Dissertation of the Degree of Master of Sports Science

Effect of Exercise-Induced Neutrophil Maturation on Muscle Regeneration *in vitro*

운동에 의해 유도된 호중구의 성숙이
In vitro 상에서 근육 재생에 미치는 영향

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Abstract

Skeletal muscle forms the largest organ system in the human body and it is essential for generating strength and movement. Skeletal muscle is prone to injury in daily life, including mechanical trauma, heat stress, ischemia, and other pathogenic conditions, but it is uniquely able to adapt and remodel for protection.

The immune system is essential for muscle repair and regeneration. It is important in determining the rate and outcome of the healing process at injury sites. Among immune cells, neutrophils are an important first line of defense in the innate immune system. In the early stages of muscle regeneration, neutrophils initiate a cascade to remove the damaged muscle fibers and secrete cytokines to recruit other immune cells to the injury site. Therefore, controlling neutrophil mobilization and function may be an effective strategy to promote muscle regeneration.

Physical exercise can mediate immune components that successfully control tissue regeneration, including affecting functional changes in neutrophils. Acute moderate- or high- intensity exercise and chronic moderate intensity exercise can improve neutrophil chemotaxis and phagocytosis. To investigate the maturation of neutrophils after 4 weeks of mouse treadmill exercise, we isolated mouse neutrophils from bone marrow and maturation markers were compared. In the exercise group, significantly higher *LTF* and *CXCR2* mRNA levels were identified compared to the sedentary group. Following this, we performed a wound healing assay to evaluate whether treatment with exercise-activated

neutrophils is effective for muscle regeneration *in vitro*. Exercise-activated neutrophils improved wound healing of mouse muscle cells; we further confirmed this at human cell level. For neutrophil-like cells treated with dexamethasone (dex), exercise mimetics had significantly higher *LTF*, *fMLP*, and *CD11b* mRNA levels compared to the control. Furthermore, neutrophil-like cells treated with dex improved wound healing of human muscle cells.

This is the first study showing that exercise affects neutrophil maturation and that exercise-induced neutrophils contribute to muscle regeneration *in vitro*.

Keywords: neutrophil, exercise, maturation, muscle regeneration, wound healing
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Contents

I. Introduction

1. Need for research

Skeletal muscle is the largest organ in the human body (Pedersen, 2013) and it enables humans to move and perform daily activities (J.Antonio, D.Kalman, 2008). Skeletal muscles play an essential role in respiratory mechanics and help to maintain posture and balance, as well as protecting vital organs. Development and maintenance of skeletal muscle is critical for movement, health, and issues associated with quality of life (LA et al., 2017). Skeletal muscle is susceptible to various injuries in daily life, such as mechanical trauma, thermal stress, myotoxic agents, ischemia, neurological damage, and other pathogenic conditions. (Yang & Hu, 2018a). However, muscle is uniquely able to adapt and remodel to protect against such stresses, which is known as muscle regeneration. Muscle regenerative ability declines with aging and disease, causing deteriorating muscle function, which can result in multiple diseases (Barberi et al., 2013; Muñoz-Cánoves et al., 2020; Yamakawa et al., 2020). In particular, skeletal muscles become smaller and weaker with age. This loss of muscle mass results in a reduced capacity to generate force and to perform daily tasks (Close et al., 2005).

The immune system plays a crucial role in tissue repair and regeneration. Indeed, the immune response to tissue injury often determines the degree of scarring, as well as the structure and function of the restored tissue (Julier et al., 2017a). The inflammatory response is central to linking the initial muscle injury response to the proper timing of muscle injury recovery. Furthermore, different

types of immune cells and cytokines play important roles in the muscle regeneration process (Smith et al., 2008; Tidball, 2005). Therefore, controlling immune components *via* physical exercise can mediate successful tissue regeneration (J. Chen et al., 2022; da Silveira et al., 2021).

When muscle injury occurs, muscle fiber membranes are damaged and cellular contents and chemotactic factors are released from the cells, which mobilize different types of immune cells (Tobin et al., 2021; Yang & Hu, 2018a). Neutrophils are important immune cells that act in the early stages of muscle regeneration after injury (Pizza et al., 2005). Although they are the first immune cells to act after tissue injury, their role in muscle healing has been somewhat overlooked. Controlling neutrophil mobilization and function could be an effective strategy to promote muscle regeneration (Julier et al., 2017a). The three main roles of neutrophils in repairing damaged tissue after injury are as follows: the first is as primary phagocytes, which remove cellular debris from the site of injury. The removal of cellular debris promotes tissue regeneration and connective tissue deposition (Wang, 2018a). Second, neutrophils act as effectors that promote angiogenesis and regeneration by releasing cytokines, such as growth factors, vascular endothelial growth factor, and matrix metalloproteinase (Dalli et al., 2013). Third, neutrophils that have fulfilled their role undergo apoptosis and are removed by macrophages, reducing the inflammatory response (Poon et al., 2014).

Neutrophils are produced in the bone marrow. Granulocyte-macrophage progenitors are converted into myeloblasts and are involved in neutrophil production, followed by a maturation process that includes promyelocytes,

myelocytes, metamyelocytes, band cells, and finally mature neutrophil stages (Ng et al., 2019). Previous studies show that mature neutrophils have many proteins, including growth factors and proangiogenic factors, while mature neutrophils have a higher phagocytic capacity than immature neutrophils (Evrard et al., 2018a). These properties of mature neutrophils are consistent with their major role of repairing damaged tissues.

Exercise affects functional changes in neutrophils. Acute moderate- or high-intensity exercise and chronic moderate intensity exercise can improve neutrophil chemotaxis and phagocytosis (Barry et al., 2017; Simpson et al., 2015; Syu et al., 2012). Despite many studies on neutrophils associated with acute and chronic exercise, studies on the effects of neutrophils altered by exercise on muscle regeneration are few; more are needed in the future.

Studies suggest that physical exercise can improve neutrophil function, including phagocytosis and chemotaxis (Bartlett et al., 2017, 2020; Simpson et al., 2015; Syu et al., 2012) and that neutrophil maturation leads to an increase in neutrophil function (Evrard et al., 2018a; Ng et al., 2019). However, there are few studies confirming the effect of exercise on neutrophil maturation. In addition, the beneficial role of neutrophils in muscle regeneration (Julier et al., 2017b; Oprescu et al., 2020; Wang, 2018b) is relative to the role of neutrophils enhanced by exercise. However, there is no study on whether exercise-activated neutrophils are effective for muscle regeneration. Therefore, this study focused on biochemical change in neutrophils after physical exercise. Following this, we evaluated whether exercise-induced neutrophils were effective in muscle regeneration *in vitro*.

2. Purpose of research

First, we assessed whether exercise promotes neutrophil maturation. Second, by treating damaged muscle cells with exercise-activated neutrophils, we assessed whether this has a beneficial effect in the early phase of muscle recovery. Lastly, we used human cell lines to see if the same effect is produced at the human cell level.

3. Hypotheses

In order to clarify the purpose of this study, the following research hypotheses were established.

First, exercise will promote neutrophil maturation in mice.

Second, neutrophils activated by exercise will improve muscle regeneration *in vitro*.

Third, these results will be confirmed in experiments with human cell lines.

II. Study Background

1. Skeletal muscle regeneration

Skeletal muscle is one of the most abundant tissues in the human body. It accounts for 40–45% of the total body mass and is essential for generating force and movement (Close et al., 2005; Liu et al., 2018). Skeletal muscle has numerous physiological functions that extend beyond movement to a variety of other vital roles, including signal transduction (Pedersen & Febbraio, 2012).

Muscle is sensetive to various injuries in daily life, such as mechanical trauma, thermal stress, neurological damage, ischemia, myotoxic agents, and other pathogenic conditions. (Yang & Hu, 2018a). Skeletal muscle is subjected to considerable stress during everyday use. However, muscle is uniquely able to adapt and remodel to protect against such stresses. Failure in such processes can be catastrophic, particularly in older individuals as skeletal muscles become smaller and weaker as we age. This loss of muscle bulk results in a reduced capacity to generate force and ability to undertake everyday tasks (Close et al., 2005).

Skeletal muscle regeneration is a complex process comprising multiple steps. Recent findings suggest that inflammatory responses may be central to bridging initial muscle injury responses and timely muscle repair. Various immune cells and cytokines have crucial roles in the muscle regeneration process (Yang & Hu, 2018a).

2. Exercise and immune cells

The acute immune response to exercise depends on the intensity and duration of effort. Early exercise immunology investigations focused on large perturbations of basic leukocyte subsets associated with the physiological stress of athletic endeavor (Nieman, 1997; Tvede et al., 1989). Increasing attention is currently being directed to enhanced immunosurveillance of distinct immune cell subtypes during exercise bouts of less than 60 min, which have potential prevention and therapeutic value.

Regular exercise involves the regulation of immune cells, particularly T and NK cells, which are the most responsive cells during exercise. Exercise increases the propotion of regulatory T cells and contributes to a stable immune environment (Yeh et al., 2006). Additionally, exercise enhances anti-tumor functions by promoting cytotoxic T cell development. The release of myokines, such as IL-6 or IL-15, during exercise enhances T cell survival and proliferation (Gebhardt & Krüger, 2022). Furthermore, exercise-induced IL-6 affects mobilization, redistribution, and activation of NK cells (Walsh et al., 2011).

3. Neutrophils

3.1 Neutrophils

Neutrophils, also known as polymorphonuclear leukocytes, are the most abundant cell type in human blood. They are produced in the bone marrow in large numbers, approximately 10^{11} cells per day. Under homeostatic conditions, neutrophils enter the circulation, migrate to tissues to complete their functions, and are finally eliminated by macrophages, all within one day. Neutrophils are important effector cells in the innate immune system (Maydas et al., 2014). They constantly patrol organisms for signs of microbial infection, and when found, these cells respond quickly by trapping and killing the invading pathogens. Three important antimicrobial functions are known for neutrophils: phagocytosis, degranulation, and the release of nuclear material in the form of neutrophil extracellular traps (NETs). Until recently, these functions were thought to be their only purpose. However, current research in several fields of neutrophil cell biology shows that neutrophils possess a much diverse repertoire of functional responses beyond simply killing microorganisms. Neutrophils respond to multiple signals by producing cytokines and other inflammatory factors that influence and regulate inflammation and the immune system (Nauseef & Borregaard, 2014; Scapini & Cassatella, 2014). Now neutrophils are recognized as transcriptionally active complex cells (Ericson et al., 2014) that produce cytokines (Tecchio & Cassatella, 2016), modulate the activities of neighboring cells, contribute to resolving inflammation (Greenlee-Wacker, 2016), regulate macrophages for long-term immune responses (Chen et al.,

2014), actively participate in several diseases, including cancer (Uribe-Querol & Rosales, 2015; Mishalian et al., 2017), and even participate in innate immune memory (Netea et al., 2016).

3.2 Neutrophils and muscle regeneration

Neutrophils make up the majority of the white blood cells and are considered an important first line of defense of the innate human immune system. Neutrophils capture or destroy microorganisms within the body through phagocytosis, degranulation, and NETs, and also act as mediators of inflammatory responses (Rosales, 2018).

In the early stages of muscle regeneration, cell membrane damage causes cell contents and chemotactic factors to flow out of the cell. This phenomenon attracts neutrophils, which remove the damaged muscle fibers from the site of injury. The removal of these muscle fiber remnants speeds up muscle regeneration. Neutrophils also secrete a variety of cytokines that attract more immune cells, such as macrophages. Neutrophils can trigger the initiation of cellular responses that control muscle stem cell activation, proliferation, and differentiation (Yang & Hu, 2018; Ziemkiewicz et al., 2021). Therefore, neutrophils are important for regulating muscle regeneration.

3.3 Neutrophils and exercise

Neutrophils and lymphocytes predominantly migrate due to exercise, and in the early stages of stress recovery, neutrophil levels in the blood continue to rise. A single bout of moderate-intensity exercise increases neutrophil chemotaxis (Barry et al., 2017), and neutrophil phagocytic capacity is improved immediately after high- and moderate-intensity exercise (Bartlett et al., 2017). Neutrophil oxidative burst is strongly influenced by exercise intensity and duration, which increases at moderate intensity and decreases at high intensity (Simpson et al., 2015). The ability to attach to the endothelium is a first step in neutrophil migrations to the site of infection or damage. However, one-time training can improves neutrophil chemotaxis and phagocytic abilities but not their ability to attach to the endothelium (Ortega, E., 2003).

Although there are multiple studies on the effect of single bouts of exercise on neutrophils, little is known about the effects of chronic exercise on neutrophil function, so further studies are needed (Kandola & Stubbs, 2020).

III. Materials and Methods

1. Cell culture and differentiation

The human promyelocytic HL-60 cell line was obtained from the Korean Cell Line Bank. Cells were cultured in RPMI-1640 medium (WelGENE, Daegu, Korea) supplemented with 10% fetal bovine serum (FBS) in a humidified atmosphere containing 5% CO2 at 37° C. For differentiation, 1% dimethyl sulfoxide (DMSO) (Sigma, MO, USA) was added to $5x10^5$cells/ml of RPMI-1640 medium followed by incubation for 6 days in a humidified atmosphere at 37° C without changing the medium.

Human skeletal muscle myoblasts (HSMMs) were obtained from Lonza. HSMM were cultured at 37° C in a humidified atmosphere with 5% CO_2 in SkGM-2 medium (Lonza, MD, USA). HSMM differentiation medium was prepared by adding 2% horse serum (HS) to DMEM-F12 medium.

Mouse muscle myoblasts C2C12 (ATCC, VA, USA) were cultured in DMEM medium supplemented with 10% FBS. For differentiation, when fully confluent, the medium was changed to a differentiation medium consisting of DMEM supplemented with 2% HS.

2. HL60 differentiation with dexamethasone treatment

Dexamethasone (Sigma, MO, USA) was dissolved in ethanol to a concentration of 200mM then diluted in RPMI-1640 to $25x10^5$ times (500 nM) their final concentration prior to addition to the cell cultures.

3. Wound healing assay

HSMMs were grown to approximately 80% confluence in a 24-well plate under standard culture conditions; SkGM™-2 Skeletal Muscle Cell Growth Mediaum-2 BulletKit™ (Lonza, MD, USA). Initially a disposable plastic tip (5-200 μl) was used in a scratching motion to create the wound. To quantify wound healing *in vitro*, the percentage of wound closure, rather than cell number, was determined over a 7-h period. This was chosen because it eliminated the process of proliferation as a confounding factor during wound closure. Images were taken at initial wounding as well as at 1, 3, 5, and 7 h postwounding. The wound area was calculated by tracing along the border of the wound using Motic 2.0 image analysis software, and the percentage of wound closure was calculated using the following equation.

Scratch wounds were created in confluent monolayers of C2C12 cells using the IncuCyte Woundmaker Tool (Sartorius, Gottingen, Germany). after washing away suspended cells,

4. Quantitative PCR analysis

Total RNA was isolated from mouse primary neutrophils and neutrophil-like cells (dHL-60) using the TRIzol reagent (Sigma, MO, USA). The RNA was converted into cDNA as it is more stable. For the conversion of cDNA, 1000 ng/ μl of RNA was utilized. cDNA was synthesized using the Accupower ® CycleScript RT PreMix (Bioneer, Daejeon, Korea).

To quantify the relative expression of *MPO, LTF, fMLP*, and *CD11b* genes in response to exercise (dexamethasone or treadmill exercise), quantitative PCR was carried out. The primers used in PCR are liested in Table 1, 2. First 2 μl of the converted cDNA was mixed with 18 μl pre-mix (2 μl primer mix, 7 μl nuclease-free water, 10 μl SYBR green). The pre-mix without cDNA was used as the negative control. The C_t value of individual genes were normalized using the reference gene (*beta-actin*). Analysis was carried out using the $2^{-\Delta\Delta Ct}$ method. The data is presented as relative gene expression.

Table 1. primer sequences used for real-time quantitative PCR analysis (mouse).

Target gene	Forward Primer (5' – 3')	Reverse Primer (5' – 3')
MPO	GAGGCCCGGAAGATTGTAGG	TGGGCCGGTACTGATTGTTC
LTF	AGCAGTCGTGAAGAACAGCA	ACACGAGCTACACAGGTTGG
fMLP	ATTGCACTGGACCGCTGTAT	TCCAGGGGGAGAAGTCGAAA
CD11b	CTTCGGGCAGTCTCTGAGTG	CCTCCCCAGCATCCTTGTTT
Ly6G	TGCCCCACTACTCTGGACAA	AGGACTGAAACCAGGCTGAA
CXCR2	GGGTCGTACTGCGTATCCTG	AGACAAGGACGACAGCGAAG
Beta-actin	GGCTCCTAGCACCATGAAGA	AGGGTGTAAAACGCAGCTCAG

Table 2. primer sequences used for real-time quantitative PCR analysis (human).

Target gene	Forward Primer (5' – 3')	Reverse Primer (5' – 3')
MPO	TTTGACAACCTGCACGATGAC	CGGTTGTGCTCCCGAAGTAA
LTF	CTCCCCAGGTGTGTTGGG	TAAGCAGATGGATGGGCAATC
fMLP	GTTGACGGTGAGAGGCATCA	CACGGATTCTGACTGTGGCT
CD11b	GGGCTCTGCTTCCTGTTTG	CTGCGTTATTGGCTTCACC
Beta-actin	GCCTTCCTTCCTGGGTATGG	TTCTGCATCCTGTCGGCAAT

5. Animal experiments

This study aimed to clarify the effect of treadmill exercise-induced alterations in neutrophils on skeletal muscle regeneration. The neutrophils donor group was divided into the sedentary groups (N = 8) and exercise groups (N = 8). All groups were made up of 11-week-old C57BL/6J male mice.

5.1 Animal care

Seven-week-old wild-type C57BL/6N male mice were obtained from the Central Lab. Animal Inc., (CLA). All mice were housed in a controlled environment at 22 - 25° C with a 12:12 h light dark cycle. Mice were fed water and food *ad libitum*. All experiments were approved by the Institutional Animal Care and Use Committee (IACUC) of Seoul National University, IACUC-SNU-211217-3-1.

5.2 Treadmill exercise protocol

In all procedures involving training the mice were randomly allocated into the sedentary or trained experimental groups. The trained group was subjected to exercise on a treadmill (Daejong, Daejeon, Korea) throughout a 4 week period, with 5 weekly sessions at the same time of day (19:00). The exercise protocol consisted of the following steps: (1) 8 m/min for 5 min for warm-up, (2) 60 min for the main exercise. The aerobic training load from the first to the last week was equivalent to 10, 12, 14, and 16 m/min during the 4 weeks (Figure 1). The treadmill exercise protocol was formulated based on previous research (Yoon et al., 2021). All of the exercised mice completed the 65-min treadmill protocol.

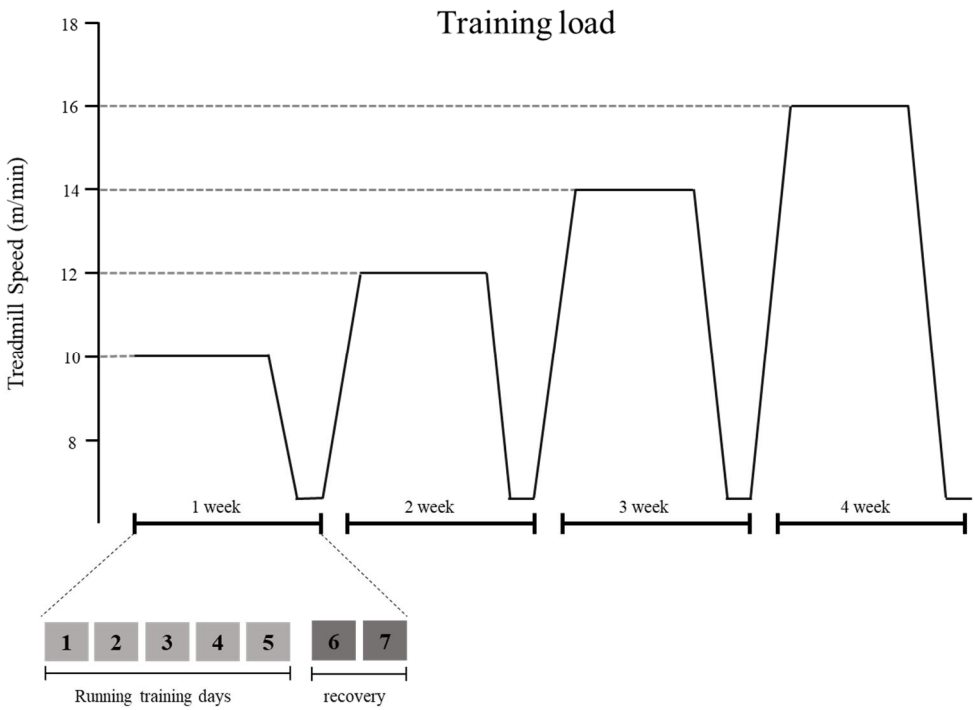

Figure1. Schematic design for the exercise protocol

6. Neutrophil isolation

Neutrophils were isolated from mouse bone marrow using Histopaque-based density gradient centrifugation. Mouse bone marrow cells were layered over Histopaque 1077 (Sigma, MO, USA) and Histopaque 1119 (Sigma, MO, USA), and centrifuged for 30 min at 872 g. Neutrophils were collected from the Histopaque 1077 and Histopaque 1119 interface and washed with RPMI 1640 supplemented with 10% FBS and centrifuged for 7 min at 427 g.

In the *in vitro* experiments, HL-60 cells were used for confirming the mechanism by which exercise affected the function of neutrophils and muscle cell regeneration.

7. Statistical analysis

Statistical analysis was performed using Graph Pad Prism v.7 software (Graph Pad Software Inc., CA, USA). All diagrams and data present the mean ± standard error (SEM). A two-way ANOVA test was performed to reveal whether neutrophils were affected by exercise in the experimental groups and to identify statistically significant changes in cellular response between the treatment and control groups. A p-value < 0.05 was accepted as statistically significant.

IV. Results

1. Exercise promotes neutrophil maturation in mice

During development in the bone marrow, neutrophils undergo sequential maturation steps. Immature neutrophils can be distinguished from mature neutrophils by granular protein expression and enhanced transcriptional activity (Kim et al., 2017; Theilgaard-Mönch et al., 2005). To explore whether exercise promoted neutrophil maturation, granular mRNA expression was quantified. *MPO* mRNA levels were not significantly different between the two groups (Figure 2A, $p > 0.05$) but we observed an incremental change in *LTF* mRNA levels in the neutrophils of the exercise group ($p < 0.05$) compared to the sedentary group (Figure 2B). *fMLP* and *CD11b* mRNA levels were not significantly different between the two groups (Figure 2C, D, $p > 0.05$).

In mouse neutrophils, the expression of surface markers changes during maturation. Classically, Ly6G and CXCR2 are used as maturation markers of neutrophils in mice (Capucetti et al., 2020; Evrard et al., 2018b). Therefore, to confirm whether exercise promotes maturation of mouse neutrophils, we measured *Ly6G* and *CXCR2* mRNA expression levels in neutrophils from the exercise and sedentary groups, respectively. *Ly6G* showed higher neutrophil values in the exercise group compared to the sedentary group, but the difference was not significant (Figure 3A, $p > 0.05$). *CXCR2* mRNA expression levels were significantly higher in the neutrophils of the exercise group ($p < 0.05$) than the sedentary group (Figure 3B).

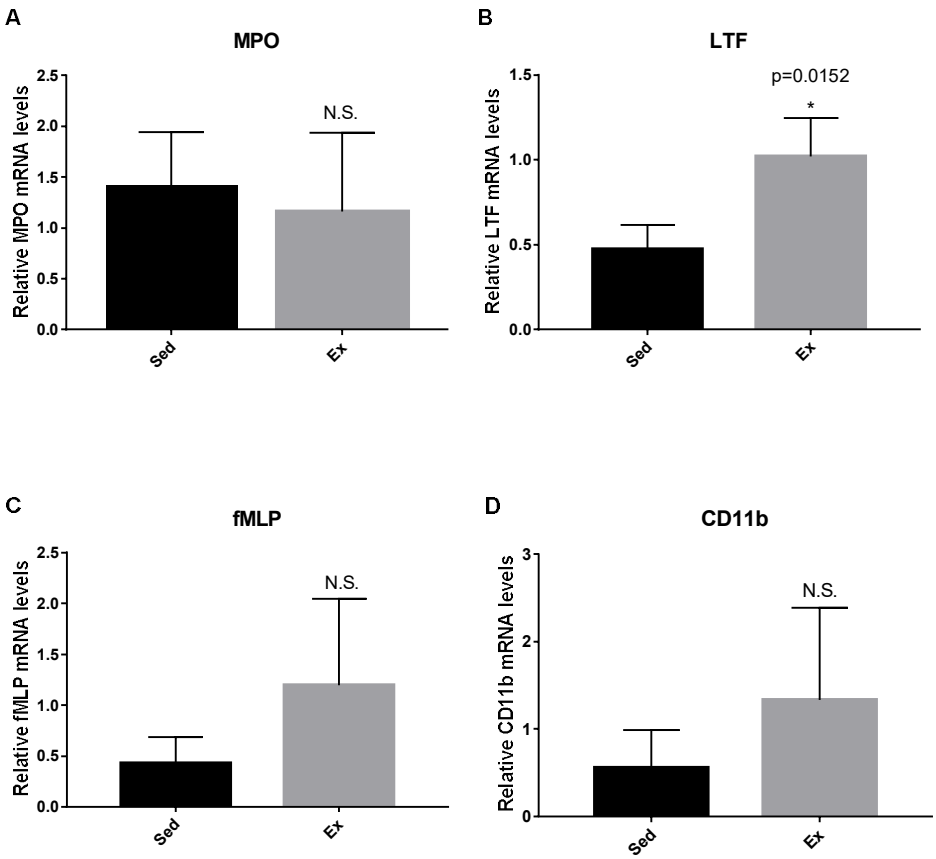

Figure 2. Effect of 4 weeks of treadmill exercise on granule markers expressed at each stage of neutrophil maturation. **(A)** *MPO*, **(B)** *LTF*, **(C)** *fMLP*, and **(D)** *CD11b* mRNA expression levels in mouse neutrophils *$p < 0.05$, N.S. = not significant.

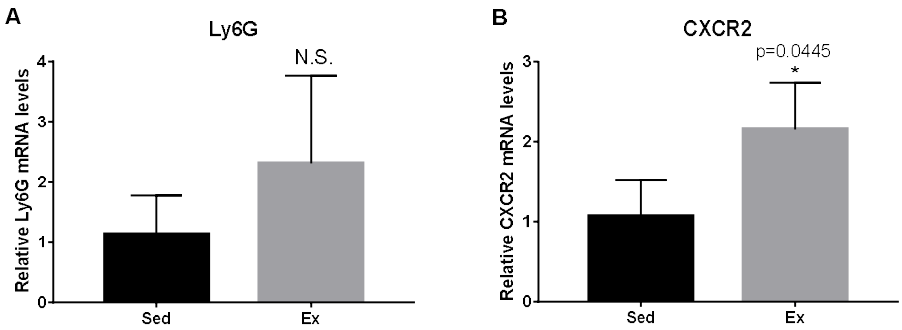

Figure 3. Effect of 4 weeks of treadmill exercise on surface markers of mouse neutrophils. **(A)** *Ly6G* and, **(B)** *CXCR2* mRNA expression levels in mouse neutrophils. *$p < 0.05$, N.S. = not significant.

2. Neutrophils from exercised mice enhanced C2C12 wound closure

Myoblast migration is essential for muscle development, regeneration, and repair (Musarò, 2014). To assess the effect of exercise-induced neutrophils on muscle regeneration *in vitro*, wound healing assays were performed, as the most appropriate method for analyzing myoblast migration during skeletal muscle repair.

Two-way ANOVA analysis revealed differences between groups with time after scratch[박1]. In particular, there was a significant difference in wound closure (%) over time ($F_{(4,21)}$ = 1230.40, $p < 0.001$) in wound closure (%) by group ($F_{(2,24)}$ = 32.34, $p < 0.001$). Post-hoc comparisons showed that wound closure (%) of C2C12 was significantly faster that the control when treated with exercise-induced neutrophils at 4 ($p < 0.05$), 8 ($p < 0.001$), 12 ($p < 0.001$), 16 ($p < 0.001$), and 20 h ($p < 0.001$) post-wounding. The wound closure (%) of C2C12 treated with neutrophils of sedentary mice was significantly higher at 8 ($p < 0.05$), 12 ($p < 0.001$), 16 ($p < 0.001$), and 20h ($p < 0.001$) after wounding compared to the control. Furthermore, wound closure (%) of C2C12 treated with neutrophils of exercised mice 4 ($p < 0.05$), 8 ($p < 0.05$), 12 ($p < 0.05$), and 20 h ($p < 0.05$) after wounding was significantly higher compared to those treated with neutrophils of sedentary mice.

These resulsts suggest that exercise-induced neutrophils have beneficial effects on muscle regeneration *in vitro*.

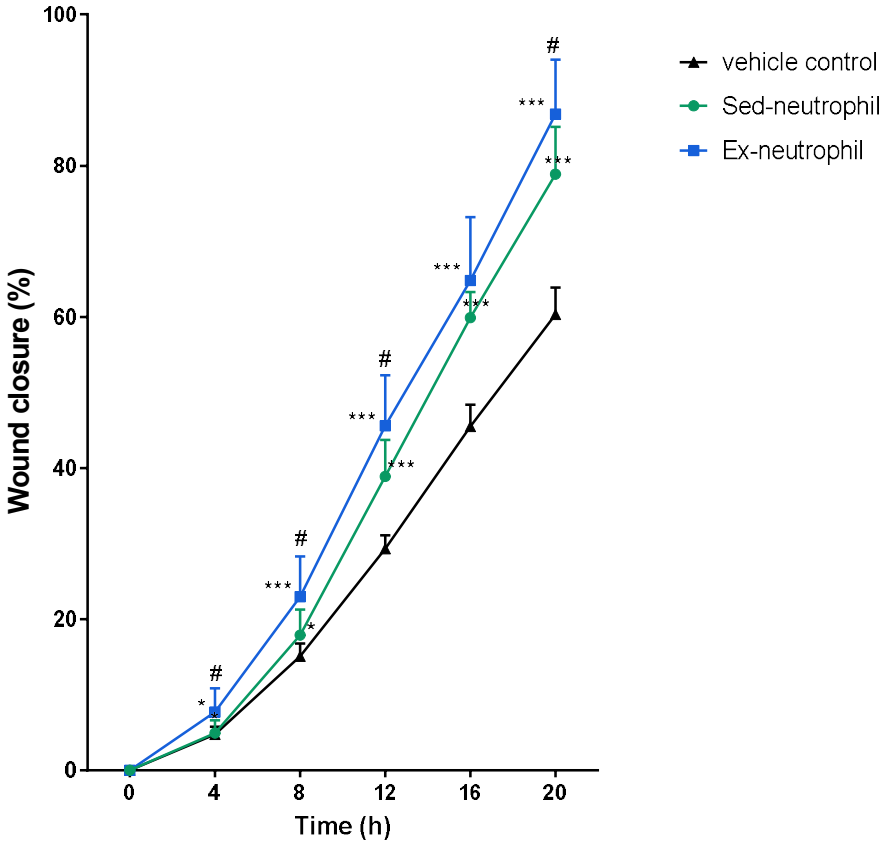

Figure 4. Summary percentage wound closure at different time points during C2C12 scratch wound assay. *$p < 0.05$ versus control, ***$p < 0.001$ versus control, #$p < 0.05$ versus sedentary neutrophil treatment.

3. Exercise mimetics promotes neutrophil-like cell (dHL-60) maturation

We confirmed that exercise mimetics promotes neutrophil-like cell maturation using human cell lines: HL-60 (human promyeloblasts) and HSMM (human skeletal muscle myoblasts). HL-60 was used after differentiation into neutrophil-like cells (dHL-60).

Cortisol is a glucocorticoid hormone secreted by the adrenal cortex in response to physical, psychological, or physiological stressors (Range et al., 2012). One stressor that significantly alters circulatory cortisol levels is physical exercise (Richmond & Rogol, 2016). Cortisol is a common biomarker used to analyze exercise responses in both athletes and the general population (Anderson & Wideman, 2017). Accumulated studies show that cortisol levels increase as a result of chronic (Gerber et al., 2013; Inoue et al., 2015) and acute exercise (Chang et al., 2008; Neto et al., 2013) in both adults humans and mice. In Particular, moderate- to high-intensity exercise increases circulatory cortisol levels likely due to a combination of hemoconcentration and adrenocorticotropic hormone and, hypothalamic-pituitary-adrenal axis stimulus (Gentili et al., 2008). Therefore, dexamethasone (dex), a type of glucocorticoid was administered to dHL-60 to mimic the effects of exercise.

Terminal granulopoiesis is characterized by sequential formation of different granule types and segmentation of the nucleus starts at the myeloblast/promyelocyte stage and ends with mature neutrophils. Granule types not only differ in the time point at which they are formed, but also in their

specific content (Fiedler & Brunner, 2012). To verify whether dexamethasone promotes dHL60 maturation, the granule markers expressed at each stage were confirmed *via* PCR.

MPO mRNA expression levels were higher in the dHL-60 group than the dHL-60 with dex group ($p < 0.05$). *LTF* mRNA expression levels were higher in the dHL-60 with dex group than in the dHL-60 group ($p < 0.01$). For *fMLP* and *CD11b,* expression levels were significantly higher in the dHL-60 with dex group compared to the dHL-60 group ($p < 0.001$, $p < 0.05$, respectively; Figure 5C, D).

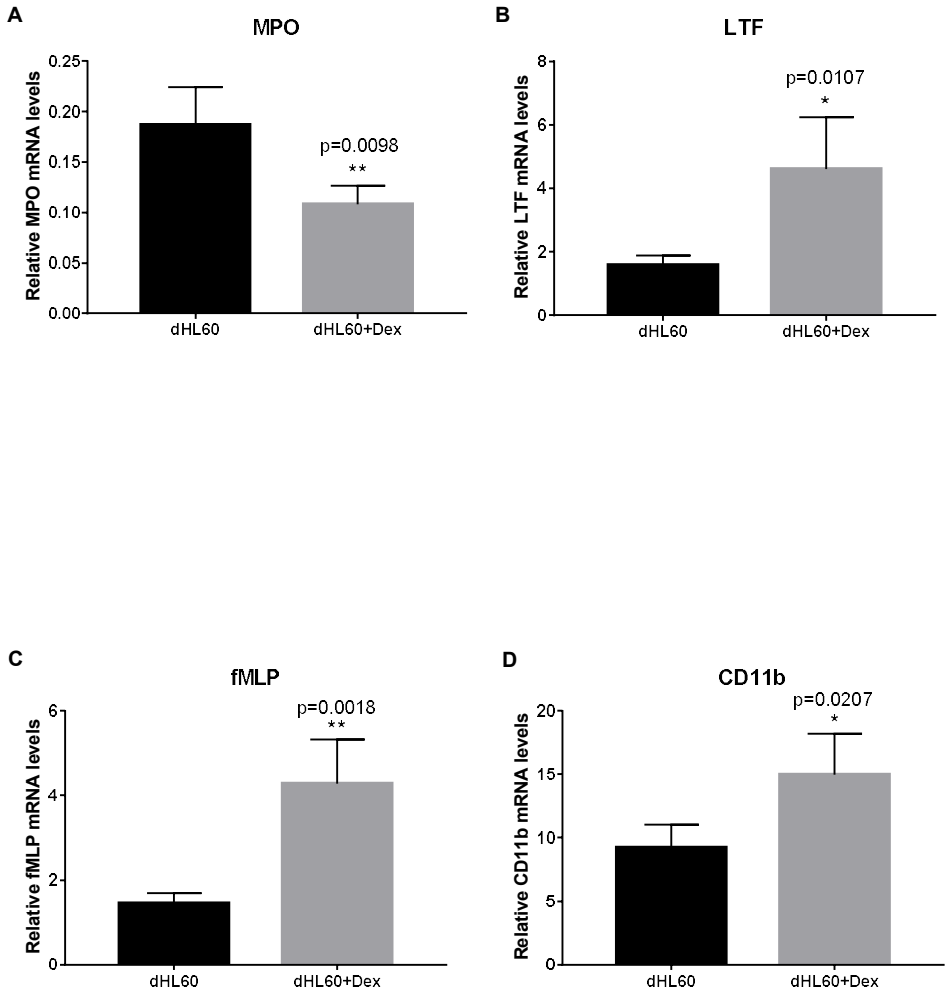

Figure 5. Effect of dexamethasone on granule markers expressed at each stage of neutrophil-like cell (dHL-60) development. **(A)** *MPO* **(B)** *LTF*, **(C)** *fMLP*, and **(D)** *CD11b* mRNA expression levels in dHL-60. *$p < 0.05$, **$p < 0.01$.

4. dHL-60 cells treated with dexamethasone enhance HSMM wound closure

The impact of dHL-60 treated with dexamethasone on HSMMs *in vitro* was evaluated using a scratch wound assay to assess cell migration.

Two-way ANOVA analysis revealed differences between groups with time after scratch[박2]. In particular, there were significant differences in wound closure (%) over time ($F_{(5,10)}$ = 87.69, $p < 0.001$) and in wound closure (%) by group ($F_{(1,10)}$ = 5.60, $p < 0.05$). Post-hoc comparison showed that wound closure (%) of HSMMs treated with dHL60 with dex occurred after scratch wound, 24 (p = 0.0298), 30 ($p < 0.05$) and 36 h ($p < 0.001$) was significantly higher compared to dHL60 alone.

The HSMM wound healing assay results suggest exercise-induced neutrophils have beneficial effects on muscle regeneration *in vitro* not only in a mouse cell model but also in a human cell model.

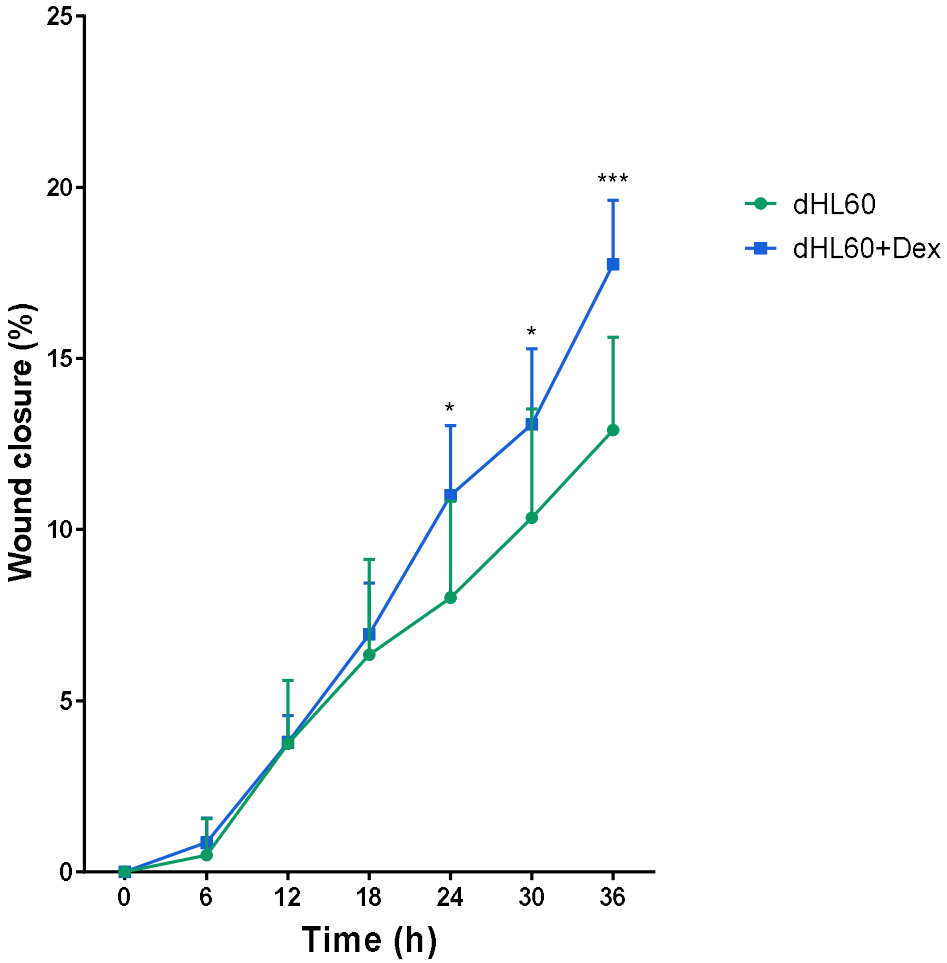

Figure 6. Percentage wound closure at different time points during HSMM scratch wound assay. *$p < 0.05$, ***$p < 0.001$

V. Discussion

Muscle regeneration is an important homeostatic process in adult skeletal muscle that maintains its ability to regenerate in response to a variety of damaging stimuli, even after development (Blaauw & Reggiani, 2014; Forcina et al., 2019; Seale et al., 2000). Appropriate muscle regeneration can recover muscle function after severe, repetitive muscle injuries. Skeletal muscle regeneration is strongly influenced by interactions with immune cells and is highly dependent on the inflammatory response (Tidball, 2017; Ziemkiewicz et al., 2021). In particular, neutrophils initiate a cascade to recruit other immune cells to the site of injury (Wang, 2018b; Yang & Hu, 2018b). However, muscle regenerative ability declines with age and disease, causing muscle function deterioration, which can develop into multiple diseases (Barberi et al., 2013; Muñoz-Cánoves et al., 2020; Yamakawa et al., 2020). Exercise enhances skeletal muscle regeneration by promoting various elements such as satellite cell (Sambasivan et al., 2011), signal pathways, senescence in fibro-adipogenic progenitors (Saito et al., 2020), and the immune system. Exercise also activates various immune cells, including neutrophils. Previous studies examining the effects of exercise on neutrophils can be roughly divided into changes in number and changes in function. The results of studies related to exercise and changes in neutrophil count can be summarised thus: acute high-intensity resistance exercise increases neutrophil concentration (Miles et al., 1998) and acute endurance exercise almost doubles it (Davison, 2011). As for research related to chronic exercise and changes in neutrophil count, some studies show no change (Barry et al., 2017), while others found a significant decrease (Michishita et al.,

2010), Although only temporarily. In studies related to exercise and neutrophil function, acute exercise increased chemotaxis (Barry et al., 2017) and phagocytosis (Syu et al., 2012), and in the oxidative burst function, it increased at moderate intensity but decreased at high intensity (Simpson et al., 2015). In addition, chronic moderate-intensity exercise increases chemotaxis, phagocytosis, and citrate synthases activity (Syu et al., 2012).

Although the extent of functional differences between immature and mature neutrophils remains an open question in the field, there are several studies showing that neutrophil function improves with maturation (Blanter et al., 2021; Mackey et al., 2021). Neutrophil differentiation involves the acquisition of neutrophil-specific granular components at various stages of neutrophil maturation (Lehman & Segal, 2020). It is also well documented that exercise improves neutrophil function (Bartlett et al., 2017, 2018, 2020; Padilha et al., 2022), However, changes in neutrophil maturation after exercise have not been explored. Therefore, in this study, we examined the effect of exercise on mouse neutrophil maturation using surface and granule markers that are altered by maturation. This study showed that 4 weeks of treadmill exercise accelerated the maturation of bone marrow-derived neutrophils in mice.

Myoblast migration is essential for muscle development, regeneration, and repair (Musarò, 2014). Skeletal muscle regeneration requires myoblast migration to the site of injury and within the wound to promote cell alignment in preparation for differentiation, fusion, and eventual healing (Goetsch et al., 2013). The scratch wound healing assay proved ideal for investigating the two-dimensional migration of skeletal muscle myoblasts *in vitro* and was specifically

optimized in our laboratory (Goetsch & Niesler, 2011). This study shows that neutrophils altered by exercise enhanced myoblast scratch wound healing *in vitro*.

At the human cell level, we used HL-60, human myelocytes, and HSMMs, human skeletal muscle myoblasts cell lines to assess whether neutrophil-like cell maturation is promoted by exercise and whether exercise-altered neutrophil-like cells are effective in myoblast wound healing. According to many previous studies, exercise increases cortisol levels in both humans and mice (C. Chen et al., 2017). Cortisol is the most important glucocorticoid hormone, and dexamethasone is a type of glucocorticoid and a commonly used chemical in experiments in vitro. Cortisol is also a possible cause of exercise-induced neutrophil change (Peake, J. M. 2002). Therefore, in this study, to create an experimental in vitro model to assess neutrophil-like cells (dHL-60) modified by exercise, dexamethasone treatment was used to stimulate exercise. There are several limitations to using this procedure to mimic the effects of exercise in vitro. However, this model is a useful tool to overcome time and situational difficulties of in vivo experiments (Carter & Solomon, 2019). This study shows that maturation was accelerated in neutrophil-like cells treated with dexamethasone, and was effective for human myoblast scratch wound closure *in vitro.* Experiments using human cell models compund the results found using mouse neutrophils. These findings imply that mouse and human neutrophils may accelerate neutrophil maturation *via* the effects of exercise, and exercise-induced neutrophils have a beneficial effect on skeletal muscle regeneration.

In summary, we observed that exercise or an exercise-mimetic environment

induced by dexamethasone promotes the maturation of neutrophils or neutrophil-like cells. We suggest that neutrophil or neutrophil-like cells induced by exercise or exercise mimetics could accelerate skeletal muscle cell regeneration *in vitro*. Further studies to validate these effects *in vivo* and to elucidate the detailed mechanisms of the effects of exercise-induced neutrophils on skeletal muscle regeneration will be of great interest.

VI. Conclusion

In conclusion, 4 weeks of treadmill exercise accelerated the maturation of bone marrow-derived neutrophils in mice. Neutrophils altered by exercise enhanced myoblast scratch wound healing *in vitro*. In addition, maturation was accelerated in neutrophil-like cells treated with dexamethasone, and was effective for human myoblast scratch wound closure *in vitro*. Therefore, the study suggests that exercise-induced neutrophils have beneficial effect on muscle regeneration *in vitro*.

VII. Reference

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Abstract (Korean)

골격근은 인체에서 가장 큰 기관계를 형성하며 힘과 움직임을 생성하는 데 필수적이다. 골격근은 일상생활에서 발생하는 기계적 외상, 열 스트레스, 허혈 및 기타 감염 상황 등에 의해 손상되기 쉽지만 이러한 스트레스에 대한 보호기전으로 스스로 적응하고 재형성하는 고유한 능력을 가지고 있다.

면역 체계는 근육 회복 및 재생에 필수적이다. 또한 손상 부위 치유 과정의 속도와 결과를 결정하는 데 있어서 중요하다. 면역 세포 중에서 호중구는 선천성 면역체계의 중요한 첫번째 방어선이다. 근육 재생의 초기 단계에서 호중구는 손상된 근육 섬유를 제거하고 손상 부위에 다른 면역세포를 동원하기 위해 사이토카인을 분비한다. 따라서 호중구 동원과 기능을 조절하는 것은 근육 재생을 촉진하는 효과적인 전략이 될 수 있다.

신체 운동은 호중구의 기능적 변화에 영향을 미치며 조직 재생을 성공적으로 제어하는 면역 구성 요소를 중재할 수 있다. 일회성의 중강도 또는 고강도 운동과 만성적 중강도 운동은 호중구의 주화성과 식균 작용을 개선할 수 있다. 본 연구는 4 주간의 마우스 트레드밀 운동 후, 호중구의 성숙도를 조사하기 위해 골수에서 호중구를 분리하고 성숙 마커를 비교했다. 운동 그룹에서 *LTF* 및 *CXCR2* mRNA 수준이 비운동 그룹에 비해 유의하게 높은 것으로 확인되었다. 그 후, 운동에 의해 변화된 호중구가 *in vitro* 상에서 근육 재생에 효과적인지 평가하기 위해 wound

healing assay를 수행했다. 운동 유발 호중구는 마우스 근육 세포의 상처 치유를 촉진시켰다. 우리는 이것을 인간 세포 수준에서 추가로 확인했다. 운동 모방체로 설정한 덱사메타손으로 처리된 호중구 유사 세포의 경우, 대조군에 비해 *LTF, fMLP* 및 *CD11b* mRNA 수준이 상당히 높았다. 또한 덱사메타손으로 처리된 호중구 유사 세포는 인간 근육 세포의 상처 치유를 촉진시켰다.

따라서 본 연구는 운동이 호중구 성숙에 영향을 미치고 운동 유발 호중구가 *in vitro* 상에서 근육 재생에 기여한다는 것을 보여주는 첫 번째 연구이다.