ID: IRB-2021-082 Effects of BFR-training on Stem Cells and Immune Cells in Human Skeletal Muscle

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Background

Murry et al in 1986 has shown a myocardium protection in the animals experienced intermittent ischemic reperfusion, indicated by less infarct sizes against the later ischemic insult compared with the control animals [1]. This landmark experiment exposed anesthetized animals to 4 episodes of 5 min coronary artery occlusions followed by a 5-min period of reperfusion before a 40-min ischemic insult (sustained occlusion of the coronary artery). Reperfusion following blood flow restriction (BFR) is currently known to induce an acute inflammation with a burst of reactive oxygen species (ROS) [2]. The mechanism behind this beneficial outcome induced after the acute ROS/inflammatory stress has yet to be fully clarified. Nevertheless, this principle has been widely applied for enhancement of training adaptation in sports. An increasing body of evidence has shown similar gains to higher intensity resistance training in muscular strength and hypertrophic adaptations following the combination of BFR with lower intensity resistance exercise [3]. Recently, a short period of BFR has been proven to lower pain [4] and enhance aerobic performance [5].

Rationale

Physical fatigue is defined by reduced performance below the optimal condition. This is closely associated with the capability of a human body to recover after physical challenges. A substantial improvement in running performance after $p16^{INK4a+}$ senescent cell clearance in mice suggests that slow recovery and loss of fitness is contributed by senescent cell accumulation in tissues [6]. Cellular senescence is a condition in which a normal replicable cell loses its capability to proliferate after a limited number of cell divisions [7], resulting in a longer recovery time after tissue damage. The $p16^{Ink4a}$ protein is a widely used senescent cell marker expressed in stem cells and immune cells, which inhibits cell division during early senescence in concert with $p21^{CIP1}$ [8, 9].

Since most of cells in human body are short-lived [10], $p16^{Ink4a+}$ senescent cells can be found in many tissues of young adults [11, 12]. In muscle tissues, myofibers are mostly long-lived, whereas endothelial cells in capillary surrounding myofibers age rapidly with a short half-life of ~2 weeks [13]. Local tissue stem cells are required for immediate replacement of these aged cells. In 2018, we have firstly reported existence of $p16^{INK4a+}$ senescent cells surrounding myofibers in young men aged 20-25 y [11]. These $p16^{INK4a+}$ cells are colocalized with $CD34^{+}$ signals in skeletal muscle, suggesting that these senescent cells are bone marrow-derived endothelial progenitor cells. Recent studies have shown that $CD34^{+}$ cells have the potential to regenerate both capillary endothelial cells and muscle stem cells ($PAX7^{+}$ satellite cells) [14-17]. To expand our current understanding in the field, we will further perform a double immunostaining to quantitate the amount of $CD34^{+}/PAX7^{+}$ cells (myogenic endothelial progenitor cells) in human skeletal muscle following BFR-training at various nutritional supplemented conditions.

DNA damage is the major cause of cellular senescence, reflected by increases $p16^{INK4a}$ expression and γ-H2AX levels in mammalian tissues [18, 19]. These increases have been considered as an inherent feedback mechanism to prevent replication of mutated DNA and therefore genetic stability can be preserved for normal cell turnover in tissues. Nevertheless this inhibition slows down recovery after tissue damage [20]. Exercise acutely increases DNA damage of blood cells ~ 5 h following exercise. A complete post-exercise recovery in 24 h suggests an enhanced DNA repair activity [21]. Determination of DNA repair in human skeletal muscle

requires two biopsy time points for DNA damage assessments during recovery. Available documentation in human skeletal muscle is rare [22].

Oxidative DNA damage (8-hydroxy-2-deoxyguanosine, 8-OHdG) in human skeletal muscle is known to associate with physical fatigue [23]. Mitochondrial DNA to nuclear DNA ratio declines significantly after three bouts of high-intensity exercise in 24 h, pointing to a possibility of increased mitochondrial DNA clearance [24]. More evidence is needed to determine whether the DNA damage following exercise is in nuclear DNA or in mitochondria DNA. In the study, we will examine the intramuscular location and the types of DNA damage in muscle cross-section following a BFR-training.

In response to DNA damage, nicotinamide adenine dinucleotide (NAD^+) is consumed up to 500-fold due to increased rate of PAR synthesis for DNA repair [25, 26]. NAD^+ is a substrate of PARP-enzymes for mono/poly-ADP-ribosylation and sirtuins for deacetylation [27]. Low NAD^+ compromises DNA repair capacity and mitochondria ATP production [27]. Increasing NAD^+ by vitamin B3 precursor supplementation rescues DNA damage and neuronal cell death in DNA repair-deficient mice [28]. β-NMN has been shown to replenish NAD+ in cultured cells [29]. No human study is currently available to answer the question on whether β-NMN supplementation can optimize a training recovery in human skeletal muscle.

Senescent cell accumulation in tissues enhances baseline inflammation [30]. Inflammation is a local immune response responsible for clearing unhealthy cells which involves with two groups of immune cell infiltration. The first group is the phagocytic type of immune cells migrating to unhealthy cells and releasing ROS for cell lysis [31]. These early-invading phagocytes reach their highest concentration within 24 h after injury and declines thereafter. The second groups of infiltrated cells are the cells responsible for muscle regeneration which will stay in the regenerating tissue until resolution of inflammation. Inflammation is essential for muscle regeneration and hypertrophy after damage [32]. We have previously shown a ~10% muscle loss when inflammation of exercised muscle is inhibited after doxorubicin injection [33].

Senescent cells release cytokines and chemokines (termed SASP) leading to an enhanced response of the tissues to a variety of inflammatory mediators [30]. Therefore, senescent cell accumulation in the body can exhaust the whole-body immune capacity due to higher baseline demand. In humans, senescent cells are constantly eliminated by the immune system [34, 35]. However, the specific immune cells involving this task is largely unexplored. In this study, we will provide human tissue evidence to identify the types of immune cells (MPO^+, CD11b, $CD4^+$, $CD8^+$, and $HMGB1^+$ cells) that are in direct contact with the $p16^{INK4a+}$ senescent cells in human skeletal muscle after BFR-training.

ROS released during phagocytic phase of inflammation [36] has been found essential for stem cell migration to the site of damage and further development in the injured tissue [37]. Low ROS levels support long-term stem cell repopulating ability in quiescent state [38]. High ROS levels stimulate stem cell differentiation and myeloid differentiation. However, exceedingly high ROS levels can exhaust stem cell pool in the muscle tissue and bone marrow [38]. Therefore, ROS elevation is transient in nature during most of challenged conditions. Taken together, ROS elevation during inflammation may play a role in exercise-induced

adaptation. It remains unknown how antioxidant supplementation may influence the senescent cell clearance during inflammation in human skeletal muscle following BFR-training.

Purpose of the study

In this proposed study, we aims 1) to assess cellular senescence ($p16^{INK4a}$ expression), DNA damage (strand break, 8-hydroxy-2-deoxyguanosine, and γ-H2AX), $PAX7^{+}/CD34^{+}$ stem cells, other bone marrow cells (e.g. Stro-1 and MPO+ cells), and myofiber regeneration (centronucleation and eMHC) in human skeletal muscle after sprinting exercise under two BFR pre-conditioning protocols; 2) to examine the effects of β-NMN supplementation on cellular senescence ($p16^{INK4a}$ expression), DNA damage (strand break, 8-hydroxy-2-deoxyguanosine, and γ-H2AX), $PAX7^{+}/CD34^{+}$ stem cells, and myofiber regeneration (centronucleation and eMHC) in human skeletal muscle following a session of BFR-training; 3) to examine the effects of antioxidant supplementation vitamin C on cellular senescence ($p16^{INK4a}$ expression), DNA damage (strand break, 8-hydroxy-2-deoxyguanosine, and γ-H2AX), $PAX7^{+}/CD34^{+}$ stem cells, and myofiber regeneration (centronucleation and eMHC) in human skeletal muscle following a session of resistance training. Immune cell infiltration into the challenged muscle tissue will also be examined to identify potential effector for senescent cell clearance in humans.

Materials and Methods

For participant recruitment, exclusion criteria are those who had smoking history, inflammatory conditions, or engaging in vigorous exercise in 24 h before the trials. For the three proposed studies, anaerobic performance assessments (time-to-exhaustion at 90% VO_{2peak} and Wingate test) will be conducted on separate occasions to avoid potential influence between muscle tissue collection and performance measurement. Participants will be refrained from taking any over-the-counter medications, sports nutrition supplements, prescription medications, antioxidant nutritional supplements for 72 h prior to experimental trials. The entire project will conform to the Declaration of Helsinki. University of Taipei Institutional ethical approval will be obtained before conducting the studies. All participants will be informed about the procedures and potential risks associated with the study. Written informed consent will be obtained before beginning of the study.

Study 1 (First year): This study aims to assess the effects of BFR-preconditioning (180 mmHg) on cellular senescence (p16INK4a expression), DNA damage (strand break, 8-hydroxy-2-deoxyguanosine, and γ-H2AX), PAX7+/CD34+ stem cell number, MPO+ cells, Stro-1+ cells, other myeloid cells and myofiber regeneration in human skeletal muscle following sprinting exercise. A randomized counter-balanced crossover study (conformed to CONSORT 2010) of BFR-preconditioning at 180 mmHg and 20 mmHg (Sham control) [40] will be conducted for the same training participants with a washout period of >3 weeks between trials. Muscle and blood samples will be taken at baseline (3 weeks before test), 0 h after and 24 h after exercise using multiple biopsies. Participants aged 20-30 y (N= 24) will be recruited by posts and social media. All participants are required to familiarize with the experimental procedures and lab equipment before beginning of the trials. Participants will complete a test to obtain VO_{2peak} and maximal aerobic power using a graded exercise test protocol on a cycle ergometer (Monark 839E, Stockholm, Sweden) one week before exercise challenge.

Blood flow restriction (BFR): A pre-exercise BFR protocol with a pressure inflow at 180 mmHg [40] has been demonstrated to enhance endurance performance compared with the sham condition (20 mmHg). **BFR protocol**: Prior to the acute exercise challenge, participants completed three intermittent sessions of BFR (180 mmHg) or Sham (20 mmHg) using a medical sphygmomanometer and cuff (CK-110; Spirit, New Taipei, Taiwan), each followed by a 5-minute recovery period. Participants lay in a supine position with legs extended and relaxed. The cuff was placed around the upper thighs, just below the hip line. During BFR, the cuff was inflated to 180 mmHg for 5 minutes to induce arterial occlusion.
The primary outcome was the number of bone marrow cells in skeletal muscle tissue (immune cells and stem cells). Secondary outcomes included the expression of inflammatory cytokines (TNF-α and IL-10), the abundance of NOX-2^{+} cells, and 8-OHdG signals in skeletal muscle tissue. At the initial session, participants were familiarized with the cycling sprint protocol and overall experimental procedures. To minimize potential nutritional influences on exercise recovery, no dietary intake was allowed after 8 pm. Participants consumed a 250 kcal standard drink (Ensure; Abbott Nutrition, Taipei, Taiwan) 12 h and 1 h before each trial. Participants were instructed to maintain their habitual diet and were allowed to drink water ad libitum before and after the exercise. Dietary intake during the 72 h preceding each trial was recorded and verified by a registered dietitian. Muscle biopsies were obtained from the vastus lateralis at baseline (3 weeks before the trial), immediately post-exercise (0 h), and 24 h after the exercise.

Acute exercise protocol
The exercise protocol was designed as a low-volume, high-intensity challenge suitable for a broad population. Five minutes after the final BFR session, participants performed a 3-minute warm-up at 50 W. This was followed by five 10-second maximal sprints on a cycle ergometer (Monark 894E Peak Bike, Varberg, Sweden) at a braking resistance equivalent to 7.5% of body weight, with 50-second rest intervals between sprints. Total work output was recorded to assess whether performance was affected by the BFR intervention.

Study 2 (second year). This study aims to examine the effects of β-NMN on cellular senescence ($p16^{INK4a}$ expression), DNA damage (strand break, 8-hydroxy-2-deoxyguanosine, and γ-H2AX), $Pax7^{+}$/$CD34^{+}$ stem cell count, Stro-1+ Cells, MPO+ cells, other bone marrow cells and myofiber regeneration in human skeletal muscle following the BFR-sprinting intervention. A placebo (PLA)-controlled randomized counter-balanced crossover study (conformed to CONSORT 2010) will be conducted for the same participants with a washout period of >3 weeks between trials. Muscle and blood samples will be taken at baseline (3 weeks before test), 0 h after and 24 h after exercise using multiple biopsies.

Nicotinamide mononucleotide (β-NMN) supplementation: The β-NMN (1200 mg, GeneHarbor, Biotechnologies Ltd, Hong Kong) dosage has been shown to improve aerobic capacity in runners [42]. Both PLA and β-NMN will be delivered by a drink containing soy protein isolate (0.12 g per kg weight) in 300 ml of water. Supplements will be consumed 1 night and 1 h before exercise and immediately after exercise. The study participants and members of the study team involving in the collection and analysis of outcomes will be blinded to the treatment condition. Participants aged 20-30 y (N= 24) will be recruited by posts and social media. Each participant will complete a 5-min warm-up at a comfortable workload. All participants are

required to familiarize with the experimental procedures and equipment before beginning of the trials. Participants will complete a test to obtain VO2peak using a graded exercise test protocol on a cycle ergometer (Monark 839E, Stockholm, Sweden) one week before exercise challenge. To evaluate the effects of NMN supplementation on inflammation and mitochondria in skeletal muscle after BFR-exercise, a double-blind, placebo-controlled crossover study was conducted, with a 3-week washout period between conditions. Participants were randomly assigned in a balanced order to receive either NMN or a Placebo. Experimenter and participant were blind with supplements (assigned as supplement 1 and 2). NMN and Placebo capsules were identical in appearance, size, and color to minimize placebo effects. Experimenters and participants were blinded to group assignments throughout the study.

During the first visit, a baseline muscle biopsy of the right vastus lateralis was obtained three weeks before the one-repetition maximum (1-RM) squat strength test. The BFR-exercise challenge was administered two weeks after the 1-RM test. Muscle biopsies were collected from the right leg immediately after the exercise challenge and from the corresponding location on the left leg 24 hours later. Following a 4-week washout, participants crossed over to the alternate condition, and the protocol was repeated. NMN supplementation: Participants received oral gelatin capsules containing 300 mg of NMN (AbinoNutra™ NMN, Shelton, Connecticut, USA) or 300 mg of cornstarch (Placebo), following protocols consistent with recent studies 22. Capsules were taken four times daily—once after each of the three main meals and once before bedtime, beginning six days prior to the BFR-exercise challenge. This regimen provided a total daily dose of 1200 mg. The same protocol was maintained on the seventh day, which included the 24-hour post-exercise recovery period. To control for the potential influence of breakfast variation on post-exercise recovery, participants consumed a standardized liquid meal replacement one hour before the BFR-exercise (0900-1000 am): a can of Ensure® Original (Abbott Laboratories, Illinois, USA), providing 250 kcal (6 g fat, 34 g carbohydrates, 9 g protein), along with the supplement capsule in the morning before each muscle biopsy (pre-exercise baseline, 0 h and 24 h after exercise). Although this is not a typical meal for healthy individuals, the liquid formulation allows rapid delivery of nutrients and supplements prior to the experimental challenge. Additionally, to minimize dietary variation after exercise, a standardized lunch was provided between 12:00 and 13:00 for each crossover trial (773 kcal total; 56% carbohydrate, 29% fat, 15% protein by energy). Blood flow restriction (BFR): Before the resistance exercise challenge, 3 episodes of BFR were applied a standard sphygmomanometer and cuff (CK-110; Spirit, New Taipei, Taiwan). Before BFR, participants sat comfortably on the bed with legs extended in a relaxed position. The blood pressure cuff was placed around the upper thighs just below the hip line. During BFR, the cuff was inflated to 180 mmHg for 5 minutes to occlude arterial inflow (total occlusion). This process was repeated three times for each limb, with a 5-minute interval between each pressurization, followed by a 5-minute period of rest after reperfusion (cuff release) at the same location. Five minutes following the last session of BFR, the participant performed a 5-min dynamic warm-up exercise before leg squatting. Each participant completed a set of warm-up exercises followed by a set of 8 repetitions (50% of 1-RM).

Resistance Exercise: Resistance exercise was imposed to participants approximately 10 min after BFR (including the 5-min warm-up) at 1000-1100 am. The back squat exercise comprised 4 sets, each with 8 repetitions (70% of 1-RM), with a 90-second rest interval between sets on a Smith machine (Cybex,

Minnesota, USA). It was recommended that participants perform squats by allowing their hips to touch an adjustable multi-angle fitness bench to reach a knee angle less than 90° (parallel to the ground). Participant's compliance was 100%. A trained supervisor monitored the exercise challenge programs for all participants in the weight training room to ensure consistency of the motion.

Study 3 (third year): This study aims to examine the effects of antioxidant supplementation on the resistance exercise-induced change in cellular senescence (p16INK4a expression), DNA damage (strand break, 8-hydroxy-2-deoxyguanosine, and γ-H2AX), PAX7+/CD34+ stem cell count, and myofiber regeneration (centronucleation and eMHC), gene activation of human skeletal muscle. A PLA-controlled randomized counter-balanced crossover study (conformed to CONSORT 2010) will be conducted for the same participants with a washout period of >3 weeks between trials. Muscle and blood samples will be taken at baseline (3 weeks before test), 0 h after and 24 h after exercise using multiple biopsies. Supplementation: After a 12-hour overnight fast, participants were randomly assigned to either Placebo or Vitamin C trial in a counterbalanced order. In this study, Vitamin C (500 mg per capsule; California Gold Nutrition, Los Angeles, California, USA) was used, with an equal weight placebo containing corn starch. Participants were unable to distinguish between Vitamin C and Placebo capsules. Participants ingested a total of 2,000 mg of vitamin C in capsule form at three time points: 2 h before (1,000 mg), 2 h after (500 mg), and 6 h after (500 mg) the squat exercise. This method builds on findings of elevated plasma Vitamin C concentration 2 h after a 1,000 mg dose (Thompson et al., 2001) and a meta-analysis reporting that doses > 500 mg reduce exercise-induced lipid peroxidation, while doses ≤ 500 mg show no effect (Righi et al., 2020). To minimize the influence of pre-exercise nutrition on study outcomes, participants were provided with a standardized breakfast 2 h before exercise consisting of one can of Ensure® Less Sweet Flavor Nutrition Shake (Abbott Laboratories, Lake County, IL, USA). The shake contained 250 calories, 6 g of fat, 41.5 g of carbohydrates, and 9 g of protein. Exercise challenge: In this study, back squats on a Smith machine (Cybex, Illinois, USA) were utilized to challenge vastus lateralis muscle. A dynamic stretching routine was completed by each participant, followed by a structured warm-up set of 8 repetitions (50% 1-RM) squat. The exercise bout consisted of 4 sets of 8 repetitions (70% 1-RM) squat, with 90-s rest intervals between sets. Participants were instructed to lower the barbell slowly until their mid-thighs were parallel to the ground during the eccentric phase for each repetition. To ensure consistency across participants, a qualified trainer supervised and guided the exercise protocol in a controlled training environment. The weight lifted for 70% 1-RM squat ranged from 0.72 to 1.19 kg/kg body weight. Blood sampling and analysis: Venous blood was collected from the forearm by a qualified nurse at pre-exercise, immediately after exercise (0 h), and 24 h post-exercise. For hematological assessment, 2 to 3 mL of whole blood was drawn into EDTA-coated tubes. A Complete Blood Count (CBC) and white blood cell (WBC) differential were subsequently performed using a Beckman Coulter DxH 900 hematology analyzer (Beckman Coulter, Brea, California, USA). Antioxidant supplementation: Participants will attend to the laboratory and evenly randomize into one of the two groups: PLA and antioxidant (1 gram of vitamin C) in a counter-balanced fashion. A 200-ml standardized drink (Herbalife Formula One shake, California, USA) containing soy protein isolate (0.12 g per kg weight) will be prepared to orally deliver PLA and antioxidant capsule to participants [41]. Each supplement will be administered in 2 doses (half 1 h before exercise and half immediately after exercise corresponding to previous study [43]. The PLA group will be given the same number of doses. Participants aged 20-30 y (N=

24) will be recruited by posts and social media. Each participant will complete a 5-min warm-up at a comfortable workload. All participants are required to familiarize with the experimental procedures and equipment before beginning of the trials. Participants will complete a test to obtain VO2peak using a graded exercise test protocol on a cycle ergometer (Monark 839E, Stockholm, Sweden) one week before exercise challenge.

Before exercise, all participants will be provided a standard isocaloric diet (Ensure, Abbott, Taiwan) 12 h and 1 h prior to each trial to avoid potential dietary effect on outcome variables. Multiple biopsies in vastus lateralis will be conducted by an experienced physician at baseline, 0 h after and 24 h after BFR-exercise for muscle sample collection. Participants will be allowed to drink water *ad libitum*. Cellular senescence markers including $p16^{Ink4a}$ will be measured by real-time PCR. The $p16^{Ink4a+}$ and $p21^{+}$ cell count will also be counted by IHC on serial cross-sections of muscle tissue. Phagocytes will be assessed by surface markers of neutrophil ($MPO^{+}/CD11c^{+}$) and M1 macrophage ($CD68^{+}/iNOS^{+}$/MARCO). Myogenic endothelial progenitor cells ($Pax7^{+}/CD34^{+}$) of exercised muscle will be measured together with lymphocytes ($CD4^{+}$ and $CD8^{+}$) on serial IHC staining. DNA damage (strand break and 8-hydroxy-2-deoxyguanosine) will be assessed by IHC double staining on the same muscle cross-section. γ-H2AX will also be measured.

Performance assessments

Participants will visit the laboratory for 2 preliminary tests (one familiarization and one baseline test) and 2 experimental crossover trials. Sessions will be divided by a minimum of 7 d to avoid fatigue and eliminate the potential effects of the possible adaptation caused by interventions. All trials will be performed at the same time of day for each pair of participants to avoid potential circadian rhythm effects. Temperature (23°C ± 1°C) and humidity (45-55%) will be maintained throughout each trial. During the first visit, lean mass will be assessed by a whole-body scan of DXEA. The thigh circumference will be recorded. All participants will familiarize with the ischemic reperfusion based on the BFR protocol as aforementioned.

To assess anaerobic exercise performance, Wingate test and time-to-exhaustion cycling at 90% VO_{2peak} will be conducted on a cycle ergometer. All participants need to familiarize with the test procedures and bike setup. Participants will perform a 5-min self-paced warm-up, rest 2 min on the cycle ergometer, and then perform the time trial. They will be verbally encouraged throughout the time-to-exhaustion test at 90% VO_{2peak} on a computer-controlled electrically braked cycle ergometer (Monark 839E, Stockholm, Sweden). To control the self-paced warm-up in subsequent visits, power output will be continuously monitored by an experimenter for participants and strictly reproduce before each time trial under the supervision of the experimenter. At the end of the last performance trial, participants will report Rating of Perceived Exertion (RPE) scores using the Borg 10-point scale to assess subjective perceived exertion.

Measurement of new markers may be included when new finding indicates unexpected outcomes. The amount of muscle regeneration will be assessed by muscle cross-section using centrally nucleated fiber based on H&E staining and embryonic myosin heavy chain (eMHC). Both IHC and PCR will be performed based on our previously established laboratory procedure [41].

Blood sample analyses

Blood samples will be collected before and after exercise via a 20-G polyethylene catheter (Jelco, Tampa, FL, USA) installed in an antecubital vein. The total numbers of leukocyte will be quantitated using an automated hematology analyzer (Sysmex XT-2000, Sysmex Corp., Kobe, Japan) according to manufacturer's instructions. During recovery, blood samples will be collected at baseline, 0 h, and 24 h after exercise.

Muscle biopsy

Muscle samples will be taken at baseline (3 weeks before test), 0 h after and 24 h after exercise using multiple biopsies. Exercise challenge will be started at 0930 am. Participants will be asked for urination right before exercise. Muscle biopsy will be conducted based on our previous published procedure (Hou et al., 2015; Yang et al., 2018) by a certified physician under local anesthesia (2% lidocaine) using 18-G Temno disposable needles (Cardinal Health, McGaw Park, Illinois, USA) inserted into the vastus lateralis positioned at 3 cm depth, ~20 centimeter proximal to kneecap. To prevent the potential interference of previous needle biopsy on the observation of next muscle tissue, the first muscle biopsy (baseline) on the vastus lateralis of right leg will be performed 3 weeks before exercise challenge. Two additional muscle biopsies will be consecutively conducted for the same participants 0 h and 24 h after the exercise challenge for each trial. The muscle biopsy after exercise (0 h) will be taken always on the same location of left leg. The next muscle biopsy (24 h) will be taken again on the right leg at the same distance from kneecap. Muscle tissue will be quickly removed from the needle, cleaned of excess blood (when rarely needed), aligned into a perpendicular direction on a thin paper, and disposed immediately into a conical vial containing 10% formalin. Paraffin-embedded tissue will be sectioned no later than 3 h following muscle sample collection for IHC and HE tissue analysis.

Muscle samples (devoid of subject name by number code) will pair-analyzed by our international collaborators to ensure consistency of the finding. Muscle samples of participants will be further analyzed by Professor Kazushige Goto for more scientific discovery (Ritsumeikan University, Japan), such as irisin expression, irisin location (myofiber-, stem cells, or infiltrated immune cell-origin) protein associated with iron storage and transport, and fiber type analysis.

Immunohistochemistry (IHC) and Hematoxylin and Eosin (HE) staining

The IHC and HE stains will be quantitated according to our standard procedure developed previously (Yang et al., 2018). Serial sections will be conducted to detect cell senescence in muscle tissue (Gu & Kitamura, 2012; Helman et al., 2016). Double IHC for $Pax7^{+}/CD34^{+}$ cells will be conducted on the same muscle cross-section using DoubleStain IHC Kit: M&R on human tissue (DAB & AP/Red)(Abcam, Taipei, Taiwan). A certified pathologist in Toson Technology will conduct tissue fixation, cutting, staining, and IHC staining. HE stains will be used to determine cell infiltration and centronucleation of the muscle cross-section. Intramyofiber and interstitial cells will be counted. The number of leukocytes will be expressed as area (per field). Muscle paraffin sections (2 μm thick) will be labeled by antibodies against specific antigens for IHC. The slides will be reviewed at a magnification of Å~200 and Å~400. Cells markers will be quantified and expressed as positive signal positive signal number/total skeletal muscle field area (mm^2) and number/total skeletal muscle fiber number (%). Approximately 500 muscle fibers will be included for analysis. The

standard criterion of positive cell is brown precipitates stained on the entire cell nuclei against blue precipitates. For γ-H2AX+ cells, the criterion includes a) myofiber covering 90% of edge of sarcolemma (since nucleus is localized mostly on the edge) and (b) when dark brown the score multiplies by 2, brown multiply by 1 for calculation. Stained muscle cross-section will be pair-analyzed by two specialists (Taiwan and Japan) from two universities. If correlation of pair analysis is lower than 0.7, criteria will be re-discussed, and analysis will be conducted again.

Paraffin-embedded tissue sections on poly-1-lysine-coated slides will be deparaffinized and rinsed with 10 mM Tris-HCl (pH 7.4) and 150 mM sodium chloride. Peroxidase will be quenched with methanol and 3% hydrogen peroxide and slides will then be placed in 10 mM citrate buffer (pH 6.0) at 100 ÅãC for 20 min. After incubation for 1 h at room temperature, slides will be washed 3 times with phosphate-buffered saline (PBS). Universal Quick Kit (PK 8800) (Vector Laboratories, Burlingame, CA, USA), providing Biotinylated, Universal Pan-Specific secondary antibody will be used for IHC analysis. At last, the slides will be photographed with the BX50 Olympus microscope (Tokyo, Japan). Negative controls will be obtained by performing all of the immunohistochemistry steps but leaving out the primary antibody. All glass slides will be digitized with an Motic Easyscan Digital Slide Scanner (Motic Hong Kong Limited, Hong Kong, China) at ×40 (0.26 μm/pixel) with high precision (High precision autofocus). Motic Easyscan whole-slide images will be viewed with DSAssistant and EasyScanner software at Toson Technology Co. (Zhubei City, Hsinchu, Taiwan).

Antibodies

Primary antibodies to detect cellular senescence phenotype are anti-human $p16^{Ink4a}$ (Abcam, USA) and anti-human $p21^{CIP1}$ (BD Bioscience, USA). Primary antibody to recognize DNA double-strand breaks is γ-H2AX (Abcam, USA) together with TUNEL assay. Primary antibodies to detect oxidative DNA damage will use 8-Hydroxyguanosine (8-OHdG) antibody (Thermo Fisher Scientific, USA). Primary antibodies to detect phagocyte infiltration are neutrophil MPO (Abcam, USA), M1 Phagocytic macrophage (CD68+/iNOS/MARCO) (Cambridge, USA), and M2 macrophage CD163 (Cambridge, USA). Anti-human CD34 (Ventana Medical Systems, USA) and anti-human PAX7 (Millipore MABD20, Darmstadt, Germany) will be used to detect myogenic endothelial progenitor cells. Anti-human embryonic myosin heavy chain (MYH3, Myosin heavy chain 3), TOM20 (mitochondria marker), CD68, iNOS, MARCO, HMGB1, CD11b, CD4, and CD8 antibodies from Abcam USA will also be used for IHC on muscle cross-sections.

DNA damage

DNA strand break will be identified by a fluorometric TUNEL detection kit (Mebstain Apoptosis Kit Direct, #8445) at 3'-OH ends of strand break (Medical & Biological Laboratories, Woburn, MA, USA) according to the manufacturer's instructions for both muscle cross-sections. TUNEL and DAPI-positive nuclei staining will be captured under a fluorescence microscope (Olympus BX51, Olympus Corporation, TKY, Japan). The number of TUNEL and DAPI-positive nuclei will be counted from overall cross-section at 20X objective magnification. Only the TUNEL-positive nuclei overlapping fiber nuclei will be quantified as nuclei with strand break.

The TUNEL labeling on 3'-OH ends of DNA strand break will be visualized and quantified as the number of TUNEL-positive nuclei per 500 muscle fibers and per field area (mm^2). We will distinguish whether the post-sprinting DNA damage is associated with DNA strand break in the nuclei (using TUNEL assay) or mitochondrial DNA damage. Immunohistochemical analysis of TOM20 antibody will be used to colocalize mitochondrial DNA damage site on the same muscle cross-section.

Quantitative PCR

The frozen muscle sample (<30 mg) will be transferred into a 1.5-ml tube containing 600 µl of β-Mercaptoethanol (β-ME) with buffer RLT on ice. Then the muscle sample will be homogenized immediately using Polytron (model PT 3000D) for 30 s at 9000 rpm (about the 50% of the maximum speed). RNA extraction from muscle samples will use RNeasy kit (QIAGEN 74104), RNase-Free DNase Set (QIAGEN 79254), and Proteinase K (QIAGEN 19131) following the muscle homogenization. One µg of RNA in a total volume of 20 µl will be reverse-transcribed to cDNA using iScript cDNA Synthesis Kit (#170-8890) (Bio-Rad, Hercules, CA, USA). RNA will be extracted from ~15 mg of biopsied muscle using the RNeasy kit (QIAGEN 74104) after a 60-s homogenization in QIAzol Lysis Reagent (QIAGEN, Hilden, Germany, 79306). One microgram of RNA in a total volume of 20 µl will be reverse-transcribed to cDNA using iScript cDNA Synthesis Kit (#170-8890) (Bio-Rad, Hercules, CA, USA). Real-time PCR will be performed using CFX Connect Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA), PrimePCR™ Probe Assay (Bio-Rad, Hercules, CA, USA) and Statistical analysis. Specific probes and primers will be synthesized by Bio-Rad, Taiwan according to Bio-Rad database.

Near-infrared spectroscopy

For monitoring tissue oxygenation status in leg muscle during BFR, a wireless NIRS apparatus (Artinis Medical Systems, Netherlands) will be installed on the distal part of the right vastus lateralis muscle belly (approximately 10 cm above the proximal edge of the patella), parallel to muscle fibers. This device allows quantitation of changes in the absorption of near-infrared light by oxyhemoglobin (HbO2) and deoxyhemoglobin (HHb). Black bandages will be used to prevent leakage of background light to the NIRS device. The pressure cuff will be placed right above the wireless NIRS device. We will use total hemoglobin change Δ[THb] as an estimate of change in tissue blood volume [44]. This NIRS device uses two continuous wavelengths (760 and 850 nm) based on a modified form of the Beer–Lambert law with a differential optical path length factor of 4.95 to obtain micromolar changes in tissue oxyhemoglobin HbO2 (Δ[HbO2]) and deoxyhemoglobin HHb (Δ[HHb]). Total hemoglobin (Δ[THb] is calculated as sum of [HbO2] and [HHb]. Tissue saturation index (TSI = [HbO2]/[THb]) will be used as an index of tissue oxygenation [45]. The NIRS data will be recorded at 10 Hz during resting and BFR-exercising conditions. When the signal is stabilized, 1 min of baseline values will be recorded during BFR treatments. NIRS signals will be analyzed 2-min posttreatment for a duration of 1 min to assess the BFR effect on resting total hemoglobin (Δ[THb] in µM).

Statistical Analysis

All results will be presented as mean ± standard error (SE). Type 1 error equal or less than 5% for comparing mean difference will be considered significant. Two-way ANOVA with repeated measure will be used to

determine the main effects and interactive effect of time (Pre and Post) and intervention. Pair t test will be used to determine percent changes after exercise between nutritional interventions. Cohen's *d* effects sizes will be calculated and interpreted as small (*d* = 0.2), medium (*d* = 0.5), and large (*d* = 0.8) [46]. Pearson's correlation will be used to determine the magnitude of association between variables.

References

1. Murry CE, Jennings RB, and Reimer KA. Preconditioning with ischemia: a delay of lethal cell injury in ischemic myocardium. Circulation. 1986; 74(5):1124-1136.
2. Wu L, Xiong X, Wu X, Ye Y, Jian Z, Zhi Z, and Gu L. Targeting oxidative stress and inflammation to prevent ischemia-reperfusion injury. Front Mol Neurosci. 2020; 13(28).
3. Hwang PS, and Willoughby DS. Mechanisms behind blood flow-restricted training and its effect toward muscle growth. J Strength Cond Res. 2019; 33 Suppl 1:S167-s179.
4. Hughes L, and Patterson SD. The effect of blood flow restriction exercise on exercise-induced hypoalgesia and endogenous opioid and endocannabinoid mechanisms of pain modulation. J Appl Physiol (1985). 2020; 128(4):914-924.
5. Bennett H, and Slattery F. Effects of Blood Flow Restriction Training on Aerobic Capacity and Performance: A Systematic Review. J Strength Cond Res. 2019; 33(2):572-583.
6. Baker DJ, Wijshake T, Tchkonia T, LeBrasseur NK, Childs BG, van de Sluis B, Kirkland JL, and van Deursen JM. Clearance of p16Ink4a-positive senescent cells delays ageing-associated disorders. Nature. 2011; 479(7372):232-236.
7. Hayflick L, and Moorhead PS. The serial cultivation of human diploid cell strains. Exp Cell Res. 1961; 25:585-621.
8. Stein GH, Drullinger LF, Soulard A, and Dulić V. Differential roles for cyclin-dependent kinase inhibitors p21 and p16 in the mechanisms of senescence and differentiation in human fibroblasts. Mol Cell Biol. 1999; 19(3):2109-2117.
9. Waaijer ME, Parish WE, Strongitharm BH, van Heemst D, Slagboom PE, de Craen AJ, Sedivy JM, Westendorp RG, Gunn DA, and Maier AB. The number of p16INK4a positive cells in human skin reflects biological age. Aging Cell. 2012; 11(4):722-725.
10. Spalding KL, Bhardwaj RD, Buchholz BA, Druid H, and Frisén J. Retrospective birth dating of cells in humans. Cell. 2005; 122(1):133-143.
11. Yang C, Jiao Y, Wei B, Yang Z, Wu JF, Jensen J, Jean WH, Huang CY, and Kuo CH. Aged cells in human skeletal muscle after resistance exercise. Aging (Albany NY). 2018; 10(6):1356-1365.
12. Ressler S, Bartkova J, Niederegger H, Bartek J, Scharffetter-Kochanek K, Jansen-Dürr P, and Wlaschek M. p16INK4A is a robust in vivo biomarker of cellular aging in human skin. Aging Cell. 2006; 5(5):379-389.
13. Erben RG, Odörfer KI, Siebenhütter M, Weber K, and Rohleder S. Histological assessment of cellular half-life in tissues in vivo. Histochem Cell Biol. 2008; 130(5):1041-1046.
14. Tamaki T, Akatsuka A, Ando K, Nakamura Y, Matsuzawa H, Hotta T, Roy RR, and Edgerton VR. Identification of myogenic-endothelial progenitor cells in the interstitial spaces of skeletal muscle. J Cell Biol. 2002; 157(4):571-577.
15. Palladino M, Gatto I, Neri V, Stigliano E, Smith RC, Pola E, Straino S, Gaetani E, Capogrossi M, Leone G, Hlatky L, and Pola R. Combined therapy with sonic hedgehog gene transfer and bone marrow-derived

endothelial progenitor cells enhances angiogenesis and myogenesis in the ischemic skeletal muscle. J Vasc Res. 2012; 49(5):425-431.
16. Okada M, Payne TR, Zheng B, Oshima H, Momoi N, Tobita K, Keller BB, Phillippi JA, Péault B, and Huard J. Myogenic endothelial cells purified from human skeletal muscle improve cardiac function after transplantation into infarcted myocardium. J Am Coll Cardiol. 2008; 52(23):1869-1880.
17. Hollemann D, Budka H, Löscher WN, Yanagida G, Fischer MB, and Wanschitz JV. Endothelial and myogenic differentiation of hematopoietic progenitor cells in inflammatory myopathies. J Neuropathol Exp Neurol. 2008; 67(7):711-719.
18. Shapiro GI, Edwards CD, Ewen ME, and Rollins BJ. p16INK4A participates in a G1 arrest checkpoint in response to DNA damage. Mol Cell Biol. 1998; 18(1):378-387.
19. White RR, Milholland B, de Bruin A, Curran S, Laberge RM, van Steeg H, Campisi J, Maslov AY, and Vijg J. Controlled induction of DNA double-strand breaks in the mouse liver induces features of tissue ageing. Nat Commun. 2015; 6:6790.
20. Sherr CJ. Cancer cell cycles. Science. 1996; 274(5293):1672-1677.
21. Tryfidou DV, McClean C, Nikolaidis MG, and Davison GW. DNA Damage Following Acute Aerobic Exercise: A Systematic Review and Meta-analysis. Sports Med. 2020; 50(1):103-127.
22. Radák Z, Apor P, Pucsok J, Berkes I, Ogonovszky H, Pavlik G, Nakamoto H, and Goto S. Marathon running alters the DNA base excision repair in human skeletal muscle. Life Sci. 2003; 72(14):1627-1633.
23. Radák Z, Pucsok J, Mecseki S, Csont T, and Ferdinandy P. Muscle soreness-induced reduction in force generation is accompanied by increased nitric oxide content and DNA damage in human skeletal muscle. Free Radic Biol Med. 1999; 26(7-8):1059-1063.
24. Marcuello A, González-Alonso J, Calbet JA, Damsgaard R, López-Pérez MJ, and Díez-Sánchez C. Skeletal muscle mitochondrial DNA content in exercising humans. J Appl Physiol (1985). 2005; 99(4):1372-1377.
25. Croteau DL, Fang EF, Nilsen H, and Bohr VA. NAD(+) in DNA repair and mitochondrial maintenance. Cell Cycle. 2017; 16(6):491-492.
26. Ying W, Alano CC, Garnier P, and Swanson RA. NAD+ as a metabolic link between DNA damage and cell death. J Neurosci Res. 2005; 79(1-2):216-223.
27. Wilk A, Hayat F, Cunningham R, Li J, Garavaglia S, Zamani L, Ferraris DM, Sykora P, Andrews J, Clark J, Davis A, Chaloin L, Rizzi M, et al. Extracellular NAD(+) enhances PARP-dependent DNA repair capacity independently of CD73 activity. Sci Rep. 2020; 10(1):651.
28. Hou Y, Lautrup S, Cordonnier S, Wang Y, Croteau DL, Zavala E, Zhang Y, Moritoh K, O'Connell JF, Baptiste BA, Stevnsner TV, Mattson MP, and Bohr VA. NAD(+) supplementation normalizes key Alzheimer's features and DNA damage responses in a new AD mouse model with introduced DNA repair deficiency. Proc Natl Acad Sci U S A. 2018; 115(8):E1876-e1885.
29. Mehmel M, Jovanović N, and Spitz U. Nicotinamide riboside-The current state of research and therapeutic uses. Nutrients. 2020; 12(6).
30. Budamagunta V, Manohar-Sindhu S, Yang Y, He Y, Traktuev DO, Foster TC, and Zhou D. Senescence-associated hyper-activation to inflammatory stimuli in vitro. Aging (Albany NY). 2021; 13(15):19088-19107.

31. St Pierre BA, and Tidball JG. Differential response of macrophage subpopulations to soleus muscle reloading after rat hindlimb suspension. J Appl Physiol (1985). 1994; 77(1):290-297.
32. Tidball JG. Regulation of muscle growth and regeneration by the immune system. Nat Rev Immunol. 2017; 17(3):165-178.
33. Huang S-C, Wu J-F, Saovieng S, Chien W-H, Hsu M-F, Li X-F, Lee S-D, Huang C-Y, Huang C-Y, and Kuo C-H. Doxorubicin inhibits muscle inflammation after eccentric exercise. J Cachexia Sarcopenia Muscle. 2017; 8(2):277-284.
34. Prata LGPL, Ovsyannikova IG, Tchkonia T, and Kirkland JL. Senescent cell clearance by the immune system: Emerging therapeutic opportunities. Semin Immunol. 2018; 40:101275-101275.
35. Kay MM. Mechanism of removal of senescent cells by human macrophages in situ. Proc Natl Acad Sci U S A. 1975; 72(9):3521-3525.
36. Forman HJ, and Torres M. Reactive oxygen species and cell signaling: respiratory burst in macrophage signaling. Am J Respir Crit Care Med. 2002; 166(12 Pt 2):S4-8.
37. Ludin A, Gur-Cohen S, Golan K, Kaufmann KB, Itkin T, Medaglia C, Lu XJ, Ledergor G, Kollet O, and Lapidot T. Reactive oxygen species regulate hematopoietic stem cell self-renewal, migration and development, as well as their bone marrow microenvironment. Antioxid Redox Signal. 2014; 21(11):1605-1619.
38. Ito K, Hirao A, Arai F, Matsuoka S, Takubo K, Hamaguchi I, Nomiyama K, Hosokawa K, Sakurada K, Nakagata N, Ikeda Y, Mak TW, and Suda T. Regulation of oxidative stress by ATM is required for self-renewal of haematopoietic stem cells. Nature. 2004; 431(7011):997-1002.
39. Kojima C, Yamaguchi K, Ito H, Kasai N, Girard O, and Goto K. Acute effect of repeated sprint exercise with blood flow restriction during rest periods on muscle oxygenation. Front Physiol. 2021; 12(1067).
40. Paradis-Deschênes P, Joanisse DR, and Billaut F. Ischemic preconditioning improves time trial performance at moderate altitude. Med Sci Sports Exerc. 2018; 50(3):533-541.
41. Lee TXY, Wu J, Jean WH, Condello G, Alkhatib A, Hsieh CC, Hsieh YW, Huang CY, and Kuo CH. Reduced stem cell aging in exercised human skeletal muscle is enhanced by ginsenoside Rg1. Aging (Albany NY). 2021; 13(12):16567-16576.
42. Liao B, Zhao Y, Wang D, Zhang X, Hao X, and Hu M. Nicotinamide mononucleotide supplementation enhances aerobic capacity in amateur runners: a randomized, double-blind study. J Int Soc Sports Nutr. 2021; 18(1):54.
43. Gomez-Cabrera M-C, Domenech E, Romagnoli M, Arduini A, Borras C, Pallardo FV, Sastre J, and Viña J. Oral administration of vitamin C decreases muscle mitochondrial biogenesis and hampers training-induced adaptations in endurance performance. Am J Clin Nutri. 2008; 87(1):142-149.
44. Van Beekvelt MC, Colier WN, Wevers RA, and Van Engelen BG. Performance of near-infrared spectroscopy in measuring local O(2) consumption and blood flow in skeletal muscle. J Appl Physiol (1985). 2001; 90(2):511-519.
45. Ferrari M, Mottola L, and Quaresima V. Principles, techniques, and limitations of near infrared spectroscopy. Can J Appl Physiol. 2004; 29(4):463-487.
46. Cohen J. (2013). Statistical power analysis for the behavioral sciences: Academic press).