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Effect of exercise-induced Neutrophil maturation on skeletal muscle repair *in vitro*

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ABSTRACT

Neutrophils as first line defender initiate a cascade of healing process immediately after muscle injury. At muscle injury site, neutrophils remove damaged muscle fibers and recruit other immune cells and these functions show in mature neutrophils. In the previous study, physical exercise can mediate neutrophils' functional changes such as phagocytosis and chemotaxis, though there is no research on how exercise-induced neutrophils contribute the muscle regeneration.

In this present study, we investigated the maturation of neutrophils after 4 weeks of mouse treadmill exercise and assessed wound healing assay to evaluate whether treatment with exercise-activated neutrophils is effective for skeletal muscle repair *in vitro*. In the exercise group, significantly higher mRNA levels of maturation markers compared to the sedentary group and exercise-activated neutrophils improved wound healing of mouse muscle cells.

To confirm at the human cell level, based on the well-known fact that exercise increases circulating cortisol levels, neutrophil-like cells were treated with dexamethasone (dHL60 + dex) as exercise mimetics. dHL60 + dex had significantly higher mRNA levels of neutrophil maturation marker and improved wound healing of human skeletal muscle cells compared to the control. These findings suggest that exercise affects neutrophil maturation and that exercise-induced neutrophils contribute to skeletal muscle repair *in vitro*.

1. Introduction

Skeletal muscle is the largest organ in the human body [1] and it enables humans to move and perform daily activities [2]. Skeletal muscles play an essential role in respiratory mechanics and help to maintain posture and balance, as well as protect vital organs. Development and maintenance of skeletal muscle are critical for movement, health, and issues associated with quality of life [3]. 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 [4,5]. However, muscle is uniquely able to adapt and remodel to protect against such stresses, which is known as muscle regeneration. However muscle regenerative ability declines with aging and diseases such as obesity, diabetes and myopathies, causing muscle function deterioration, which can result in multiple diseases [6,7]. In particular, skeletal muscles become smaller and weaker with age and this loss of muscle mass results in a reduced capacity to generate force and to perform daily tasks [8].

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 [9]. 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 [10,11]. Therefore, controlling immune components *via* physical exercise can mediate successful tissue regeneration [12,13].

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 [4,14]. Neutrophils are important immune cells that act in the early stages of muscle regeneration after injury [15]. 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

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an effective strategy to promote muscle regeneration [9]. 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 [16]. Second, neutrophils act as effectors that promote angiogenesis and regeneration by releasing cytokines, such as growth factors, vascular endothelial growth factor, and matrix metalloproteinase [17]. Third, neutrophils that have fulfilled their role undergo apoptosis and are removed by macrophages, reducing the inflammatory response [18].

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 [19]. Neutrophil granule production begins with immature neutrophils and continues to the mature neutrophil stage: azurophilic (primary) granule, specific (secondary) granule, gelatinase (tertiary) granule and secretary granule each *MPO*, *LTF*, *fMLP*, *CD11b* [20]. Previous studies show that neutrophil function is related to granule production and that neutrophil function such as antimicrobial, phagocytosis and transmigration improves as granule production increases [21]. These properties of mature neutrophils are consistent with their major role in repairing damaged tissues.

Exercise affects neutrophil counts and increases neutrophil concentrations during and after exercise [22–24]. In an aerobic exercise study, neutrophil counts were significantly reduced after exercise in people with chronic inflammatory diseases [25]. Whether this effect is detrimental or beneficial depends on the context [26]. Exercise specifically affects functional changes in neutrophils. Acute moderate- or high-intensity exercise and chronic moderate-intensity exercise can improve neutrophil chemotaxis and phagocytosis [27–29]. 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 [27,29–31] and that neutrophil maturation leads to an increase in neutrophil function [19,32]. However, few studies are confirming the effect of exercise on neutrophil maturation. In addition, the beneficial role of neutrophils in muscle regeneration [33–35] 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.

We hypothesized that the exercise promotes neutrophil maturation in mice and neutrophils activated by exercise improve skeletal muscle repair *in vitro*. Moreover these hypotheses tried to validate with experiments using human cell lines. To test that idea, we isolated mouse neutrophils after 4 weeks of treadmill exercise and assessed the neutrophil maturation markers. To assess whether exercise-induced neutrophils have a beneficial effect in the early phases of muscle regeneration *in vitro*, wound healing capacity was monitored in muscle cells treated with exercise-induced neutrophils. Lastly, we confiremd whether regenerative capacity of muscle cells was enhanced by neutrophil-like cells in exercise mimetic conditions at the human cell level.

2. Methods

Ethical approval

All experimental procedures were conducted according to the protocol approved by the animal ethical review board of Seoul National University (IACUC-SNU-211217-3-1) and adhered to the relevant guidelines and regulations concerning the management and handling of experimental animals. This study is reported in accordance with the ARRIVE guidelines (https://arriveguidelines.org).

2.1. Animal study

For the animal experiments, male C57BL/6 (B6) mice (7 weeks) were obtained from the Central Lab. Animal Inc. (Seoul, Korea). All mice were housed in a controlled environment at 22–25 °C with a 12:12 h light dark cycle. In the experiment, mice were randomly allocated into the sedentary (n = 8) or exercise experimental groups (n = 8). The exercise 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 (Fig. 1a). The treadmill exercise protocol was formulated based on previous research [24]. All of the exercised mice completed the 65-min treadmill protocol.

2.2. Neutrophil isolation

Neutrophils were isolated from mouse bone marrow using Histopaque-based density gradient centrifugation [36]. 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.

2.3. HL-60 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.

2.4. HSMM, C2C12 cell culture

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).

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.

2.5. Cortisol analysis

Blood cortisol levels were measured using an ELISA kit (LSbio, WA, USA). Briefly, blood samples collected with EDTA were centrifuged at 1000×*g* for 15 min. After collecting supernatants for assay, dilute the sample with the reagent provided by the kit in accordance with the manufacturer's instructions. Both standard and sample dilutions were performed in 96-well plates. They were incubated at 37 °C for 40 min after mixing 50 μl capture antibody to each well. Washing was then performed with wash buffer three times, followed by the addition of 100 μl HRP-conjugate to each well and incubated for 30 min at 37 °C. After washing five times, add 90 μl of TMB substrate to each well and incubate in the dark for 20 min at 37 °C. Finally, the addition of 50 μl stop solution. The amount of cortisol was determined using the light absorbance of standard samples and their respective standard curves.

2.6. dHL60 with dexamethasone treatment

Dexamethasone (Sigma, MO, USA) was dissolved in ethanol to a

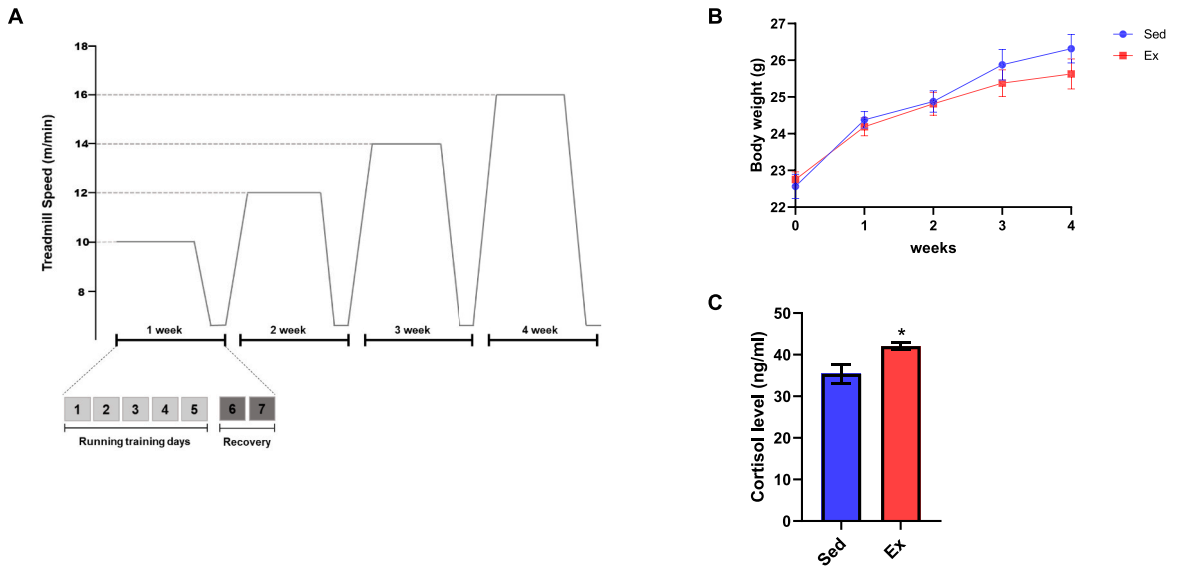

Fig. 1. Schematic design for the exercise protocol and changes in body weight and cortisol concentration. **A.** Experimental design for 4 weeks of treadmill exercise, **B.** Change in body weight over 4 weeks period (n = 8 per group), p = 0.692 **C.** Effect of treadmill exercise on blood cortisol level in mice (n = 4 per group), *p < 0.05, Sed, sedentary group, Ex, exercise group.

concentration of 200 mM then diluted in RPMI-1640 to 25x 10^5 times (500 nM) their final concentration prior to addition to the cell cultures.

2.7. Wound healing assay

C2C12, HSMM were each grown to approximately 80% confluence in a 96-well cell culture plate under standard culture conditions. Initially scratch wounds were created in confluent monolayers of C2C12, HSMM cells using IncuCyte Woundmaker Tool (Sartorius, Gottingen, Germany). Remove the debris by washing the cells once with 1 ml of the growth medium and then treat primary mouse neutrophils, dHL60 with standard couture medium of C2C12, HSMM respectively. To quantify wound healing *in vitro*, the percentage of wound closure, rather than cell number, was determined. Images were taken at initial wounding as well as at 4-h intervals up to 20 h post-wounding for C2C12 and 6-h intervals up to 36 h for HSMM. 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 wound healing assay protocol equation [37].

2.8. Quantitative RT-PCR

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 Tables 1 and 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	AGGAGTCGTGAAGAACAGCA	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

2.9. 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 followed the Turkey comparison test. A *p*-value <0.05 was accepted as statistically significant.

3. Result

3.1. Characterization of mice after treadmill exercise

Divided into sedentary group and exercise group, the exercise group perfomed treadmill exercise for 4 weeks (Fig. 1A). Both groups of mice gained weight over the 4 weeks, but there was no difference between the

groups (p = 0.692) (Fig. 1B).

Cortisol is a glucocorticoid hormone secreted by the adrenal cortex in response to physical, psychological, or physiological stressors [38]. We measured blood cortisol levels to determine whether this training protocol could induce chronic exercise effects in mice. Blood cortisol levels were significantly higher in the exercise group than in the sedentary group (Fig. 1C).

3.2. Exercise promotes neutrophil maturation in mice

After treadmill exercise for 4 weeks and then neutrophils were isolated from the bone marrow of each group. 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 (Fig. 2A) [20,39]. To explore whether exercise promoted neutrophil maturation, granular mRNA expression was quantified. *Mpo* mRNA levels were not significantly different between the two groups (Fig. 2B) but we observed an incremental change in *Ltf* mRNA levels in the neutrophils of the exercise group compared to the sedentary group (Fig. 2C). *fMlp* and *Cd11b* mRNA levels were not significantly different between the two groups (Fig. 2D and E).

In mouse neutrophils, the expression of surface markers changes during maturation. Classically, *Ly6G* and *Cxcr2* are used as maturation markers of neutrophils in mice [40,41] and previous studies using this marker was referenced [42]. Therefore, to confirm whether exercise promotes the 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 (Fig. 2F). *Cxcr2* mRNA expression levels were significantly higher in the neutrophils of the exercise group than the sedentary group (Fig. 2G).

3.3. Neutrophils from exercised mice enhanced C2C12 wound closure

Myoblast migration is essential for muscle development, regeneration, and repair [43]. To assess the effect of exercise-induced neutrophils on skeletal muscle repair *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. 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 than 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 (Fig. 3A). 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 for 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 (Fig. 3A).

These results suggest that exercise-induced neutrophils have beneficial effects on skeletal muscle repair *in vitro*.

3.4. 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 in the blood was confirmed to increase in response to exercise, a physiological stressor (Fig. 1C). 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 [20]. To verify whether dexamethasone

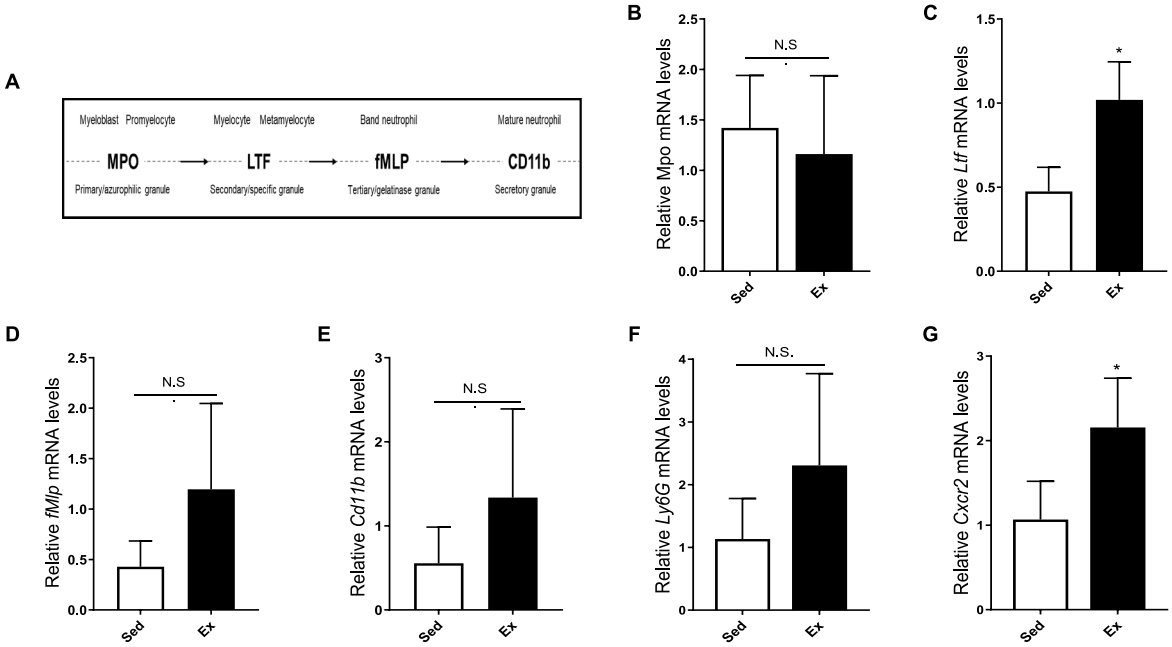

Fig. 2. Maturation marker mRNA change on mouse neutrophils after 4 weeks of treadmill exercise. **A.** Formation of different granules during neutrophil maturation, **B.** *Mpo*, **C.** *Ltf*, **D.** *fMlp*, **E.** *Cd11b*, **F.** *Ly6G* and, **G.** *Cxcr2* mRNA expression levels in mouse neutrophils from bone marrow, Sedentary group (n = 3), Exercise group (n = 4) **B-G.** normalized by *Beta-actin*, respectively. Sed, sedentary mouse neutrophils, Ex, exercise mouse neutrophils, *$p < 0.05$, N.S. = not significant.

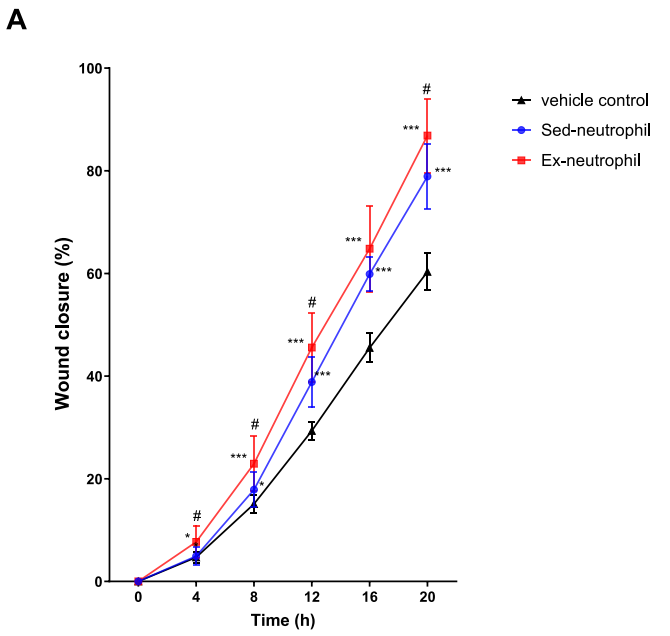

Fig. 3. Summary percentage wound closure at different time points during C2C12 scratch wound assay (n = 9 per group). *$p < 0.05$ versus control, ***$p < 0.001$ versus control, #$p < 0.05$ versus sedentary neutrophil treatment.

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 (Fig. 4A). *LTF* mRNA expression levels were higher in the dHL-60 with dex group than in the dHL-60 group (Fig. 4B). For *fMLP* and *CD11b,* expression levels were significantly higher in the dHL-60 with dex group compared to the dHL-60 group (Fig. 4C and D).

3.5. 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. 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 ($F_{(1,10)} = 5.60$, $p < 0.05$). The 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 (Fig. 5A).

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

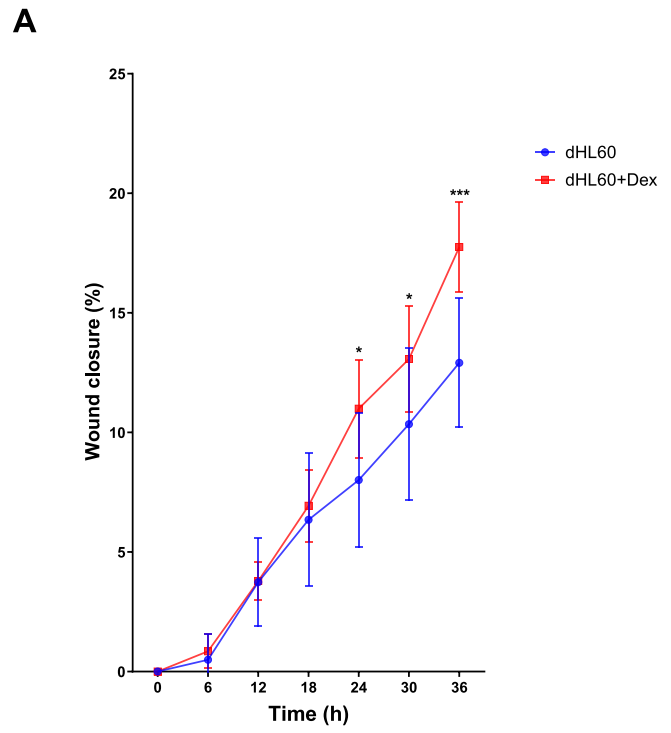

Fig. 5. Percentage of wound closure at different time points during HSMM scratch wound assay (n = 8 per group). *$p < 0.05$, ***$p < 0.001$.

4. Discussion

Muscle regeneration is an important homeostatic process that maintains its ability to regenerate in response to a variety of damaging stimuli [44]. 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 [45,46]. In particular, neutrophils initiate a cascade to recruit other immune cells to the site of injury [34,47].

Exercise enhances skeletal muscle regeneration by promoting various elements such as satellite cells [48], signal pathways [49], senescence in fibro-adipogenic progenitors [50], 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

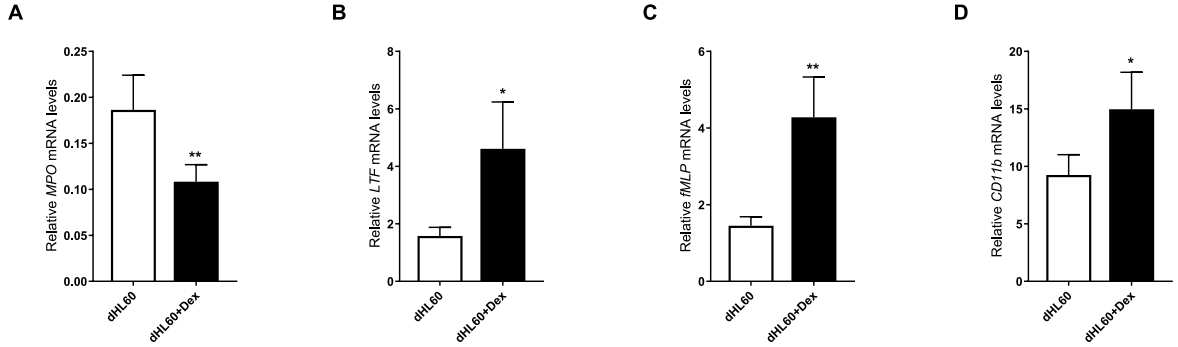

Fig. 4. Effect of dexamethasone on granule markers expressed at each stage of neutrophil-like cell (dHL-60) development. **A.** *MPO,* **B.** *LTF*, **C.** *fMLP* and **D.** *CD11b* mRNA expression levels in dHL-60, **A-D.** normalized by *Beta-actin*, respectively, Dex, dexamethasone *$p < 0.05$, **$p < 0.01$.

results of studies related to exercise and changes in neutrophil count are controversial. Acute high-intensity resistance exercise increases neutrophil concentration [51] and acute endurance exercise almost doubles it [52]. In chronic exercise changes in neutrophil count, some studies show no change [28]. While others found a significant decrease [25], although only temporarily. In studies related to exercise and neutrophil function, acute exercise increased chemotaxis [28] and phagocytosis [27]. In the oxidative burst function, it increased at moderate intensity but decreased at high intensity [29]. In addition, chronic moderate-intensity exercise increases chemotaxis, phagocytosis, and citrate synthase activity [27].

This study showed that 4 weeks of treadmill exercise accelerated the maturation of bone marrow-derived neutrophils in mice. although the extent of functional differences between immature and mature neutrophils remains an open question in the field, several studies are showing that neutrophil function improves with maturation [53,54]. Neutrophil differentiation involves the acquisition of neutrophil-specific granular components at various stages of neutrophil maturation [55]. It is also well documented that exercise improves neutrophil function [30,31,56,57]. 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 shows that neutrophils altered by exercise enhanced myoblast scratch wound healing *in vitro*. Myoblast migration is essential for muscle development, regeneration, and repair [43]. 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 [58]. 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 [59].

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. In further study, it appears worthwhile to investigate the neutrophil maturation marker not only at the mRNA level but also at the protein level. Furthermore, although this study assessed neutrophils isolated from bone marrow, there is merit in extending the investigation to include the examination of neutrophils derived from blood or muscle tissues.

According to many previous studies, exercise increases cortisol levels in both humans and mice [60]. 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. 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 [61]. 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 were consistent with 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*. To confirm the effect of exercise-induced neutrophils on muscle regulation in vivo, further studies seem necessary to establish a mouse model for skeletal muscle injury, followed by the injection of exercise-induced neutrophils into the injury model. Evaluation of the effect of muscle regeneration in vivo will be possible through histological analysis through skeletal muscle tissue staining and analysis of myogenesis factors at the mRNA level and protein level. When considering the significant role of muscle stem cells in muscle regeneration, it appears crucial to assess the relationship with muscle stem cells in vivo, particularly by recruiting other immune cells such as macrophages or secreting cytokines, even though exercise-induced neutrophils may not directly impact muscle stem cells.

CRediT authorship contribution statement

Jae Yeon Park: Writing – review & editing, Writing – original draft, Project administration, Methodology, Investigation, Formal analysis, Conceptualization. **Tae Yeon Kim:** Methodology. **Song Won Woo:** Methodology. **Hyo Youl Moon:** Writing – review & editing, Supervision, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

Acknowledgments

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