

TIME COURSE ALTERATIONS OF SATELLITE CELL EVENTS IN RESPONSE TO LIGHT MODERATE ENDURANCE TRAINING IN WHITE GASTROCNEMIUS MUSCLE OF THE RAT

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ABSTRACT: This study investigated satellite cells and their related molecular events adapted to light moderate endurance training in the white gastrocnemius muscle of the rat. The white gastrocnemius muscle of male Sprague-Dawley rats that had been trained for 4 weeks and 8 weeks, with control rats being analysed alongside them, was selected for analysis (n=3 per group). The training protocol consisted of treadmill running at 20 m · min⁻¹ for 30 min on a 0% grade, for 3 days · week⁻¹. Immunohistochemical staining coupled with image analysis was used for quantification. To provide deeper insight into the cell layer, 40 sections per rat, corresponding to 120 values per group, were obtained as a mean value for statistical comparison. The results indicated that at week 4, training effects increased the vascular endothelial growth factor (VEGF) content and c-met positive satellite cell numbers. At week 8, the training effect was attenuated for VEGF and c-met satellite cell numbers, but it increased in the muscle fibre area. Additionally, c-met positive satellite cell numbers correlated with VEGF content ($r = 0.79$, $p < 0.05$). In conclusion, this study suggests that light moderate endurance training could stimulate satellite cell activation that might be related to VEGF signalling. Additionally, the satellite cells activated by moderate endurance training might contribute to slight growth in myocytes.

KEY WORDS: c-met, exercise, VEGF

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INTRODUCTION

Satellite cells are the undifferentiated stem cells of skeletal muscle. Upon muscle injury, satellite cells can be activated and can then be responsible for repairing damaged regions, eliciting muscle fibre hypertrophy or undergoing self-renewal to re-establish quiescent satellite cell pools [8,26]. In sports science research, traditionally it was considered that satellite cell activation was caused by muscle-damaging exercise, such as resistance exercise and eccentric exercise [9,11,26,27]. In addition to these training models, in recent years, some studies have found that endurance training, which has been shown not to cause apparent muscle damage, could also activate satellite cells [7,17,30]. However, these studies did not include measurement of the time course and related molecular events. Hence, it is of limited value to draw conclusions regarding the effect of endurance training on the adaptive process in satellite cells.

Not surprisingly, the individual's adaptation to endurance training is primarily characterized by increased oxygen delivery. At the cellular and molecular level, vascular endothelial growth factor (VEGF) is considered to be the key mediator [10]. Endurance-exercise-induced VEGF expression is primarily regulated by oxygen availability [28].

Recent in vitro studies have also indicated that satellite cell activation depends on oxygen availability [5]. Therefore, it is interesting to investigate these variables in response to endurance and how they are related.

Previous studies found that satellite cell numbers increased in the plantaris muscle of rats that underwent 8 weeks of low-intensity endurance training [17]. In our present study, we wanted to investigate whether satellite cells would be activated by 8 weeks of light moderate endurance training which was mostly recommended to maintain or improve health-related factors [2]. To investigate the adaptive process, this study was designed with 4-week and 8-week training phases.

Therefore, the purpose of this study was to investigate satellite cell events' adaptation to light moderate endurance training. To provide preliminary evidence of whether light moderate endurance training could activate satellite cells, the white part of the gastrocnemius muscle was investigated, because the gastrocnemius was confirmed to be the main fast muscle recruited during treadmill exercise [18], and satellite cells are more easily activated in fast-twitch muscle fibres

compared to slow-twitch ones [11]. Furthermore, c-met was selected in this study because it is considered as a candidate marker for mediating activation of quiescent satellite cells [3]. Additionally, the relationship between c-met positive satellite cell numbers and VEGF content was also examined. Various labelled antibodies were identified by immunohistochemical (IHC) staining. The use of specific antibodies to stain particular molecular species in situ, coupled with an image analysis method, serves as a more precise tool for the quantification of protein expression in the cell layer [6,20]. We hypothesized that light moderate endurance training would increase satellite cell numbers accompanied with muscle fibre enlargement within 8 weeks, and that the endurance training induced satellite cell number increase would be related to VEGF content.

MATERIALS AND METHODS

Animals. Twelve male Sprague-Dawley (SD) rats (10 weeks old) obtained from the National Institute of Animal Care (Taiwan) were used in the experiment. Rats were individually housed in cages with rat chow and water supplied ad libitum, in a room controlled at 20–22°C, and with a constant artificial 12:12 h light-dark cycle. All experimental procedures were approved by the Taipei Medical University of Animal Care Committee.

Study design

The rats were randomly divided into a 4-week exercise training (4WT) group, a 4-week sedentary control (4WC) group, an 8-week exercise training (8WT) group, and an 8-week sedentary control (8WC) group ($n=3$ per group). Training was carried out between 10:00 and 14:00 per session. During each training session, instead of being required to run on the treadmill, the C groups were placed on a non-moving treadmill. Each of the trained groups and their control counterparts were sacrificed for tissue removal 48 h after the last exercise training period.

Training protocol

Exercise training included 3 days of habituation to the treadmill before the training programme. Rats began at a running speed of $20 \text{ m} \cdot \text{min}^{-1}$, at 0% grade for 10 min for two days. The duration was then increased by 10 min until $20 \text{ min} \cdot \text{day}^{-1}$ was achieved on the third day, followed by one day of rest. After familiarization, the training groups began a treadmill training programme for 4 or 8 weeks according to the group to which they were assigned. Rats ran at $20 \text{ m} \cdot \text{min}^{-1}$ for 30 min on a 0% grade for 3 days $\cdot \text{week}^{-1}$, and electrical stimulation was used to motivate the rats to run. The running speed is close to that of Starnes et al. [31]. According to Norton et al. [21] in the classification of exercise intensity, the range of exercise intensities which Starnes et al. [31] reported fulfils the criterion of moderate intensity [4,31]. In addition, the relative exercise intensity applied during the whole training session in our study as estimated by the standard of the previous study is within the range of light moderate intensity [23,32].

Tissue Preparation

Tissues were preserved freshly and fixed in a 10% formalin solution until paraffin-embedded tissue blocks were made. Five- μm thick cross-sections were cut using a freehand section method with a microtome (*Jung SM 2000R*, Leica, Nussloch, Germany) from each block, and were mounted on micro slides (*Menzel-Glaser*, Braunschweig, Germany) for further analysis. **IHC staining:** The slides were incubated at 60°C for 10 min, followed by dewaxing using xylene, and rehydrated by passing through degraded concentrations of ethanol. Then, slides were briefly washed and immersed in phosphate-buffered saline (PBS) buffer for 5 min, and PBS was also used 5 min between all the following staining steps. After that, slides were immersed in citrate buffer solution (pH=6.0) and were heated in a digital decloaking chamber (Biocare Medical, Concord, CA, USA) for 30 min to induce antigen retrieval. Endogenous peroxidase activity was then inhibited by 15 min of incubation in 3% H_2O_2 , followed by 3% bovine serum for 30 min in a humidified chamber to block nonspecific binding sites. Primary antibodies to c-met (dilution 1:100; Zymed Laboratories, South San Francisco, CA), anti-VEGF (dilution 1:50; Abcam, Cambridge, United Kingdom), type I myosin heavy chain (type I MHC) (dilution 1:100; Sigma Chemical, St. Louis, MO), and type II MHC (dilution 1:150; Sigma Chemical, St. Louis, MO) were added individually onto the given slides and allowed to incubate for 90 min. Slides were then washed and incubated in a secondary biotinylated goat anti-rabbit IgG antibody (Dakopatts, Glostrup, Denmark) for 20 min. Next, slides were incubated with streptavidin–horseradish peroxidase (HRP) (DAKO. LSAB kit, K0675, Carpinteria, CA) conjugated for 20 min. Finally, incubation for 2–4 min in diaminobenzidine (DAB) (Dako, Carpinteria, CA) substrate-chromogen for peroxidase was used to visualize the bound antibody. For slides that were used for image quantification, no counterstain was applied so as to simplify image colorimetric quantification.

Mean optical density measurement and myofibre morphological profile counting

In this study, histological sections on slides were imaged using a Nikon 80i Eclipse E600 microscope (Nikon, Tokyo, Japan) equipped with the Nikon Digital Sight DS-Fi1 camera system (Nikon, Kawasaki, Japan). Visualization was performed at high magnification (objective $\times 40$). On each slide sample, 40 fields of area were chosen randomly throughout histological sections, but edge areas were avoided. Image analysis using Image-Pro Plus 6.2 software for Windows (Media Cybernetics, Silver Springs, MD, USA) was performed for the following quantification.

To evaluate the IHC staining intensity (mean optical density, MOD) of VEGF, image analysis was used following a previous protocol with minor modifications [6,12,15]. First, images were converted into an eight-bit grey-scale with pixel values within the range of 0.0 (black) to 255.0 (white). On each grey image, five visually cytoplasmic stained areas were randomly selected, and a white area served as a blank reference. The pixel data were then imported to Microsoft Excel 2007

(Microsoft, Seattle, WA). The MOD value of each image was obtained according to the following formula:

$$\text{MOD} = -\frac{1}{N} \sum_{i=1}^N \log \left(\frac{I_i}{I_0} \right) \quad [29]$$

where N is equal to five, I_i is the intensity level of the pixel i, and I_0 is the intensity level of the blank background measured in each image.

For the quantification of morphological profiles, fibre area, numbers of muscle fibre, c-met positive satellite cells and fibre type distribution (%) were counted. Each fibre area was obtained using a micrometer scale encircled manually; then the area was automatically calculated by the software. The mean fibre area was determined by dividing the total area by the total number of muscle fibres. To be identified as a satellite cell, cells stained positively for c-met as oval dark staining bodies surrounding muscle fibres were counted (Fig. 1). Data of c-met positive satellite cells were expressed as the number of satellite cells per muscle fibre. To calculate the percentage of type I and type II fibres, muscle cells staining positive for each MHC isoform antibody were expressed relative to the total MHC pool.

In this study, one person performed all counting in duplicate and was blinded to the groups' identity until all counting was completed. There were no differences between counts of each dependent parameter, so the count was considered reliable. Averaged values from all the duplicated dependent parameters were used for statistical analysis.

Data analysis:

All data were expressed as the mean \pm standard error of the mean (SEM). To compare the difference of dependent parameters more precisely in cell layers [20], 40 sections per rat, corresponding to 120 values per group, were obtained as a mean value for statistical comparison. Statistical analysis was carried out by two-way analysis of variance (ANOVA) (group \times training) to assess the differences between the trained and the control groups. In addition, to determine the relationship between VEGF and c-met positive satellite cells, 3 rats per group, corresponding to 12 values, were tested by Pearson's product correlations. Statistical significance was accepted at $p < 0.05$ for all tests.

TABLE 1. MORPHOLOGICAL PROFILES OF THE GASTROCNEMIUS MUSCLE BY IMMUNOHISTOCHEMISTRY.

	4WC	4WT	8WC	8WT
C-met positive satellite cells (numbers per muscle fiber)	0.17 \pm 0.02	0.27 \pm 0.01*‡	0.13 \pm 0.01	0.25 \pm 0.01*
Mean fiber area (μm^2)	3310 \pm 899	3349 \pm 944	3079 \pm 695	3478 \pm 719*
Percentage of muscle fiber types				
Fast-twitch fibers (%)	94.27 \pm 1.08	93.36 \pm 1.26	96.30 \pm 0.75	94.81 \pm 0.95
Slow-twitch fibers (%)	5.73 \pm 1.06	6.64 \pm 2.01	3.70 \pm 0.76	5.19 \pm 0.95

Note: Values are expressed as means \pm SEM; 4WC, 4-week sedentary control rats; 4WT, 4-week exercise trained rats; 8WC, 8-week sedentary control rats; 8WT, 8-week exercise trained rats; *Significantly differs between trained groups and control counterparts ($p < 0.05$). ‡ Significantly differs between 4WC and 8WC ($p < 0.05$). Each group represents 120 values (3 rats, 40 values per rat).

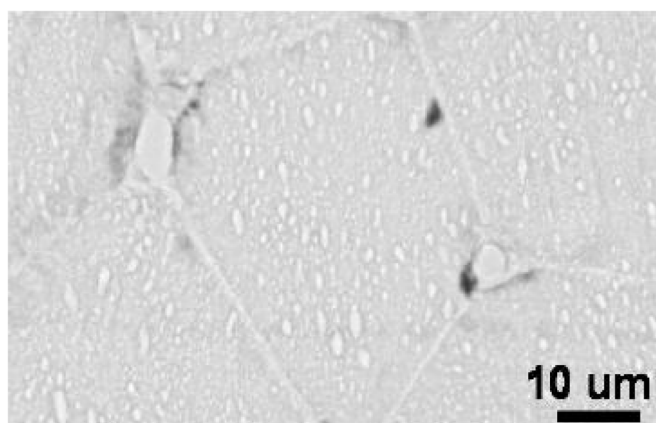


FIG. 1. C-MET ANTIBODY STAINING OF THE SATELLITE CELL.

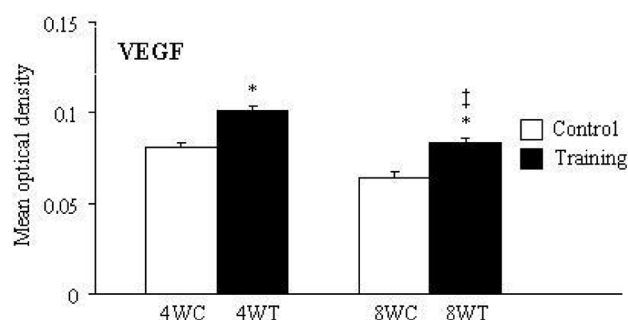


FIG. 2. MEAN OPTICAL DENSITY OF VASCULAR ENDOTHELIAL GROWTH FACTOR (VEGF) OF TRAINED RAT GASTROCNEMIUS MYOCYTE FOR 4 WEEKS (4WT) AND 8 WEEKS (8WT); AND CONTROLS FOR 4 WEEKS (4WC) AND 8 WEEKS (8WC).

Note: Each column represents the mean \pm SEM of 120 values (three rats, 40 values per rat); *Significantly differs compared to C ($p < 0.05$). †Significantly differs between 4 WT and 8 WT groups ($p < 0.05$).

RESULTS

C-met positive satellite cell numbers. As seen in Table 1, c-met positive satellite cell numbers in the 4WT group were higher than those in the 4WC group ($p < 0.05$). In the 8-week groups, c-met positive satellite cell numbers were still higher in the 8WT group than those in the 8WC group ($p < 0.05$), but in the 8WT group were significantly smaller than those in the 4WT group ($p < 0.05$).

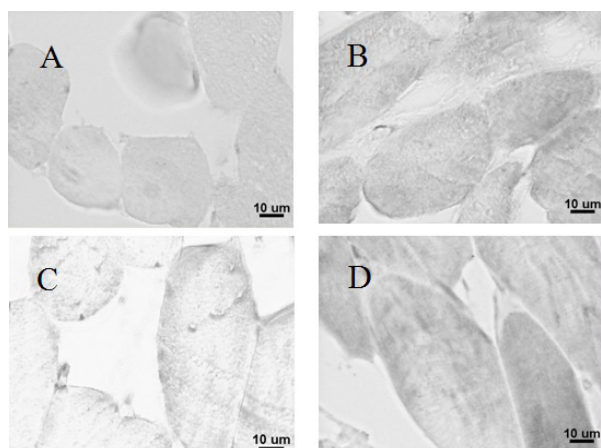


FIG. 3. REPRESENTATIVE EXAMPLES OF VEGF OF THE EIGHT-BIT GREY-SCALE IHC STAINING IMAGES SHOWN FROM THE 4-WEEK TRAINING (A), 4-WEEK CONTROL (B), 8-WEEK TRAINING (C) AND 8-WEEK CONTROL (D) GROUPS. IMAGES ACQUIRED USING 40 \times MAGNIFICATION OBJECTIVES. Note: Bar represents 10 μ m. VEGF – vascular endothelial growth factor. IHC – immunohistochemistry.

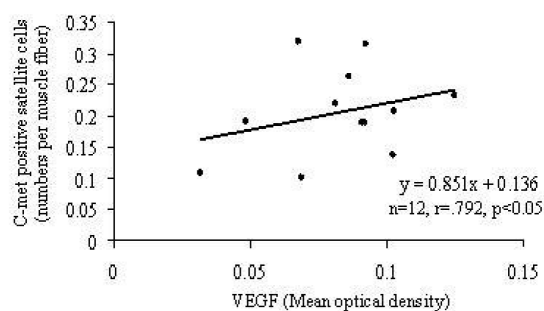


FIG. 4. PEARSON CORRELATION COEFFICIENTS BETWEEN C-MET POSITIVE SATELLITE CELL NUMBERS AND VEGF CONTENT.

Morphological profiles of muscle fibres

Table 1 shows the fast-twitch gastrocnemius as being over 90% in the four groups, and there were no statistically significant main or interaction effects of fibre type distribution ($p>0.05$). However, the 8WT group showed a greater muscle fibre area than did the 8WC group by 13% ($p<0.05$), whereas no significant differences occurred in the muscle fibre area between the 4WT and 4WC groups ($p>0.05$).

VEGF content

Figure 2 shows the VEGF contents of the four groups. As compared with the control groups, the VEGF contents were significantly higher in the trained groups than in their control counterparts ($p<0.05$), and the VEGF content in the 8WT group was significantly lower than that in the 4WT group ($p<0.05$). Representative examples of IHC staining images are illustrated in Fig. 3.

The relationship between VEGF and c-met positive satellite cells

Figure 4 shows that the c-met positive satellite cell numbers and VEGF content positively correlated with each other ($r=0.792$, $p<0.05$).

DISCUSSION

Endurance training at moderate intensity is usually recommended to improve health-related factors [2]. Few studies have investigated this training zone in relation to satellite cell activation. The present findings echo those of previous studies, affirming that endurance training effects result in increased satellite cell numbers [7,17,30]. Further, we found that in addition to long-term endurance training (>8 weeks, like the previous study), even the gastrocnemius of a rat that had undergone 4 weeks of light moderate endurance training demonstrated higher satellite numbers compared with their control counterparts. Since the repair potential of individual myofibres may depend on the abundance of the satellite cells they harbour [30], this reveals that light moderate endurance training could increase myocyte repair capacity. However, when doubling the training period to 8 weeks, the training effects were attenuated.

To study the satellite cell adaptive process, we focussed on the satellite cell number and VEGF content in response to two phases of light moderate endurance training, and investigated the relationship between them. The data show that the time course of effects of this endurance training on the c-met satellite cell numbers coincided with the training effects on the VEGF content. VEGF acts as an angiogenic signal as it increases both blood vessels and their permeability, allowing the oxygen to transport effectively. The VEGF content in response to exercise is shown to be subjected to a negative feedback mechanism once training adaptations occur [25]. In the initial 4-week period, the increase in VEGF content can be explained by there having been a better blood supply for the myocytes to work more efficiently. At week 8, the training effects that caused an increase in the VEGF content were attenuated. This suggests that these trained rats may adapt to the chronic exercise programme, and thus during each training session from 4 to 8 weeks, smaller relative exercise intensity may have occurred, with a gradual decrease in the oxygen consumption that serves as a stimulus on the myocytes. Coupled with the increased VEGF content and the satellite cell numbers then attenuated in the present study, we deduced that the adaptation to training at a constant speed is somewhat similar to the detraining effect. Our present study, similar to the studies of Kadi *et al.* [13], indicated cessation of training to be associated with termination of satellite cell activation and a gradual decrease of the satellite cell numbers.

In addition, the present study found that the VEGF content and c-met positive satellite cell numbers were highly correlated ($r=0.792$, Fig. 4) among all the rats. While exercise-induced satellite cell activation is traditionally considered to be caused by muscle injury, in the present study we used an endurance-training model not considered to cause apparent muscle injury [14,16]. Although our data do not allow us to determine the specific factor that activated satellite cells, we speculate that the common initiator of the satellite cell and VEGF might be the primary factor in activating the satellite cell by light moderate endurance training. For example, the cumulative effects of a hypoxic episode and shear stress-induced nitric oxide during endurance exercise have both been demonstrated to activate

VEGF and satellite cell numbers [5,19,24,28]. However, the exact mechanism needs to be further clarified.

An important function of the satellite cell is to prepare for muscle hypertrophy by fusing with its resident muscle fibres [1]. Endurance training increases in satellite cell numbers accompanied with both increases in myofibre area [7], or in the absence of myofibre hypertrophy [7,17], have been reported. The reason for these discrepancies may lie in the intensity and duration of the training programme, with low intensity exercise failing to cause muscle fibre hypertrophy [17], implying an intensity-dependant response. As previously described in our study, the relative intensity during each training session may be greater in the 4-week trained group than that of 8 weeks. However, the kinetics of changes in the myofibre hypertrophy seem to be independent of exercise intensity in the present study. Our data from week 4 are similar to those of Kurosaka et al. [17], who found that low intensity exercise activated satellite cells without muscle fibre enlargement. At week 8, although training effects that induced an increase in satellite cell numbers were attenuated, a 13% increase in myofibre hypertrophy by endurance training was observed. Our data from week 8 are similar to those observed in previous studies [7], indicating that endurance training effects increase both the muscle fibre area and the satellite cell numbers. It is noteworthy that the exercise intensity in our study may not be as low as that used in the study by Kurosaka et al. [17]; therefore, different results may occur over time. The existing data allow us to infer that when the exercise intensity reaches a given threshold, the endurance training induces satellite cell activation earlier than muscle fibre enlargement. The phenomenon of the kinetics of changes in the satellite cell does not always coincide with that of myofibre hypertrophy, as

was also reported by Olsen et al. [22], who estimated satellite cell numbers and myofibre hypertrophy at different time points during a 16-week training programme. Olsen et al. [22] found that the earlier increase in the satellite cell numbers caused by training then returned to the baseline, whereas the muscle fibre area remained greater than at the pre-training level. Taken together with the present results and those of previous studies, we speculate that the pre-activated satellite cells might retain their developmental history in exercised muscle. Thus, it is expected that skeletal muscle hypertrophy will occur over time after a period of remodelling events. In addition, training-induced increase in satellite cell activation might be subject to withdrawal once it has accomplished remodelling even though the myofibre enlargement consequences remain in effect.

CONCLUSIONS

In conclusion, this study suggests that light moderate endurance training could stimulate satellite cell activation, and the changes in satellite cell numbers might be related to VEGF content. The activated satellite cells would undergo a down-regulated response once training adaptation occurred, which might be in part regulated by the increase of oxygen availability. Additionally, the satellite cells activated by light moderate endurance training might contribute to slight growth in myocytes.

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