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Original Paper

Muscle Resting and TGF-β Inhibitor Treatment Prevent Fatty Infiltration Following Skeletal Muscle Injury

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Key Words
Muscle regeneration • Intermuscular adipose tissue (IMAT) • Fibro-adipogenic progenitors (FAPs) • Hindlimb unloading • Exercise

Abstract
Background/Aims: Skeletal muscle injuries are the most common type of injury occurring in sports, and investigating skeletal muscle regeneration as well as understanding the related processes is an important aspect of the sports medicine field. The process of regeneration appears to be complex and precisely orchestrated, involving fibro-adipogenic progenitors (FAPs) which are a muscle-resident stem cell population that appears to play a major role in abnormal development of fibrotic tissue or intermuscular adipose tissue (IMAT). Our present study aims to investigate whether muscle resting or endurance exercise following muscle injury may change the behavior of FAPs and subsequently impact the development of fatty infiltrations and fibrosis, two hallmarks of regeneration failure. ***Methods:*** We used the validated glycerol muscle injury model to mimic abnormal muscle regenerative conditions in mice. We challenged this specific regeneration model with hindlimb unloading or endurance exercise and, in a second set of experiments, we treated mice with decorin, a TGF-β inhibitor. ***Results:*** In this study, we demonstrated that: i) muscle resting just after injury leads to inhibition of IMAT development, ii) TNF-α mediated FAP apoptosis might be perturbed in this specific glycerol model of muscle injury, leading to IMAT development, and iii) treatment with the TGF-β inhibitor decorin decreases IMAT development and might restores FAP apoptosis. ***Conclusion:*** In addition to the potential clinical relevance of decorin treatment in situations involving muscle plasticity and regeneration, this study also demonstrates that a period of muscle resting is necessary following muscle injury to achieve efficient muscle regeneration

A. Chopard and G. Py contributed equally to this work.

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which is associated with a reduction in fatty infiltration. Unreasonably early resumption of exercise brings no gain to regeneration, further highlighting that this resting period is necessary.

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Introduction

Skeletal muscle injuries are the most common type of injury occurring in sport practice and studies investigating skeletal muscle regeneration and the processes related to it are not fully understood. In a healthy but injured muscle, a regeneration process leads to the establishment of new myofibers and to efficient repair of damaged tissue, thus restoring the original muscle integrity [1]. Successful skeletal muscle regeneration appears to be a complex and precisely orchestrated process involving multiple cell types. Of these cell types, satellite cells, localized between the sarcolemma and the basal lamina of myofibers [2], are the most commonly studied and are known to support the regeneration processes after injury. Nevertheless, a growing number of studies describing the crucial role of the interaction between the inflammatory/immune systems and muscle-resident stem cells are gradually highlighting the complexity of muscle regeneration [3-5].

The abnormal development of fibrotic and/or intermuscular adipose tissue (IMAT) deposits within skeletal muscle is a strong marker of regenerative failure. Studies have shown that increased IMAT deposition in skeletal muscle is strongly associated with decreased force production and overall muscle function [6, 7] as well as decreased mobility in older adults [8-10]. The level of IMAT deposition has also been correlated to the severity of Duchenne muscular dystrophy [11, 12]. Moreover, sizeable fatty infiltration occurs after rotator cuff injuries [13, 14] and a study by Goutallier, et al. [15] previously showed that the amount of IMAT infiltration was directly linked to decreased muscle function and to the severity of the injury. In line with these studies, Rahemi, et al. [16] generated a mathematical model arguing that IMAT inclusion was directly linked, through modifying pennation angle of fibers, to a decrease in muscle force and quality. Importantly, skeletal muscle fatty degeneration appears to be irreversible following rotator cuff tears, highlighting the importance of preventing their development.

Among the existing muscle-resident stem cells, numerous studies have highlighted the importance of fibro/adipogenic progenitors (FAPs), expressing the cell surface marker platelet-derived growth factor receptor alpha (PDGFRα or CD140a), in achieving efficient skeletal muscle regeneration. FAPs are able to rapidly proliferate following injury, participate in the phagocytosis of necrotic muscle fibers, and support satellite cell-mediated muscle regeneration [17-19]. In contrast, under pathological conditions, muscle disuse, or even in glycerol-induced muscle degeneration, FAPs have been shown to contribute to IMAT and fibrosis development [20-22]. Moreover, Uezumi, et al. [20] have demonstrated that only PDGFRα-positive cells can differentiate into adipocytes in glycerol-injected regenerating muscles thereby demonstrating the major implication of FAPs, which represent 98% of PDGFRα-positive cells in a regenerating muscle, in IMAT development.

The glycerol model of muscle injury, originally developed in rabbits [23], is now regularly used to investigate IMAT development and the related adipogenic process [18, 20, 22, 24, 25]. A study by Lukjanenko, et al. [22] showed an increase in the adipogenic response amplitude in glycerol-injected muscle compared to the classic cardiotoxin injury model, leading the glycerol model to be associated with IMAT development and accumulation. This study also highlighted that the glycerol-injected regenerating muscles showed a greater induction of anti-inflammatory cytokine mRNAs, as demonstrated by an approximately 2-fold higher expression of TGF-β1 and IL-10. The authors concluded that glycerol-injected muscles were characterized by a stronger anti-inflammatory response, suggesting an earlier M1 to M2 macrophage phenotype transition in comparison with cardiotoxin-induced muscle regeneration, thus affecting IMAT development. In light of a recent study demonstrating that TNFα released by M1 macrophages induces FAP apoptosis while TGF-β1 released by M2

macrophages promotes FAP survival [19], it is likely that pro-inflammatory processes may be dysregulated in the glycerol model. Clearly, studies are still necessary to clarify the impact of macrophages phenotype and more importantly macrophages phenotype transition during glycerol model of muscle regeneration on the IMAT development.

In a previous study, we demonstrated for the first time an almost complete inhibition of IMAT development in glycerol-injected regenerating muscles following hindlimb unloading [25]. Numerous studies have reported that hindlimb unloading leads to a pro-inflammatory environment within the muscle, with substantial macrophage infiltration [26-28], leading us to investigate the role of inflammatory processes on the inhibition of IMAT development observed in this situation. Aside unloading, it is well accepted that regular exercise has anti-inflammatory effects on skeletal muscle, suggesting that physical activity *per se* may suppress systemic low-grade inflammation that is often a hallmark of chronic diseases [29]. Moreover the levels of numerous circulating cytokines implicated in both inflammatory and anti-inflammatory pathways also fluctuate following exercise [30, 31]. However, the question of whether an early increase of muscle activity following injury, in the form of endurance exercise, could limit or exacerbate IMAT development in regenerating glycerol-injected muscle has not yet been addressed.

Consequently, this study was first designed to test the effect of early and regular endurance exercise on IMAT development in the glycerol-injected regenerating muscle. Secondly, we investigated the underlying mechanisms involved in the IMAT inhibition previously observed with hindlimb unloading, treating glycerol-injected muscle with decorin, a myokine known to interact with Transforming Growth Factor (TGF-β) family members. As highlighted above, the macrophages phenotype transition is a crucial stage during muscle regeneration, and can be characterized by their capacity to produce specific cytokines, like TNFα and TGF-β. In our study, decorin treatment, a natural antagonist of TGF-β, has been performed in order to better characterize the role of the TNFα/TGF-β axis during glycerol-induced muscle regeneration.

Materials and Methods

Ethics statement

This study was approved by the Committee on the Ethics of Animal Experiments of Languedoc Roussillon in accordance with the guidelines from the French National Research Council for the Care and Use of Laboratory Animals (CEEA-LR-14002). This study is in adherence to the Directive 210/63/EU for animal care standards. All efforts were made to minimize animal suffering.

Animals

Experiments were carried out on at least 6-month-old C57BL6J female mice from our own colony. Animals were maintained on a 12h/12h light–dark cycle and provided with food and water *ad libitum*. Experiments were performed at 22°C.

Experimental groups and muscle sampling

Experimental procedures were performed under anesthesia using isoflurane inhalation.

In a first set of experiments, mice were injected with 25µl of 50% v/v glycerol in the right tibialis anterior (TAg), and with 25 µL of saline solution in the contralateral tibialis anterior (TA) and then subjected to exercise training (EX group, training sessions detailed hereafter).

In a second set of experiments, mice were injected with 25µl of 50% v/v glycerol in the right tibialis anterior (TAg), and with saline solution in the contralateral tibialis anterior (TA) and then multiple experimental groups were formed: hindlimb-unloaded (HU), trained (EX) and control (CTL) groups for three different time points (2, 3 and 21 days). TA-CTL muscles have been used, when permitted, as reference control in our subsequent analyses.

In a third set of experiments, mice were injected with 25µl of 50% v/v glycerol in both tibialis anterior (TAg) muscles, and two experimental groups were formed: a decorin-treated group (DECO) and a control

PBS-treated group (PBS) for each of three different time points (5, 9 and 21 days). Decorin (D8428-.5MG, Sigma-Aldrich) treatments were administered 3 and 6 days after injury with intra-muscular injections in the right TAg (50μg of decorin diluted in 25μl of PBS), while the left TAg was injected with 25μl of PBS. At the end of the protocol, for each mouse one TAg was rapidly dissected out and immediately fixed overnight in a 4% paraformaldehyde solution at room temperature, after which it was paraffin-embedded. The second TAg was dissected out and rapidly frozen in liquid nitrogen.

Training sessions

Training sessions were conducted on a motor-driven treadmill (Exer-6M Treadmill; Columbus Instruments). The training protocol started 2 days after glycerol injection. Mice rigorously performed the same training program consisting of 8 exercise sessions distributed among the remaining 19 days of the protocol. In each exercise session, mice ran at 10m/min for 10min, following by a running speed increase of 1m/min every 2min for an additional 16min (thus until 18m/min speed). The speed was then maintained at 18m/min until 45 minutes of exercise had been performed. In order to get rid of the acute exercise effects, for the early time points of our study (at 2 and 3 days post-glycerol injection), mice of the EX groups either performed an exercise session 1 day after the glycerol injection and were euthanized 1 day later (TAg-EX 2 days), or performed two sessions, 1 and 2 days after glycerol injection, and were euthanized 1 day after the final session (TAg-EX 3 days).

Paraffin-embedded histological and immunohistochemical analyses

The paraffin-embedded histological and immunohistochemical analyses were performed exactly as previously described [32].

mRNA extraction and real-time polymerase chain reaction (qPCR)

Total RNA was isolated from muscle homogenates using the RNeasy Fibrous Tissue Mini Kit following the manufacturer's instructions (Qiagen). RNA concentration was determined by spectrophotometric analysis (Eppendorf AG, Hamburg, Germany), and integrity was checked by the OD260nm/OD280nm absorption ratio (>1.7). Reverse transcription reaction was performed with 2μg of total RNA using the RevertAid First Strand cDNA Synthesis kit (Thermo Scientific) according to the manufacturer's instructions. qPCR analysis was performed in a MiniOpticon detection system (Bio-Rad, Hercules, CA) with 10μL of KAPA SYBR Fast Universal Readymix (CliniSciences), 300nM of both forward and reverse primers, 2μL of diluted cDNA template and water to a final volume of 20μL. PCRs were performed in duplicate using the following cycle parameters: 30s at 98°C, 40 cycles of 1s at 95°C and 15s at 60°C. Relative mRNA levels were normalized to rp-S9 and tubulin housekeeping gene levels, which were unaffected by the experiment. Results are expressed using the comparative cycle threshold (CT). The relative changes in the level of a specific gene were calculated with the ΔΔCT formula. Tgfb1 (NM_011577.1) primer sequences were GCAACATGTGGAACTCTACCAG for the forward primer and CAGCCACTCAGGCGTATCA for the reverse primer.

Protein isolation and Western Blotting

The Western Blot protocol was performed as previously described [32]. β-actin was used as a loading control for homemade gels whereas Stain Free technology was used with Bio-Rad precast gels [33].

Antibodies

Anti-PDGFRα (#3174), anti-perilipin (#9349), anti-FABP4 (#3544) and anti-C/EBPα (#8178) primary antibodies were purchased from Cell Signaling and used at 1:500. Anti-PPARγ (sc-7273; 1:200), anti-C/EBPβ (sc-150; 1:200) anti-TNFα (sc-52746; 1:200), and anti-β-actin (sc-81178; 1:4000) primary antibodies were purchased from Santa Cruz. Anti-mouse (sc-2005) and anti-rabbit (sc-2004) HRP-conjugated secondary antibodies were purchased from Santa Cruz and used at 1:4000.

Statistics

All values are expressed as mean ± SEM and the significance level was set as $p<0.05$. Differences between the two groups were evaluated for significance using the unpaired Student t-test or the Mann-Whitney test when data deviated from a normal distribution (Shapiro-Wilk normality test). When more than two simultaneous comparisons were made, one-way or two-way ANOVA was employed to compare

data (level of mechanical constraints or treatment and time factors). When a significant effect was indicated, a Fisher significant difference post-hoc test was performed. All statistics and graphs were made with GraphPad Prism 6 Software.

Results

Reduced muscle activity, but not endurance exercise, decreases IMAT development after injury

In our previous study [25], we demonstrated that the area occupied by IMAT 21 days after mouse tibialis anterior glycerol injection (TAg) reached 2.83% of the total muscle CSA in the CTL group, whereas hindlimb unloading (HU) almost completely inhibited IMAT development, significantly decreasing the area occupied by IMAT (0.08%, Fig. 1). In the present study we further measured IMAT deposition in an exercise-trained group (EX) and observed no differences between the TAg-EX and the previously reported TAg-CTL group (2.24% of the total muscle CSA in the EX group and 2.83% in the CTL group, Fig. 1). In regards to the results obtained from this EX group, we further performed new experiments including the three groups (CTL, EX and HU) and measured mature adipocyte markers within total muscle lysates by Western blot to confirm and support the presence or absence of IMAT.

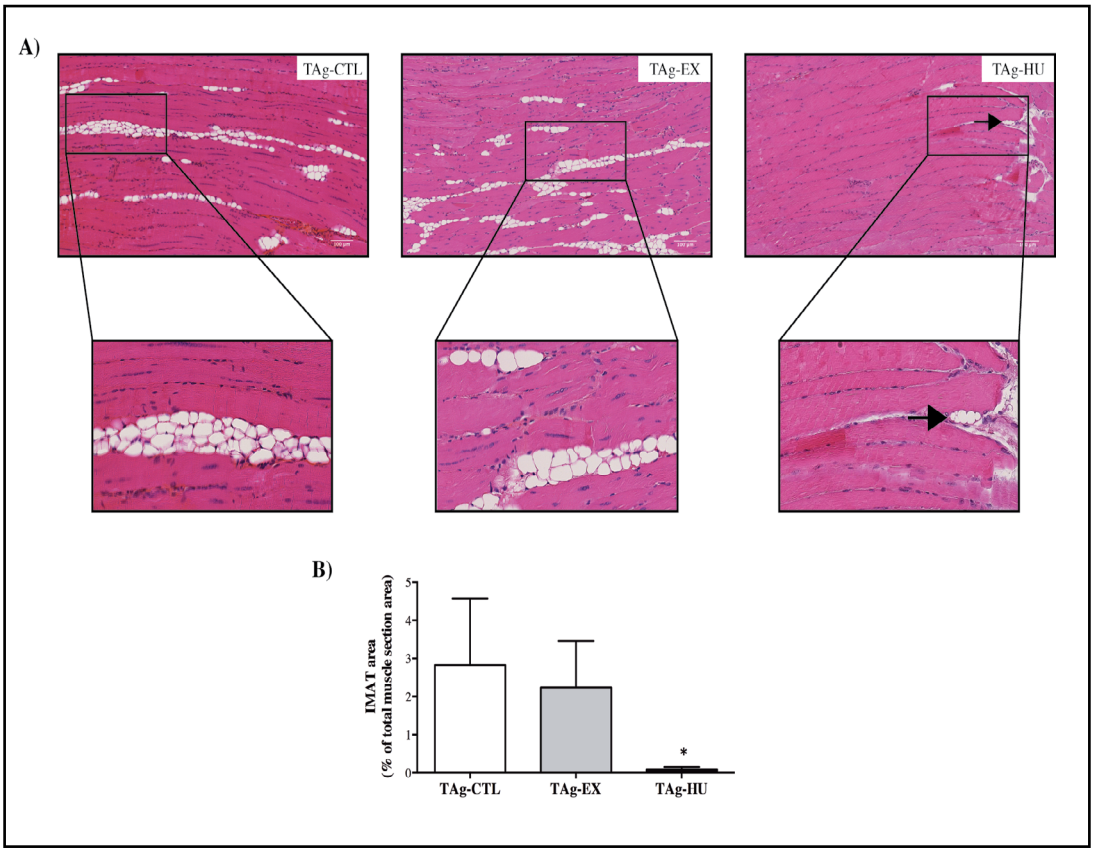

Fig. 1. Effect of different muscle activity levels on IMAT accumulation. A) Representative longitudinal paraffin-embedded muscle sections, stained with hematoxylin-eosin-saffron, from TAg of each experimental group (CTL, EX and HU), 21 days after glycerol muscle injury (N=6). Images of TAg-CTL and TAg-HU are re-used from Brioche, et al. [5] and Pagano, et al. [25] respectively. B) Quantification of intermuscular adipose tissue (IMAT) area in percentage of total muscle section area from TAg of each experimental group (CTL, EX and HU), 21 days after glycerol muscle injury (N=6). One-way ANOVA was used to compare our experimental groups, * $p<0.05$ vs. TAg-CTL.

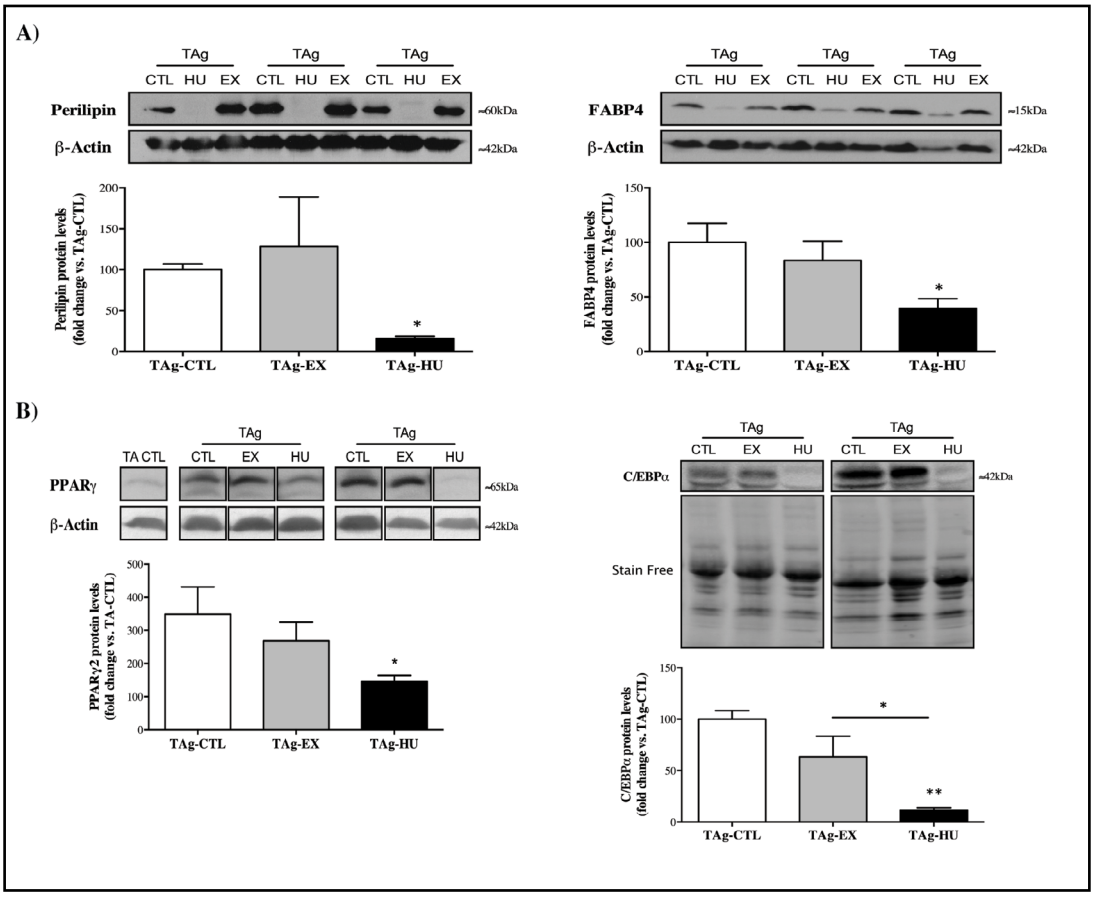

Fig. 2. Effects of different muscle activity levels on markers of IMAT accumulation. A) Perilipin and FABP4 protein levels from TAg of each experimental group (CTL, EX and HU), 21 days after glycerol muscle injury (N=6). B) PPARγ and C/EBPα protein levels from TAg of each experimental group (CTL, EX and HU), 21 days after glycerol muscle injury (N=6). One-way ANOVA was used to compare our experimental groups,* $p<0.05$ and ** $p<0.01$ vs. TAg-CTL.

As expected, we observed no differences in the adipocyte markers perilipin and FABP4 in the TAg-EX group compared to the TAg-CTL group, but a clear decrease of these markers in the TAg-HU condition, with -84% for perilipin and -61% for FABP4 when compared to TAg-CTL ($p=0.03$ and $p=0.038$ respectively, Fig. 2A). We next investigated levels of PPARγ and C/EBPα, two important transcription factors implicated in adipogenesis, and found a similar decrease in their protein expression in only the TAg-HU condition (-58% for PPARγ, $p=0.038$, and -89% for C/EBPα compared to TAg-CTL, $p=0.0011$, Fig. 2B). Altogether, these results suggest an important role of reduced muscle activity immediately following injury in the inhibition of IMAT development and accumulation in glycerol-induced skeletal muscle injury. In contrast, performing endurance exercise as soon as 48h post-injury did not inhibit adipogenesis compared to control conditions.

Glycerol-induced muscle injury likely disturbs FAP apoptosis, which may be restored by HU

FAPs are known to rapidly proliferate following injury, and reach peak expression around 3 days post-injury [18, 19]. After peak expression, FAPs enter an apoptotic phase, and return to basal levels observed in an uninjured muscle within 9 days post-injury. In our experiments we observed a clear decrease in FAP levels, represented by expression of their specific cell surface marker PDGFRα, in HU group compared to the CTL and EX groups 21 days after glycerol injection (-54%, and -57%, $p=0.0154$ and $p=0.0184$ respectively, Fig. 3A). This result was confirmed by immunohistochemical PDGFRα staining of muscle cross-sections (-74%

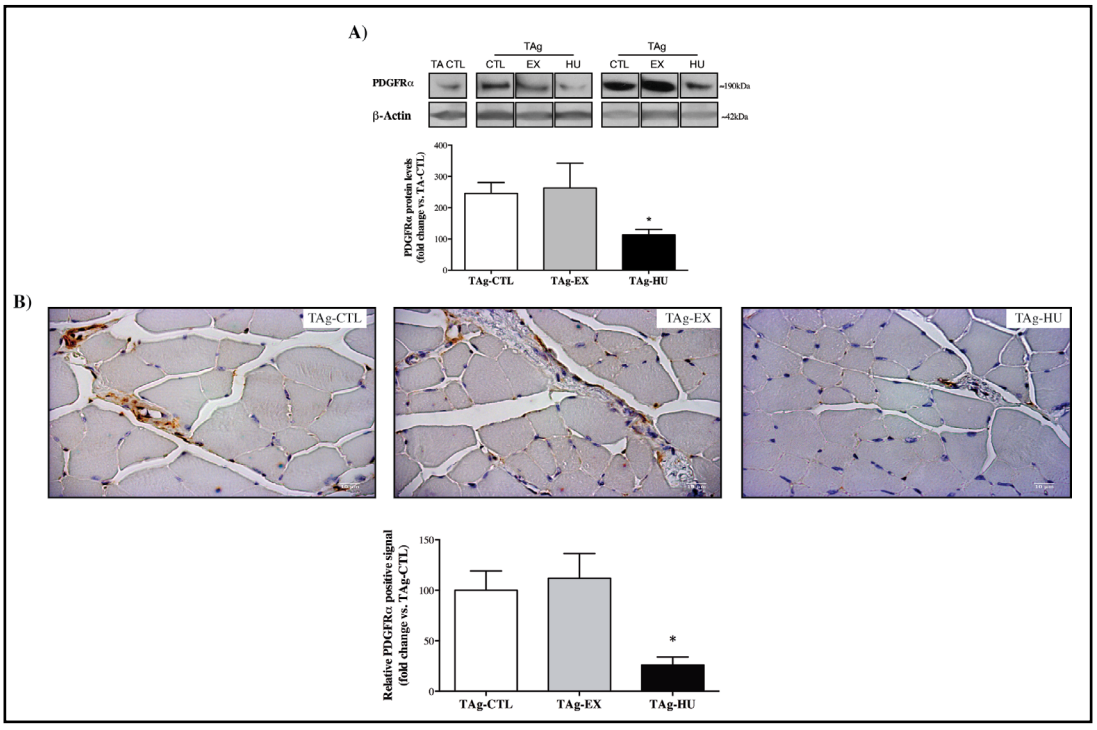

Fig. 3. Effect of different muscle activity levels on expression of FAP cell surface marker PDGFRα. A) PDGFRα protein levels from TAg of each experimental group (CTL, EX and HU), 21 days after glycerol muscle injury (N=6). B) Representative PDGFRα immunostained histological transversal paraffin-embedded from TAg of each experimental group (CTL, EX and HU), 21 days after glycerol muscle injury and quantification of the PDGFRα-positive signals (N=4). One-way ANOVA was used to compare our experimental groups,* p<0.05 vs. TAg-CTL.

compared to the TAg-CTL group, p=0.05 Fig. 3B). We thus show that, unlike in the HU group, FAP expression did not return to basal values in the CTL and EX groups, and these results all coincide with the degree of IMAT infiltration observed in each group. We next wanted to evaluate if this increase in FAP levels was due to an increase in FAP proliferation during the early stages of the regeneration process. For that purpose, we collected samples at 2 and 3 days post-glycerol injection, and found a massive and expected increase in PDGFRα signal in all groups between day 2 and 3. However, there were no differences between groups at either day 2 or 3, meaning that FAP proliferation was not affected by activity levels (Fig. 4A). Knowing that 98% of PDGFRα-positive cells represent FAPs in that specific glycerol model of regeneration [18], we therefore assumed that PDGFRα protein expression correlates with FAP levels. A result confirmed in our subsequent immunohistochemical PDGFRα staining (Fig. 4B*)*. These results might indicate a defective transition in FAPs from proliferation to apoptosis in our CTL and EX groups, which is restored by the HU condition. We next looked at levels of TNFα, a key cytokine secreted by M1 pro-inflammatory macrophages and implicated in the promotion of FAP apoptosis, and TGF-β1, released by M2 macrophages and implicated in FAP survival [19]. We found a considerable increase in the expression of TNFα within the TAg muscle of our HU group 3 days post-injury (+405% compared to the TAg-CTL and +443% compared to the TAg-EX, p=0.003, Fig. 4C). This result coincided with decreased induction of TGF-β1 mRNA in the TAg-HU group (-49% compared to the TAg-CTL, p=0.025 and -24% compared to the TAg-EX, p=0.064, Fig. 4C). These results again strongly suggest that FAP apoptosis is inhibited in our CTL and EX groups, which may directly stimulate IMAT development. To compliment the results obtained regarding IMAT development, we also quantified C/EBPβ protein expression in the early time points. Expression of this key early marker of adipogenesis did not increase in the HU group, while in the CTL and EX groups a

large increase was observed on day 3 (approximately a 3-fold increase compared to TA-CTL, p<0.001, Fig. 4D). This result highlights once again the important role of reducing muscle activity in the inhibition of adipogenesis following glycerol injury.

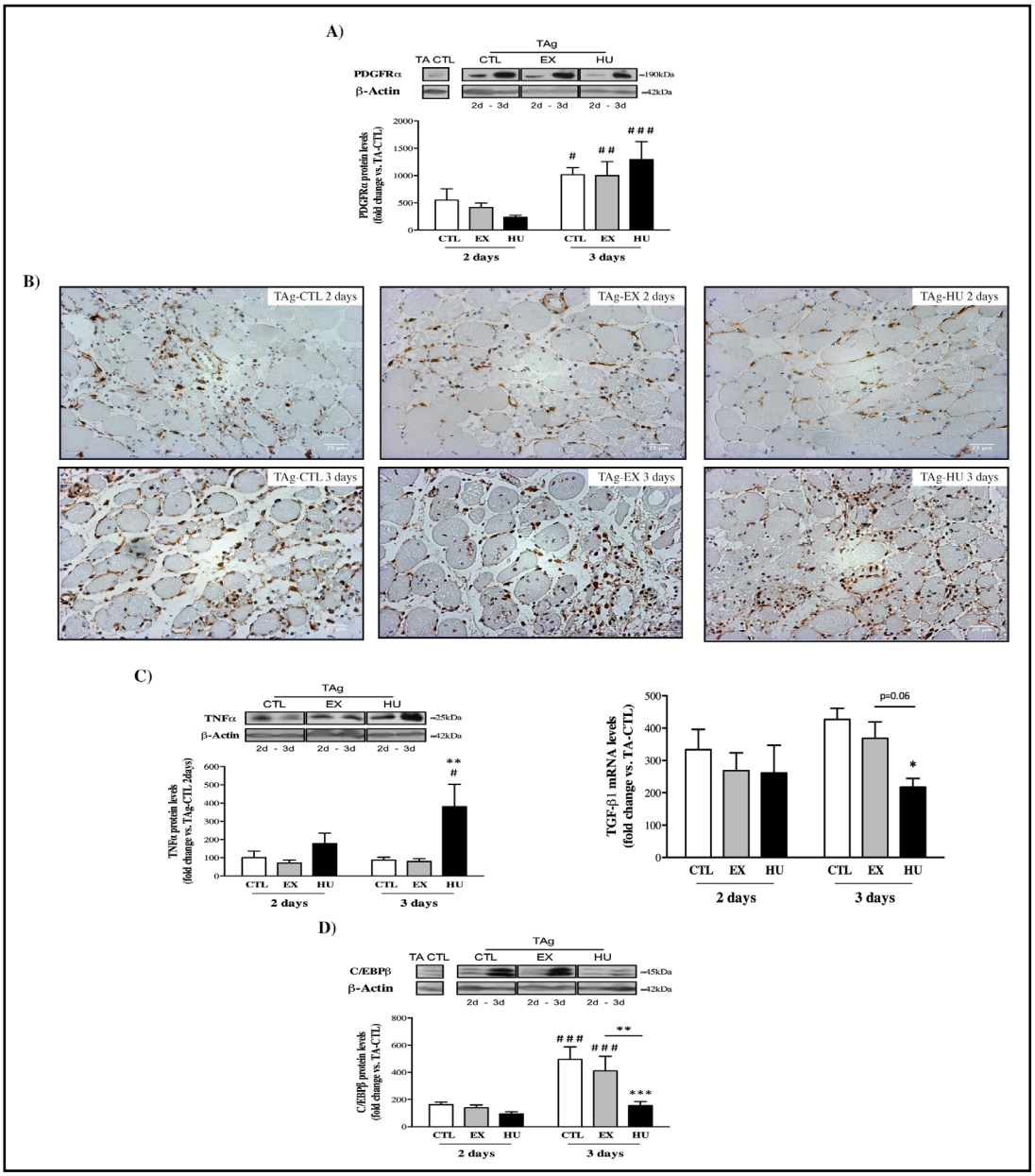

Fig. 4. Effects of different muscle activity levels 2 and 3 days after glycerol muscle injury. A) PDGFRα protein levels from TAg of each experimental group (CTL, EX and HU), 2 and 3 days after glycerol muscle injury (N=6). B) Representative PDGFRα immunostained histological transversal paraffin-embedded from TAg of each experimental group (CTL, EX and HU), 2 and 3 days after glycerol muscle injury (N=4). C) TNFα protein and TGF-β1 mRNA levels from TAg of each experimental group (CTL, EX and HU), 2 and 3 days after glycerol muscle injury (N=6). D) C/EBPβ protein levels from TAg of each experimental group (CTL, EX and HU), 2 and 3 days after glycerol muscle injury (N=6). Two-way ANOVA was used to compare our experimental groups, * p<0.05, **p<0.01 and *** p<0.001 vs the TAg-CTL group at the same time after glycerol injury (effects of muscle activity variations). # p<0.05, ## p<0.01 and ### p<0.001 vs the same group at 2 days after injury (time effect).

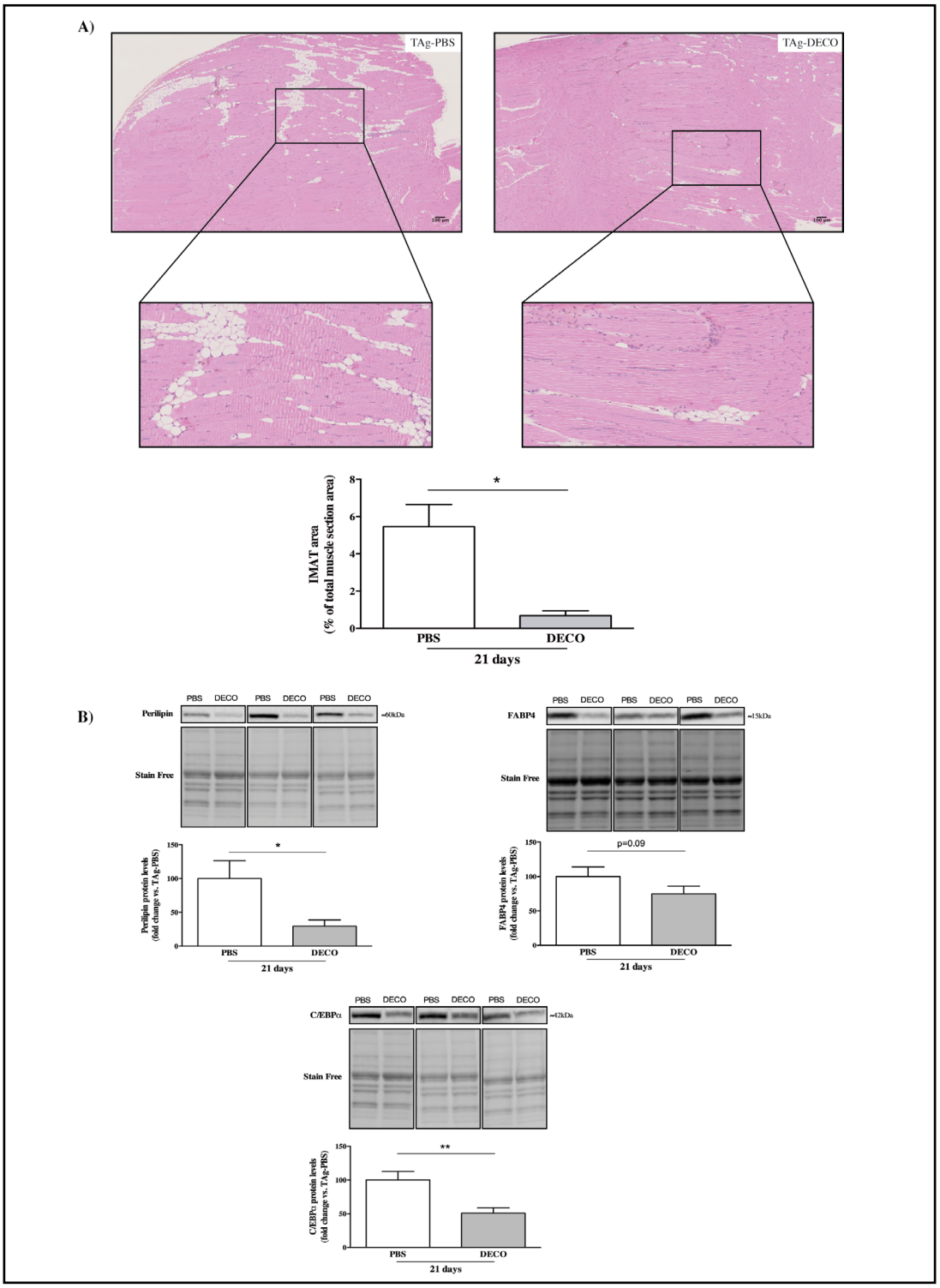

Fig. 5. Effects of decorin treatment on IMAT accumulation. A) Representative histological longitudinal paraffin-embedded muscle sections, stained with hematoxylin-eosin-saffron from TAg of each experimental group (PBS- and decorin (DECO)-treated TAg) and quantification of intermuscular adipose tissue area in percentage of total muscle section area at 5 and 9 days after glycerol muscle injury (N=9). B) Perilipin, FABP4 and C/EBPα protein levels from PBS-treated and decorin-treated TAg at 5 and 9 days after glycerol muscle injury (N=10). Unpaired Student t-test, or Mann-Whitney test when data deviated from a normal distribution, was used to compare our experimental groups, * $p<0.05$ and ** $p<0.01$.

Decorin treatment inhibits IMAT development in the glycerol model of muscle injury

In order to confirm that IMAT development in the glycerol model of muscle injury is due to a perturbed macrophage phenotype shift, and thus to an unbalanced cytokine response, we further treated injured animals with decorin, a small leucine-rich proteoglycan. Decorin is an extracellular matrix protein within all collagen-containing tissues and is known to strongly inhibit activities of the TGF-β superfamily. We chose to inject decorin at 3 and 6 days after glycerol injury based on FAP proliferation/apoptosis time course during muscle regeneration. FAP expression reaches a peak at 3 days after injury and returns to basal values within 9 days following apoptosis [18, 19]. According to our results, we suggest that FAP apoptosis may be deregulated in this glycerol model of injury leading to an increased FAP expression and the development of IMAT. Therefore, we chose to inject decorin first at day 3, exactly at the time when FAPs are supposed to enter into their apoptotic stage, and then again at day 6 with the hypothesis that decorin could restore TNFα-mediated FAP apoptosis through TGF-β1 inhibition.

In our experiments, intramuscular administration of decorin at 3 and 6 days after glycerol injury strongly inhibited IMAT deposition 21 days post-glycerol injection. More precisely, IMAT deposits reached around 6% of total muscle area in the PBS-treated group, while IMAT infiltration was largely inhibited with decorin treatment, with deposits totaling about 1% of muscle area (Fig. 5A). Interestingly, we believe that the path of the needle and the PBS injection could have worsened the glycerol injury, and be the reason why IMAT deposits reached levels as high as 6% in this specific experiment. Along with the decrease observed in IMAT accumulation, quantification of perilipin (-71%), C/EBPα (-49%) and, to a lesser extent, FABP4 (-25%) protein levels in glycerol-injured muscle treated with decorin also confirmed a reduction of adipocyte differentiation (p=0.012, p=0.004 and p=0.06 respectively, Fig. 5B). Finally, when looking at the decorin-treated and control muscles at 5 and 9 days post-glycerol injection, we observed a decrease at day 5 in the expression of the major early adipogenic factor C/EBPβ (-32%, p=0.012, Fig. 6A).

Our first experiments suggested defective FAP apoptosis in the glycerol-injected model of muscle regeneration, resulting in a higher expression of PDGFRα 21 days following injury. Subsequently, our results, with respect to PDGFRα protein expression at 5 and 9 days after glycerol injury (thus during the theoretical FAP apoptosis stage) confirm that decorin treatment is able to strongly reduce PDGFRα protein expression, a result suggesting an increase in FAP apoptosis processes. Indeed, PDGFRα expression strongly decreased, at day

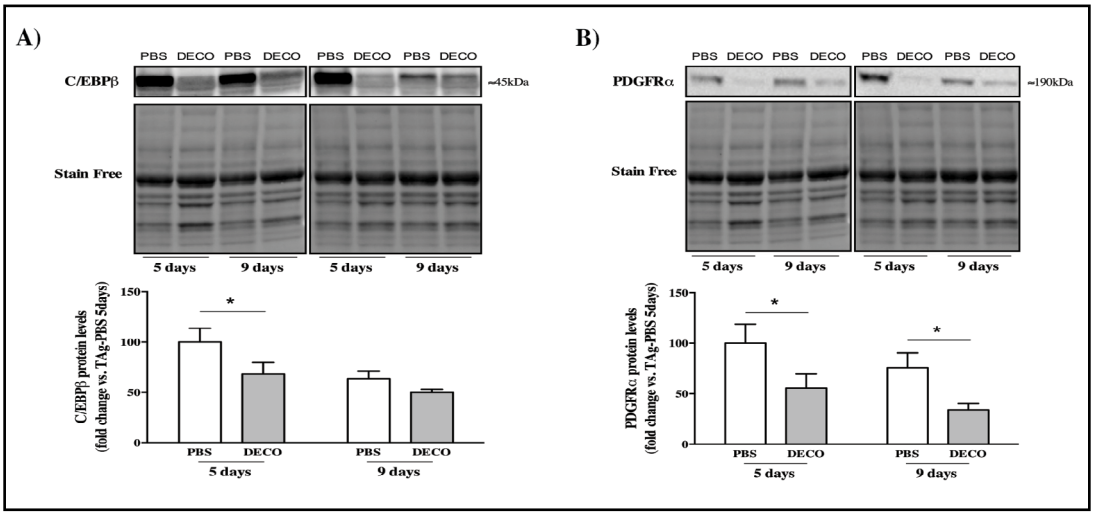

Fig. 6. Effects of decorin treatment 5 and 9 days after glycerol muscle injury. A) C/EBPβ protein levels from PBS-treated and decorin-treated TAg at 5 and 9 days after glycerol muscle injury (N=10). B) PDGFRα protein levels from PBS-treated and decorin-treated TAg at 5 and 9 days after glycerol muscle injury (N=10). Two-way ANOVA was used to compare our experimental groups, * p<0.05.

5 and 9, in our decorin-treated injured muscles compared to PBS-treated muscles (-44% and -55%, p=0.038 and p=0.04 respectively Fig. 6B).

Discussion

Using the glycerol model of muscle regeneration, we have highlighted the important benefit of immediate muscle resting following injury and emphasized the role of FAP apoptosis on IMAT development in muscle. We have also highlighted that maintaining natural inflammatory processes after muscle injury decreases IMAT development. Our previous study demonstrated inhibition of IMAT development in the HU condition 21 days following glycerol injury [25]. In the present work, we therefore studied a contrasting third group of mice who were subjected to exercise training in order to increase activity of the regenerating muscle. The percentage of IMAT deposition measured in this group 21 days after injury was almost the same that of the CTL group, and protein quantification of several key markers of adipogenesis and mature adipocytes further confirmed this result. These findings reflect once again the importance of removing or at least reducing muscle activity after injury to achieve efficient muscle regeneration. However, our lab and others have also shown perturbed muscle regrowth after regeneration processes in HU conditions [25, 34], which highlights the importance of increasing muscle activity levels as soon as possible after the necessary rest period for full recovery of muscle fiber size. Indeed, further studies are still needed to determine the ideal timeframe for transitioning between muscle resting and muscle activity after injury.

While many stem cells in the muscle environment can adopt the capacity to differentiate into adipocytes [4, 35], FAPs are currently accepted to represent the major population that appears to play a role in IMAT development. A study by Uezumi, et al. [20] clearly showed that only progenitors expressing the cell surface receptor PDGFRα were able to differentiate into adipocytes in the glycerol model of muscle injury. Importantly, the study by Heredia, et al. [17] subsequently confirmed that PDGFRα was exclusively expressed by FAPs after muscle injury. Monitoring FAP fate is therefore a major objective in promoting optimal muscle regeneration and preventing IMAT development. In healthy muscle FAPs are known to rapidly proliferate, reach peak expression around 3 days after muscle injury [18, 19], and aid regeneration by both improving muscle satellite cell activation and via their important phagocytic activity [17, 18]. After these key events, FAPs enter a period of apoptosis and their levels return to the basal values observed in uninjured muscle, a required process for efficient regeneration without abnormal fibrosis development [19]. In this study, we showed an approximately 2-fold increase of PDGFRα protein expression 21 days post-injury in the CTL and EX groups compared to the HU group. This can be interpreted either as an increase in FAP proliferation or as a deficiency in their apoptotic process, ultimately leading to IMAT development. Interestingly, our study revealed no differences in FAP proliferation at days 2 and 3 post-glycerol injury between all experimental groups, suggesting a disturbed FAP apoptosis in these groups. In accordance with the fact that TNFα, released by M1 macrophages, provokes FAP apoptosis [19], we also detected a strong increase in TNFα protein expression 3 days after injury in the HU group, but not in CTL and EX groups. These results strongly suggest that altered FAP apoptosis is a critical factor explaining, at least in part, IMAT development in the glycerol model of muscle regeneration. Interestingly, the study of Lukjanenko, et al. [22] already showed an unusual early anti-inflammatory response in the glycerol model compared to the cardiotoxin model of muscle injury. These authors revealed elevated levels of TGF-β1 three days after glycerol injury and, in line with the study conducted by Lemos, et al. [19], this strong TGF-β1 response promotes FAP survival through inhibition of TNFα-mediated FAP apoptosis. Interestingly, we also reported a reduction in mRNA levels of TGF-β1 3 days after injury in our HU group compared to the CTL and EX groups. Thus, immediately decreasing muscle activity after glycerol injury may participate in the recovery of FAP apoptosis through increased expression of TNFα.

In order to confirm involvement of the TNFα/TGF-β1 axis in the IMAT development observed in the abnormal muscle regeneration, we treated injured muscle with decorin, a small leucine-rich proteoglycan localized in the extracellular matrix of all collagen-containing tissues. This myokine [36] is a major inhibitor of the TGF-β superfamily, including TGF-β1 and myostatin, and its overexpression leads to major positive effects on muscle regeneration and limits the occurrence of abnormal fibrosis during the regeneration process [37-39]. Interestingly, decorin treatment has also been found to improve macrophage activity [40]. We therefore hypothesized that decorin treatment could inhibit IMAT development in the glycerol model of muscle regeneration. We also hypothesized that decorin could restore TNFα-mediated FAP apoptosis through TGF-β1 inhibition. Indeed, we observed inhibition of IMAT development in the decorin-treated muscles compared to PBS-treated and injured muscles, and confirmed this result by demonstrating decreased expression of key markers of mature adipocytes and adipogenesis. Analysis of the marker PDGFRα at day 5 and 9 post-injury, the critical time in which FAPs are in an apoptotic period, revealed a decreased expression with decorin treatment, possibly indicating a decreased FAP presence with decorin treatment. These results strongly suggest a restoration of the FAP apoptosis process leading to IMAT inhibition and other studies with specific proliferation and/or apoptosis measurements are needed to confirm and validate our hypothesis.

Conclusion

To conclude, this study raised three major points: i) muscle resting immediately after injury leads to prevention of IMAT development in the glycerol model of skeletal muscle injury, whereas early endurance exercise has no beneficial effect, ii) TNF-α mediated FAP apoptosis might be perturbed in abnormal muscle regeneration, leading to increased IMAT development, and iii) treatment with decorin, a TGF-β1 inhibitor decreases IMAT development in this specific model of muscle injury, certainly through an increase in FAP apoptosis. In addition to the potential clinical relevance of decorin treatment in situations involving muscle plasticity and regeneration, this study also raises questions about the appropriate timing for reestablishing muscle activity after sports injuries. From our point of view, ideal timing could be at the resolution of pro-inflammatory mechanisms, when the M1 to M2 macrophage phenotype shift occurs and the FAPs enter apoptosis, leading to a muscle environment primed to accomplish pro-regenerative and fibers regrowth functions.

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Disclosure Statement

The authors have no conflicts of interest to declare.

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