Rad, Hercules, California, USA) following the manufacturer's instructions. The reaction mixtures for reverse transcription were incubated with an initial priming step at 25 °C for 5 min, followed by reverse transcription at 46 °C for 20 min. The PCR samples were subjected to a melting curve analysis. Inactivation was carried out at 95 °C for 1 min, followed by holding at 12 °C.

All reverse-transcribed cDNA was amplified by performing RT-PCR according to the manufacturer's instructions. All samples were measured in duplicate. The reaction mixture contained the iTaq Universal Probes Supermix (Bio-Rad, Hercules, California, USA) (1–2 μl), specific primers and probes. The primers and probes used to amplify the target were supplied from the Bio-Rad PrimePCR™ probe assay according to the unique assay ID as follows: VEGF, qHsaCEP0051468; CD34, qHsaCIP0026476; Pax7, qHsaCIP0030375; and internal standard RPP30, qHsaCEP0052683. Amplification was carried out on a CFX96 Touch Real-Time PCR Detection System (Bio-Rad, Hercules, California, USA) following the standard protocol, which involved polymerase activation and DNA denaturation at 95 °C for 30 s, followed by 40 amplification cycles at 95 °C for 5 s and 55 °C for 30 s. Gene expression, normalized to the geometric mean of a housekeeping gene (RPP30), was quantified using the $2^{-\Delta\Delta Ct}$ method and expressed as fold difference relative to RPP30.

Statistical analysis

Statistical analysis was performed using SPSS 27.0 (IBM, NY, USA). A paired *t*-test was used to compare the difference between baseline and post-exercise values. Treatment difference between the placebo and *Cordyceps* trials was also compared by a paired *t*-test. Data are presented as mean ± standard error (SE). Type 1 error of $P \leq 0.05$ was considered significant and $P < 0.1$ was considered moderately significant. Cohen's *d* indicated the effect size. The value of *d* was classified into small effect (<0.5), medium effect (0.5–0.7), and large effect (≥0.8).

Results

HIIE-induced VEGF expression returned to the baseline in 24 h

Effects of HIIE on VEGF mRNA in skeletal muscle under placebo- and *Cordyceps*-supplemented conditions are shown in Fig. 1A. HIIE doubled VEGF mRNA levels in skeletal muscle in 3 h to a similar level for the placebo (+117%, $P = 0.006$, $d = 1.2$) and *Cordyceps*-supplemented conditions (+108%, $P = 0.02$, $d = 0.9$). VEGF mRNA returned to the baseline in 24 h. HIIE induced CD34 mRNA (Fig. 1B) in skeletal muscle at 24 h of

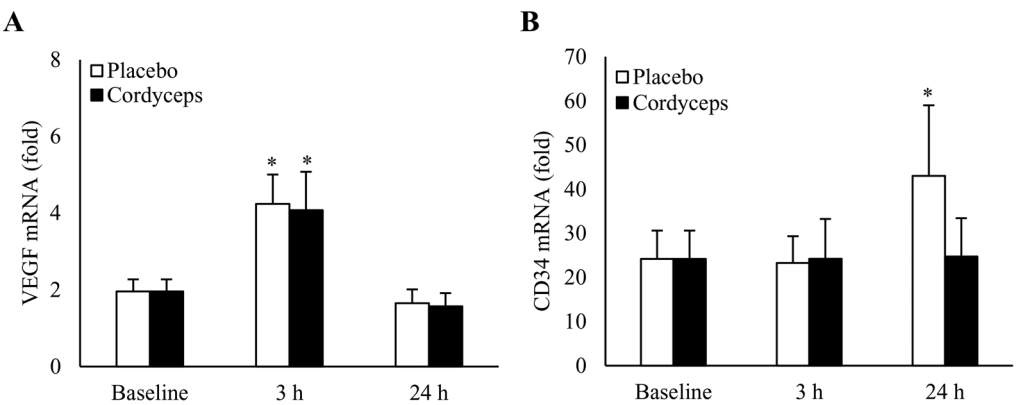

Fig. 1 PCR analysis for human skeletal muscle after high-intensity interval exercise (HIIE). The increases in VEGF mRNA returned to baseline 24 h after HIIE (A). The responses for the placebo- and *Cordyceps*-supplemented conditions were similar. CD34 mRNA in human skeletal muscle increased 24 h after HIIE and this response was absent after *Cordyceps* supplementation (B). *$P \leq 0.05$ compared to pre-exercise baseline. Data are presented as mean ± SE. Value expresses as fold difference relative to RPP30 (internal standard).

recovery (+78%, P = 0.05). No change in CD34 mRNA was observed in the *Cordyceps*-supplemented condition.

Cordyceps supplementation attenuated HIIE-induced cell infiltration

Cell infiltration in the necrotic area is indicated on the HE stains, as shown in Fig. 2. HIIE-induced necrosis (+284%) was observed at 3 h of recovery period (P = 0.04). This muscle damage response was resolved in 24 h. Under the *Cordyceps*-supplemented condition, this HIIE-induced damage response was minimal. Centronucleation is a conventional marker of damage-induced myofiber regeneration (Fig. 3). The pattern of this response after HIIE is similar to necrotic cell infiltration, but was only moderately significant. The myofiber density in the muscle tissues was 78 myofibers per mm^2.

CD34$^+$ cell homing in the necrotic area of human skeletal muscle

To further characterize specific types of infiltrated cells in the necrotic area of muscle tissues, we conducted immunofluorescence analysis using muscle cross-sections (Fig. 4), which indicated highly concentrated CD34$^+$ cells localized in the necrotic area and contributed to Pax7$^+$ cells, demonstrated by double staining of CD34$^+$/Pax7$^+$ cells. The average sizes of CD34$^+$ and Pax7$^+$ cells were 13.5 (6.0–75.8) and 8.8 (6.0–266.1) μm, respectively. Representative immunofluorescence stain images of CD34$^+$ cells (green), Pax7$^+$ cells (red), and CD34$^+$/Pax7$^+$ cells (merge, yellow) in the muscle sample are shown in Fig. 4A for non-necrotic areas and Fig. 4B for necrotic areas. Immunofluorescence images indicate that CD34$^+$ cells were highly concentrated in the necrotic areas of human skeletal muscle, regardless of exercise status.

Cordyceps accelerated recruitment of CD34$^+$/Pax7$^+$ cells to exercised muscle

HIIE induced a significant increase in CD34$^+$ cells in skeletal muscle by 38% at 24 h of recovery period (Fig. 5A) (P = 0.04, d = 0.6). This effect occurred faster after pre-exercise *Cordyceps* supplementation, leading to an earlier increase of 51% at 3 h of recovery period (P = 0.002, d = 0.9). The HIIE-induced change was accompanied by protracted increases in Pax7$^+$ cells from 3 h to 24 h after HIIE (P = 0.05, d = 0.7). *Cordyceps* supplementation resulted in a more pronounced response on Pax7$^+$ cell expansion both at 3 h (P = 0.04, d = 0.7) and 24 h post-exercise (P = 0.03, d = 0.7), with a moderate difference between treatments (P = 0.1, d = 0.5) (Fig. 5B).

To further determine the contribution of CD34$^+$ cells to Pax7$^+$ cells in exercised skeletal muscle, immunofluorescence double staining was performed (Fig. 5C). A 4-fold increase in CD34$^+$/Pax7$^+$ cells in the entire muscle cross-section was observed 24 h after HIIE (P = 0.02, d = 0.9), and this increase was accelerated by *Cordyceps* supplementation to a similar level at 3 h post-exercise (P = 0.01, d = 0.9). CD34$^+$ cells were much more concentrated in the necrotic region of myofibers. In the necrotic region (Fig. 5D), HIIE increased the contribution of CD34$^+$ cells to Pax7$^+$ cells from 5% to 11% (P = 0.01, d = 0.8), and returned to the pre-exercise baseline.

Discussion

High intensity exercise causes muscle damage and induces cell infiltration in human skeletal muscle.[8] In this study, we asked the question whether the infiltrated cells in the necrotic sites of exercised human skeletal muscle are associated with CD34$^+$ cell homing. In addition, we measured the contribution of CD34$^+$ cells to Pax7$^+$ satellite cells after HIIE by immunofluorescence co-staining. The effect of *Cordyceps* supplementation on this damage response was also assessed after HIIE. The major findings of the study are: (1) CD34$^+$ cells are highly concentrated in the necrotic area of human skeletal muscle and contributed to Pax7$^+$ satellite cells; (2) pre-exercise *Cordyceps* supplementation accelerated HIIE-induced CD34$^+$ cell infiltration in skeletal muscle; (3) CD34$^+$/Pax7$^+$ cells in skeletal muscle increased by ∼4-fold 24 h after HIIE and this response was accelerated to occur in 3 h post-exercise by *Cordyceps* supplementation; (4) pre-exercise *Cordyceps* supplementation accelerated resolution of HIIE-induced necrosis in human skeletal muscle.

Effects of *Cordyceps* supplementation in exercise-induced muscle inflammation have not previously been reported. The results of the study provide the first human evidence which demonstrates an accelerated resolution of exercise-induced muscle inflammation by pre-exercise *Cordyceps* supplementation. Cell infiltration in the necrotic area of human skeletal muscle indicated by HE stains was expanded by nearly 4-fold 3 h after the HIIE protocol from the pre-exercise baseline. Inflammation is known as an immune response essential for muscle regeneration after damage.[7,17] The fungus *Cordyceps sinensis* has been reported to increase phagocytosis of human monocytes,[2] the first phase of the inflammation program before entering the regenerative phase to heal injured skeletal muscle.[7] Thus, the early resolution of muscle necrosis by pre-exercise *Cordyceps* supplementation may have been associated with potentiation of phagocytosis before and during HIIE. The results of the study provide a scientific basis to support a pre-conditioning strategy of using a pro-inflammatory stimulus to accelerate the resolution of muscle inflammation following high intensity exercise.

To further characterize the specific type of infiltrated cells in human skeletal muscle, we identified that CD34$^+$ cells are highly concentrated in the necrotic area (disrupted myofibers) of human skeletal muscle. This result indicates that HIIE directs CD34$^+$ bone marrow stem cell homing to damaged sites of human muscles. In mice, bone marrow stem cell transplantation regenerates skeletal muscle after an irradiation-induced injury.[18] Increasing VEGF expression in tissues seems to require the recruitment of CD34$^+$ bone marrow stem cells into injured tissues from circulation.[19–22] In this study, we confirmed increased VEGF expression 3 h after HIIE concurrent with CD34$^+$ cell infiltration in human skeletal muscle. Taken

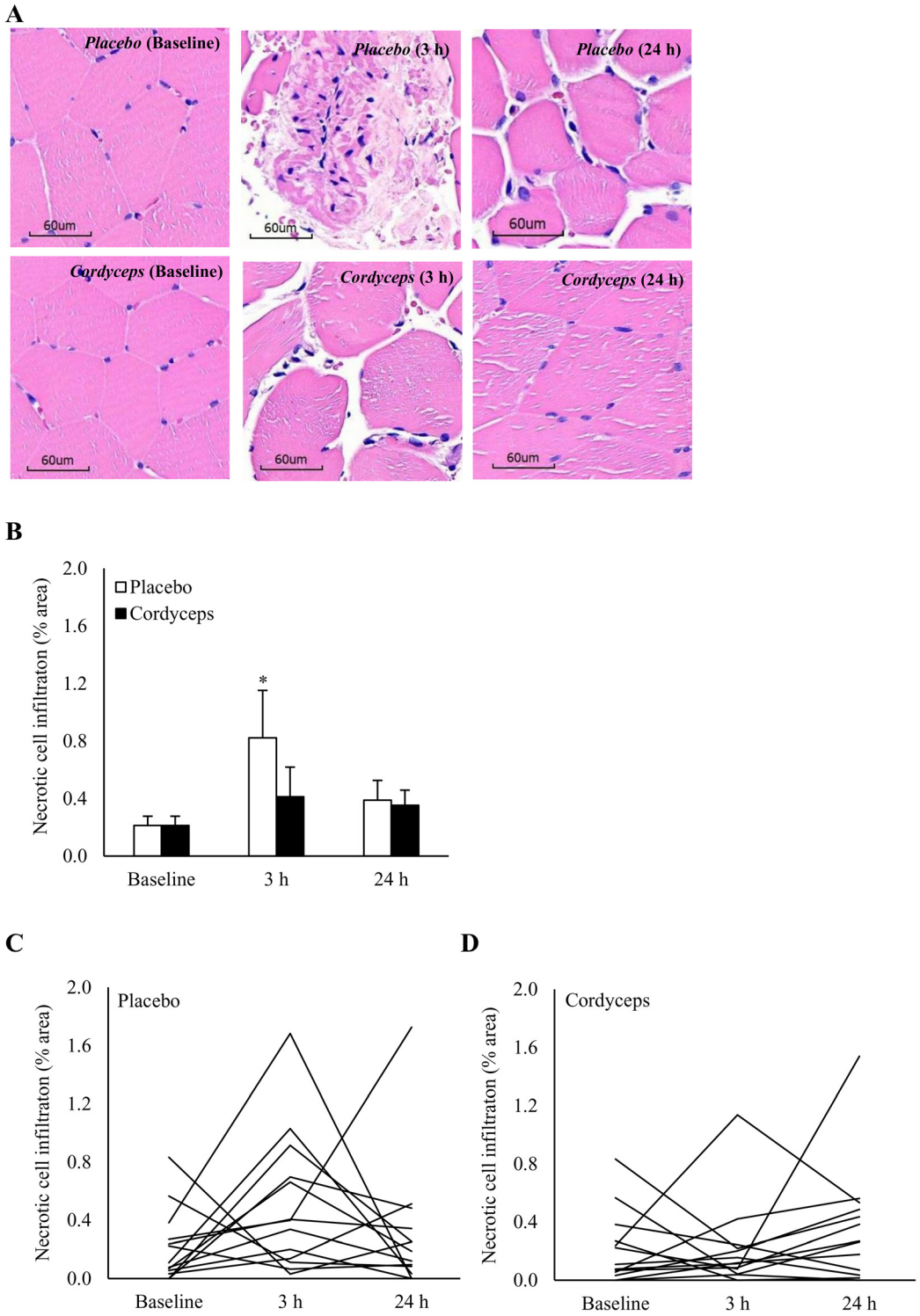

Fig. 2 Effect of pre-exercise *Cordyceps* supplementation on muscle necrosis after high-intensity interval exercise (HIIE). Representative HE stains images of necrotic cell infiltration in human vastus lateralis (A). HIIE increased the necrotic area with increased cell infiltration, which was attenuated by *Cordyceps* supplementation (B). Individual response to HIIE in the placebo-supplemented (C) and *Cordyceps*-supplemented (D) conditions. *$P \leq 0.05$ compared to pre-exercise baseline. Data are presented as mean ± SE. Scale bars = 60 μm.

together, the results of the study suggest the role of $CD34^+$ bone marrow stem cells in exercise-induced muscle regeneration in humans.

In this study, increased CD34 mRNA in human skeletal muscle was observed 24 h following HIIE. This change may be important for developing myogenic phenotype of infil-

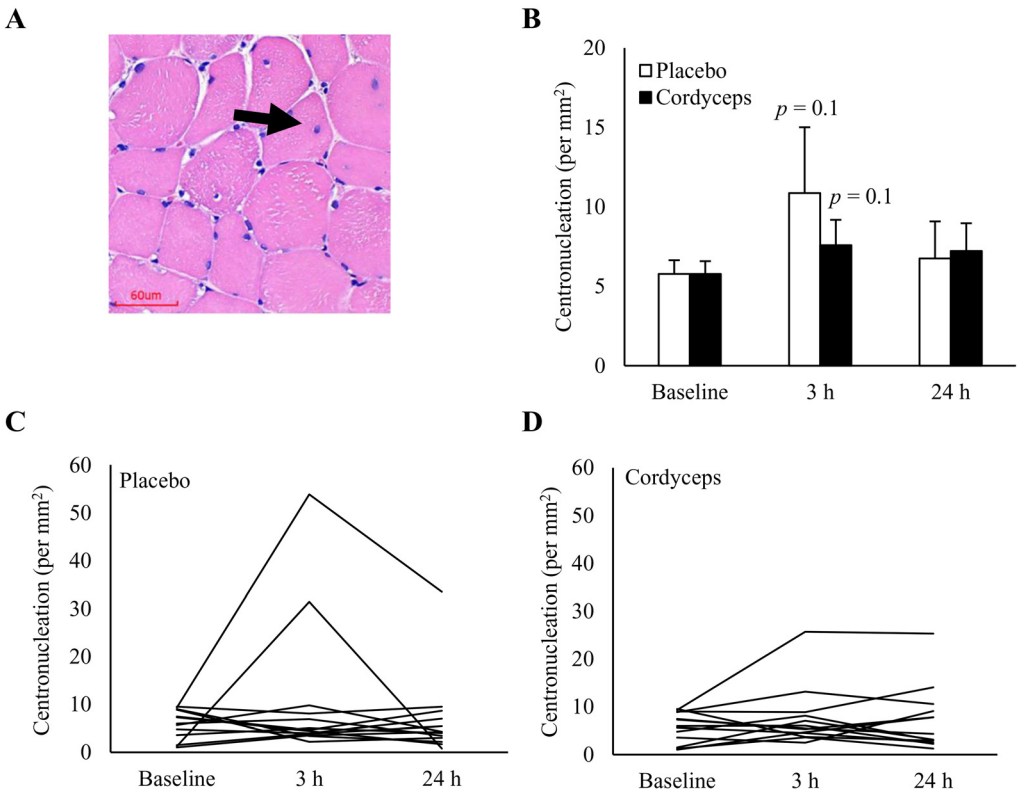

Fig. 3 Effect of pre-exercise *Cordyceps* supplementation on centrally nucleated myofibers after high-intensity interval exercise (HIIE). A representative HE staining image indicating centrally nucleated myofibers in human vastus lateralis (A). HIIE moderately increased centronucleation at 3 h of recovery (P = 0.1) (B). Individual responses to HIIE in the placebo-supplemented (C) and *Cordyceps*-supplemented (D) conditions. Data are presented as mean ± SE. Scale bars = 60 μm.

trated stem cells. This is suggested by a defective muscle regeneration after muscle injury in CD34-deficient mice.[23] In this study, we do not observe significant responses in CD34 expression 3 h and 24 h after exercise in the *Cordyceps*-supplemented trial. This may have been associated with the faster resolution of muscle inflammation. However, due to limited time points of the muscle samples, we could not rule out the possibility of an earlier response of CD34 expression in skeletal muscle between 3 h and 24 h of recovery time under the *Cordyceps*-supplemented condition.

Evidence for $Pax7^+$ satellite cells developed from $CD34^+$ bone marrow stem cells was mostly reported *in vitro* and in rodents.[13,24] In this study, we provided the first human evidence to validate the putative bone-to-muscle development, the increases of $CD34^+/Pax7^+$ cells in the damaged area of muscle tissues. $CD34^+$ bone marrow stem cells contributed to ~11% of $Pax7^+$ satellite cells in the necrotic area of the exercised muscle. $Pax7^+$ satellite cells are well-known as the major type of muscle stem cell to regenerate myofiber by fast nuclear donation into the cytoplasmic space. Mobilization of undifferentiated bone marrow stem cells is required to support muscle regeneration, since the amount of myogenic precursor exceeds resident satellite cells.[25] Exercise can transiently deplete $Pax7^+$ satellite cells and rapidly replenish them within 3 h after exercise, a process unlikely to rely on cell proliferation within such a short period.[26] The result of the study further explains the requirement of $CD34^+$ bone marrow cell homing to replenish $Pax7^+$ cell pool in skeletal muscle. $Pax7^+$ satellite cells were doubled after HIIE over the course of a 24 h recovery period, suggesting a further satellite cell expansion after the early $CD34^+$ cell homing during recovery.[27]

Pre-exercise *Cordyceps* supplementation accelerated recruitment of $CD34^+$ bone marrow stem cells to the exercised human skeletal muscle 3 h post-exercise. This increase was followed by a sustained $Pax7^+$ cell expansion in the same muscle during 3–24 h post-exercise. This result strengthens the previous report indicating the stimulatory effect of *Cordyceps* on both stem cell proliferation and differentiation.[28] This effect seems to associate with the pro-inflammatory properties of *Cordyceps*. $CD34^+$ bone marrow stem cell homing to injured tissues is known to involve with pro-inflammatory cytokine release during the early phagocytic phase of inflammation.[29] Taken together, $CD34^+$ cell recruitment to injured sites plays a crucial role in regenerating human skeletal muscle following HIIE-induced muscle inflammation.[30] This tissue remodelling can be enhanced by pre-conditioning with *Cordyceps* supplementation to explain the early resolution of HIIE-induced muscle inflammation.

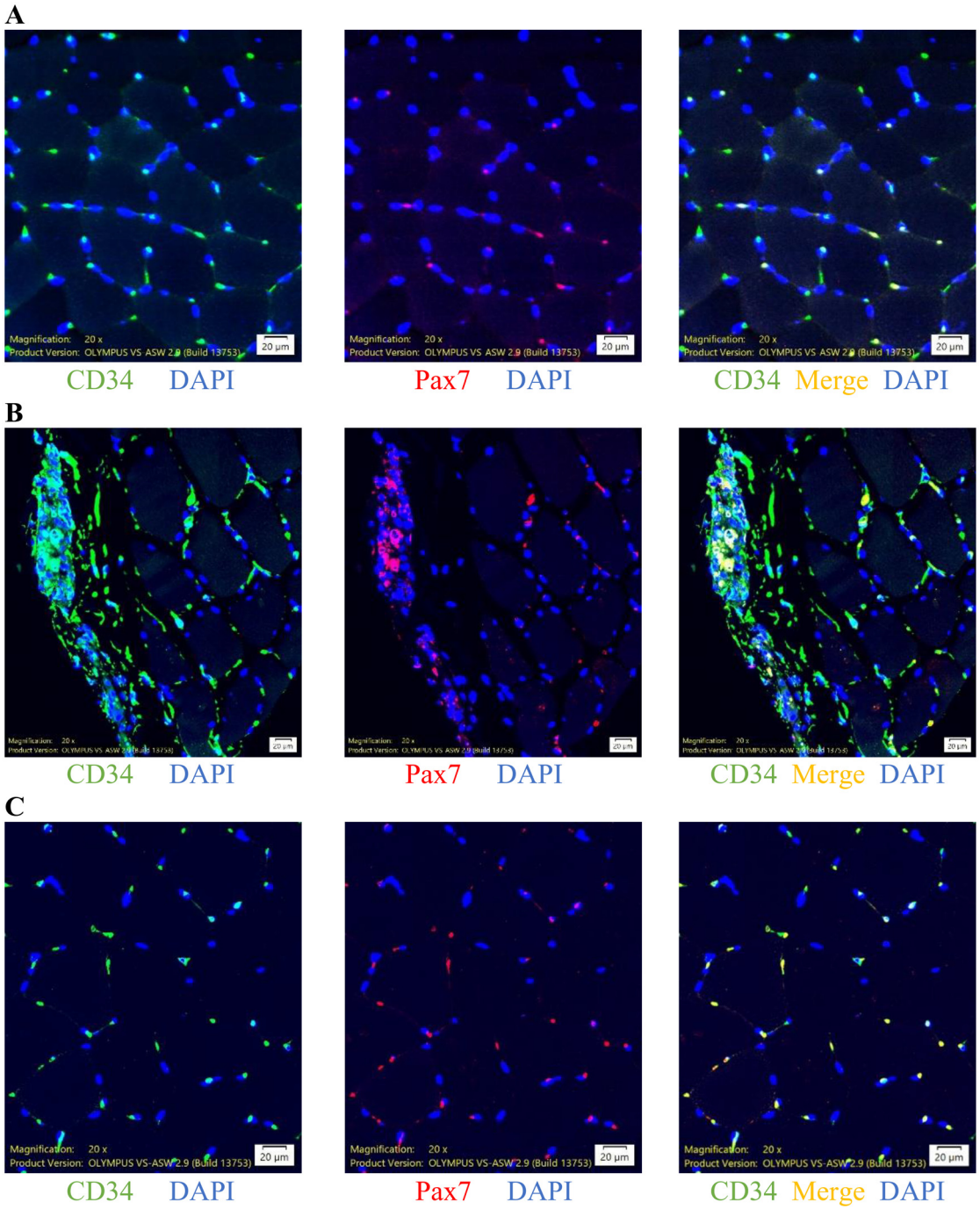

Fig. 4 Immunofluorescence co-staining for stem cells ($CD34^+/Pax7^+$) in human vastus lateralis. Representative immunofluorescence stain images localize cells expressing antigens against CD34 antibodies (green), Pax7 antibodies (red), and merge (yellow) in the muscle cross-sections. Pre-exercise muscles are illustrated in (A). Following high intensity interval exercise, stem cells ($CD34^+$, $Pax7^+$ and $CD34^+/Pax7^+$) were increased and particularly concentrated in the necrotic area of muscle at 3 h of recovery (B). This response returned to pre-exercise baseline in 24 h (C). Scale bars = 20 μm.

Limitations

It is worthy to note that $CD34^+$ cells contributed to ~11% of $Pax7^+$ cells in the necrotic area of human skeletal muscle. $CD34^+$ bone marrow stem cells are multipotent which can develop into other progenitors, such as vascular endothelial cells, neural stem cells, adipocytes, smooth muscle, and fibroblasts.[31,32] Future studies are needed to elucidate relative contributions of $CD34^+$ cells to develop specific cell types in human muscle tissues and the mediators involved in this cell-type specific regulation.[33] Furthermore, the results of the study on young participants present a limitation for knowledge gen-

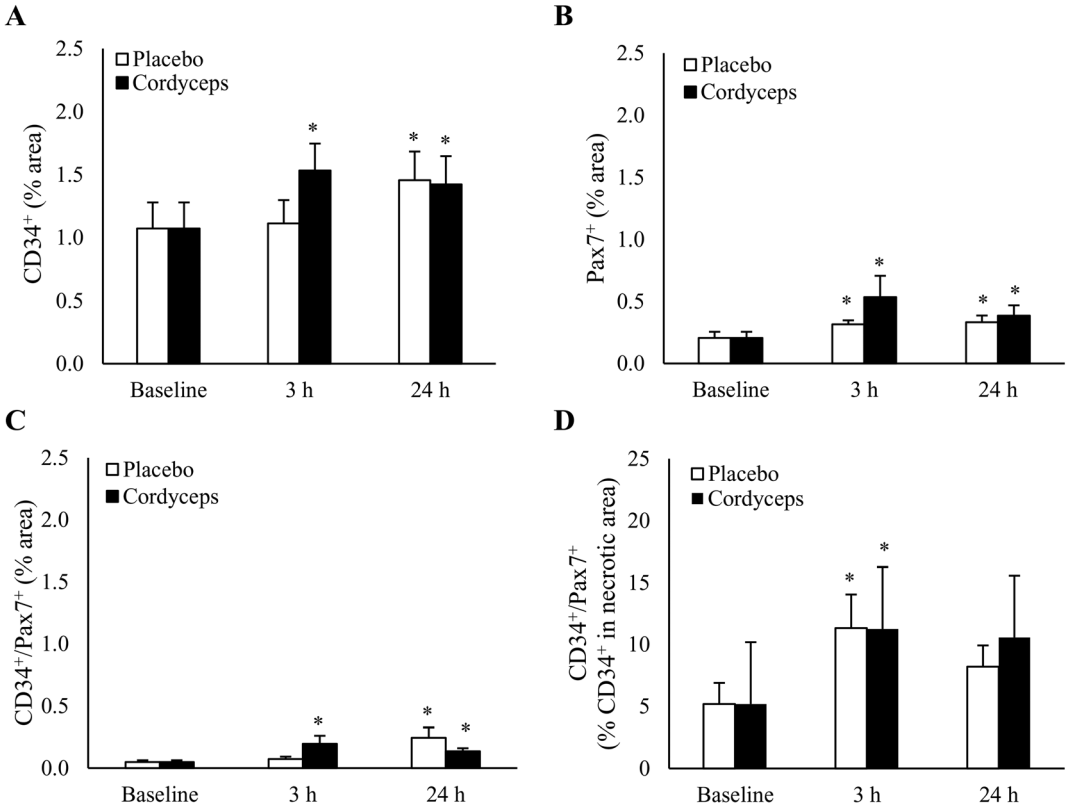

Fig. 5 Semi-quantitative analysis of the pre-exercise *Cordyceps* supplementation effect on stem cells in human skeletal muscle after high-intensity interval exercise (HIIE). *Cordyceps* supplementation accelerated early $CD34^+$ cell responses at 3 h post-exercise (A). $Pax7^+$ cell expansion post-HIIE significantly improved with *Cordyceps* supplementation (B). *Cordyceps* supplementation advanced a fourfold increase in $CD34^+/Pax7^+$ cells at 3 h, usually observed at 24 h after HIIE (C). Immunofluorescence staining suggests a similar *Cordyceps*-induced response of $CD34^+/Pax7^+$ cells in the necrotic area to that of the placebo-supplemented trial regarding total $CD34^+$ cell contribution to $Pax7^+$ cells (D). $^*P \leq 0.05$ compared to pre-exercise baseline. Data are presented as mean ± SE.

eralization to older population. Higher age is generally associated with greater levels of body weight (high cell population) and basal inflammation. Growth in weight is associated with progressive decreases in bone-to-body mass ratio, which may contribute to relative inadequacy of bone marrow cell production to regenerate cell in tissues.[34] Elevated basal inflammation typically witnessed in overweight individuals can increase background demand for $CD34^+$ cells to renew peripheral tissues of individuals with higher cell population. Therefore, it is hard to predict whether pre-exercise *Cordyceps* supplementation can also accelerate resolution of muscle inflammation for elderly, particularly those with heavier weight.

The *Cordyceps* used in this study contain adenosine and polysaccharides. It is possible that the observed acceleration in $CD34^+$ cell homing and $Pax7^+$ cell expansion is due to a synergistic effect of both components. Adenosine, known for its potent impact on the innate immune response,[35] is expected to modify the time required to resolve inflammation post-exercise. We cannot eliminate the potential influence of polysaccharides on the observed exercise response in human skeletal muscle. Previous reports indicate an increase in polysaccharide concentration in the blood due to gut leakage after high-intensity exercise,[36] potentially bolstering the immune response in skeletal muscle. However, further studies are necessary to confirm this hypothesis. Furthermore, the effect of adenosine on the protein kinases/phosphatase involving muscle adaptation to exercise requires more investigations from animal and cell studies to provide deeper insight into the observed effect related to muscle stem cell homing and expansion.[37]

Conclusions

The results of the study demonstrate the contributions of $CD34^+$ bone marrow stem cells to $Pax7^+$ satellite cells in damaged human skeletal muscle after high intensity exercise. In this study, we have further shown a substantially shortened period of muscle inflammation after exercise concurrent with accelerated increases in $CD34^+/Pax7^+$ cells in muscle tissues after *Cordyceps* supplementation. These findings suggest that the pro-inflammatory fungal-based supplement may potentiate

immune response to protect muscle against damage induced by exercise.

Abbreviations

BMI	Body mass index
CD34	Cluster of differentiation 34
DAPI	4′,6-Diamidino-2-phenylindole
HIIE	High intensity intermittent exercise
ICC	Intraclass correlation coefficient
Pax7	Paired box 7
PFA	Paraformaldehyde
RPE	Rated perceived exertion
rpm	Revolution per minute
RT-PCR	Real-time reverse transcription–polymerase chain reaction
SE	Standard error
VEGF	Vascular endothelial growth factor
W_{max}	Maximum work rate

Author contributions

CHK, CYH, and LKC contributed to the conception and design of this study; LD, YCL, and WHJ contributed to the main part of the experiment; LD, YCL, and LFL participated in the *in vivo* experiment; LD, YCL, LFL, and AN performed the tissue analysis; CHK, LD, YCL, LFL, and AN analysed the data; LD, CYH, CHK, CYH, and LKC contributed to drafting the paper and revising it strictly for vital intellectual content. All data were generated in-house, and no paper mill was used. All authors agree to be accountable for all aspects of work ensuring integrity and accuracy.

Data availability

The dataset used and/or analysed is available in the supplementary file.

Conflicts of interest

The *Cordyceps* used in the study is a fermented material supplied by TCM Biotech Taiwan and WynHealth Consultants, USA.

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