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Accelerated skeletal muscle recovery after *in vivo* polyphenol administration☆

Kathryn H. Myburgh, Maria J. Kruger, Carine Smith*

Department of Physiological Sciences, Stellenbosch University, Stellenbosch, South Africa

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Abstract

Acute skeletal muscle damage results in fiber disruption, oxidative stress and inflammation. We investigated cell-specific contributions to the regeneration process after contusion-induced damage (rat gastrocnemius muscle) with or without chronic grape seed-derived proanthocyanidolic oligomer (PCO) administration. In this placebo-controlled study, male Wistar rats were subjected to PCO administration for 2 weeks, after which they were subjected to a standardised contusion injury. Supplementation was continued after injury. Immune and satellite cell responses were assessed, as well as oxygen radical absorption capacity and muscle regeneration. PCO administration resulted in a rapid satellite cell response with an earlier peak in activation ($Pax7^+$, $CD56^+$, at 4 h post-contusion) vs. placebo groups (PLA) ($P<.001$: $CD56^+$ on Day 5 and $Pax7^+$ on Day 7). Specific immune-cell responses in PLA followed expected time courses (neutrophil elevation on Day 1; sustained macrophage elevation from Days 3 to 5). PCO dramatically decreased neutrophil elevation to nonsignificant, while macrophage responses were normal in extent, but significantly earlier (peak between Days 1 and 3) and completely resolved by Day 5. Anti-inflammatory cytokine, IL-10, increased significantly only in PCO (Day 3). Muscle fiber regeneration (MHC_f content and central nuclei) started earlier and was complete by Day 14 in PCO, but not in PLA. Thus, responses by three crucial cell types involved in muscle recovery were affected by *in vivo* administration of a specific purified polyphenol in magnitude (neutrophil), time course (macrophages), or time course and activation state (satellite cell), explaining faster effective regeneration in the presence of proanthocyanidolic oligomers.

Keywords: Grape seed; Inflammation; Satellite cell activation; Proanthocyanidin

1. Introduction

Immune cell infiltration is associated with secondary damage in chronic muscular pathology [1] as well as after acute disruption of the contractile and cytoskeletal proteins of muscle cells induced by toxin injection [2] or traumatic contusion [3]. Acute insults could range from workplace contusion injuries to motor vehicle accidents or muscle injuries in contact sports. The inflammatory response is required for removal of debris and promotion of cytokine-mediated processes involved in regeneration [3,4]. However, chronic conditions have been positively influenced by restricting the inflammatory response [5]. This dichotomy, and the relative shortage of studies on acute muscle contusion simulating trauma, necessitates more research on treatment options after acute insults.

Recently, most research on factors that could accelerate muscle regeneration has focused on stem cell therapy [6–8], satellite cell function [9–11] or manipulation of inflammation using complex experimental approaches [12,13]. The role of macrophages in the inflammatory response has received much attention [4,13]. Macrophages are important, given the critical juncture between their destructive phagocytic role compared to their anti-inflammatory [14], muscle regenerative [1,13,15] and angiogenic [16] roles. Intramuscular injection of macrophage conditioned media into injured muscle increases muscle regeneration [17]. Classically activated macrophages (M1) increase release of reactive oxygen species and promote tissue destruction, while those alternatively activated (M2a-c) suppress inflammation [18]. A focus on macrophages may play down earlier events during the destruction phase, at which time increased oxidative stress is a major player.

Free radical generation is known to accelerate progression of chronic muscle pathologies [19,20]. Similarly, the increased release of free radicals in the first 24 h after acute stretch-induced skeletal muscle injury [21] causes secondary damage [22]. Oxidative stress in skeletal muscle is increased in various other models such as electrical stimulation during claudication and is ascribed to increased enzyme-initiated oxidant production as well as to neutrophil-derived myeloperoxidase activity [23]. In a model of acute muscle injury in response to a single eccentric contraction, inhibition of the neutrophil oxidative burst and degranulation decreased microscopic damage [24]. It is also recognized that inhibiting neutrophil activation could be an early therapeutic target for reduction of myocardial ischemia–

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* Corresponding author. Department Physiological Sciences, Stellenbosch University, Matieland 7602, South Africa. Tel.: +27 21 808 4388/3146; fax: +27 21 808 3145.

E-mail address: csmith@sun.ac.za (C. Smith).

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reperfusion injury, where oxidative stress is an important mechanism of damage [25].

Plant-derived compounds, such as triterpenoids and polyphenols, are known to have anti-oxidant effects and to provide some protection against inflammatory stress [26,27]. Most *in vivo* studies have been done in animal models and indicate that these biological compounds effectively reduce pathology in various chronic conditions, e.g., pulmonary edema [28] or inflammation-associated tissue degradation in chronic musculoskeletal disorders [29]. Synthetic triterpenoids are being tested in Phase I clinical trials as potential cancer therapeutic agents [30].

Regardless of the extent of the oxidative stress and inflammatory responses, the regeneration of muscle tissue in either chronic pathology or acute damage relies on recruitment of precursor cells able to differentiate into a muscle-specific phenotype [31]. These are primarily mobilized from the satellite cell niche but also from circulating progenitors [31,32]. Satellite cells at the site of damage expand through proliferation to form a population of myoblast precursor cells [33], but also migrate from adjacent muscle fibers if a sufficient connection remains between the damaged and undamaged regions [34]. Another population of cells, the muscle-derived stem cells (MDSCs), also contributes to muscle regeneration, and in transplant experiments these survive longer than myoblasts [35]. A recent report indicates that the superior anti-oxidant capacity of MDSCs compared to myoblasts is critical for their better survival rate [36]. It is not clear whether this is related to capacity to quench neutrophil-induced oxidative stress or other forms of oxidative stress [36].

Interactions between myoblasts and immune cells have been recognized and are mediated by various cytokines, among other factors [37]. In animal models, cytokine up-regulation is a major local response to trauma [4]. IL-1β, IL-6 and TNF-α were found to be elevated in murine muscle following crush-induced damage and their increases were greater with a larger trauma [38]. These cytokines are also present in the interstitial space distal to muscle trauma [39]. *In vitro* conditioned media from injured myoblasts increases neutrophil chemotaxis [40], while macrophage-conditioned media injected at the site of muscle damage improves regeneration [17]. Mechanistic studies have begun to unravel these observations. Pro-inflammatory macrophages increase satellite cell proliferation [15], possibly due to delayed differentiation in the presence of TNF-α and IL-1β [41]. However, macrophages with an anti-inflammatory profile improve differentiation [15], while IL-1β blockade by antibody administration improves survival and proliferation of engrafted myoblasts [42]. In this study, the effect was enhanced by co-administration of anti-oxidants.

In summary, endogenous effectors including reactive oxygen species and cytokines modulate muscle repair at various stages of the destruction–regeneration continuum. Research on the role of potential treatments should take into account which cells and which stage or stages after damage are most affected by the intervention. We hypothesized that increasing circulatory as well as tissue oxygen radical quenching capacity prior to contusion-induced muscle damage would limit the magnitude of the destructive phase and promote the advance to the regenerative phase of muscle healing.

To test this hypothesis, we implemented a noninvasive method to induce contusion damage to skeletal muscle in rats supplemented with procyanidolic oligomers (PCO, also known as proanthocyanidins) or placebo. The model allows for a controllable starting point from which to evaluate the time course of subsequent events required for healing. A key feature of the study was the focus on three specific cell types, each known to have a different function, some resident in muscle tissue and others arising from other compartments, in order to determine whether there was a uniform response to PCO.

2. Methods and materials

2.1. Experimental animals

Adult male Wistar rats weighing approximately 280 g, with access to standard rat chow and tap water *ad libitum*, and exposed to a 12-h light/dark cycle (lights on at 6:30 a.m.), were used in the study. Ambient temperature was controlled at 21°C, and the housing facility was ventilated at a rate of 10 air changes per hour. All experimental protocols were approved by the Animal Research Ethics Committee of Sub-Committee B of Stellenbosch University (reference no. 2006 Smith01).

Experimental rats were randomly divided into two weight-matched groups, a placebo (PLA) and a procyanidolic oligomer-treated (PCO) group. In each group, eight rats were used per group per time point.

2.2. PCO administration

PCO rats were administered a daily dose of 20 mg/kg per day of grape seed-derived PCO by oral gavage for 14 days prior to muscle contusion, as well as up to 14 days post-contusion (dependent on sacrifice time point). The same administration procedure was followed for PLA rats, which were gavaged with sterile water.

The PCO supplement used (Oxiprovin, Brenn-O-Kem, Wolseley, South Africa) was a hydrophilic extract from the seeds of *Vitis vinifera* L., all harvested from cultivated vines in a local region (Western Cape/Winelands, South Africa). The extract (dry powder) typically contains 45% proanthocyanidins and less than 5% monomers (with the remainder constituted by the long-chain sugars and glycosides attached to the oligomers). Each batch was analysed independently for % phenolics (CPUT ARU, Cape Town, South Africa), % moisture, % ash, traces of heavy metals (BemLab, Cape Town, South Africa) and absence of microbes (Swift Microlaboratories, Cape Town, South Africa).

2.3. Muscle contusion intervention

Prior to experimental contusion-induced damage, rats were anaesthetised with 75 mg/kg ketamine and 0.5 mg/kg medetomidine in 0.9% saline, administered intraperitoneally. Contusion of the hind limb was produced by a drop-mass jig, similar to the model first described by Stratton et al. [43]. Briefly, using a purpose-made jig, a 200-g flat-bottomed, circular weight was dropped from a height of 50 cm onto the medial surface of the right gastrocnemius muscle to induce a contusion in a noninvasive manner. Rats showed similar increases in body weight over time, with a slight transient decrease in body weight (7.14±6.94 g) in both the contusion groups 1 day after inducing damage.

2.4. Sacrifice and sample collection

Death was induced by pentobarbitone sodium overdose and whole blood collected into heparinised tubes by cardiac puncture of the right ventricle. Gastrocnemius muscle was dissected from the injured leg.

2.5. Muscle histology and immunohistochemistry

For cross-sectional histology and immunohistochemistry, muscles were fixed in 10% formal saline, processed and embedded in paraffin wax. Five-micrometer-thick cross-sections were prepared (Leica Microsystems CM1850, Nussloch, Germany) and stained with haematoxylin and eosin (H&E) for qualitative histological analysis.

For immunohistochemistry, sections were adhered to poly-L-lysine (Sigma Aldrich)-coated slides, deparaffinized, fixed in 0.1% trypsin (Highveld) at 37°C for 30 min and then blocked with 5% serum for 30 min. The following primary antibodies were used: mouse anti-human CD34 (1/100 dilution; Santa Cruz), rabbit anti-human CD56 (1/100 dilution; Santa Cruz), rabbit anti-human M-cadherin (M-cad; 1/100 dilution; Santa Cruz), mouse anti-human Pax7 [1/200 dilution; Developmental Studies Hybridoma Bank (DSHB)], mouse anti-human fetal myosin heavy chain (MHC_f; 1/25 dilution; F1.652, DSHB), goat polyclonal TNF-α (1/100 dilution; Santa Cruz), goat polyclonal IL-6 (1/100 dilution; Santa Cruz), mouse anti-human HIS48 (1/200 dilution; Becton Dickinson) and goat anti-human F4/80 (1/200; Santa Cruz). Primary antibodies were left to incubate for 4 h at room temperature, after which sections were revealed with donkey anti-goat Texas Red-conjugated, donkey anti-rabbit FITC-conjugated, donkey anti-mouse FITC-conjugated, goat anti-mouse FITC-conjugated or goat anti-rabbit Texas Red-conjugated (1/250 dilution for all, Invitrogen) secondary antibodies for 40 min at room temperature. For double labeling, sections were labeled with both mouse anti-human Pax-7 (1/200 dilution; DSHB) for 4 h at room temperature and rabbit anti-human laminin (1/200 dilution; Dako Diagnostic) overnight at 4°C. Hoechst (1/200 dilution; Sigma Aldrich) were used to visualize nuclei.

2.6. Image analysis

All imaging data were obtained by analysing two serial sections (i.e., 5 μm apart) from each muscle sample, at each time point for each antibody. Six fields of view per section were imaged using a microscope (Nikon ECLIPSE E400; 400× objective used), equipped with a colour digital camera (Nikon DXM1200). Photos were used to count

positively stained satellite cells as well as the total number of muscle fibers and regenerating muscle fibers per field of view using a computer program (Simple PCI version 4.0, Compix Inc., Imaging Systems, USA). Where necessary, photos were enlarged and the color of the stain enhanced after importation into Simple PCI to assist with identification. The images presented here are only partial images of those taken at 400×. All satellite cell and MHC_f photos are of border zones (areas right next to the severely injured areas), whereas immune cell and cytokine images are of both the border zones and injured area. Satellite cell data were expressed as the number of satellite cells per myofiber, whereas MHC_f data were expressed as the percentage of regenerated myofibers (muscle fibers containing central nuclei) per total myofiber count in the viewed area. Muscle cytokine data were expressed as the percentage area staining positive for a particular cytokine, and immune cell data were expressed as the number of cells per standardized tissue area.

2.7. Oxygen radical absorbance capacity assay

Oxygen radical absorbance capacity (ORAC) assays were performed on plasma as well as skeletal muscle tissue homogenate supernatants, using the method described earlier [44], no later than 10 days after sample collection. ORAC values for muscle were standardised for protein content by determination of total protein concentration of the muscle supernatants used, by means of the Bradford assay [45]. Results for ORAC were then expressed as micromole Trolox equivalents per liter for plasma and micromole Trolox equivalents per microgram of protein for muscle.

2.8. Flow cytometry

Plasma collected from animals with no contusion (control), as well as 4 h, 3 days, 7 days and 14 days post-contusion, was analyzed for IL-6, IL-10 and TNF-α with a Cytometric Bead Array Rat Flex Set kit from BD Biosciences, according to the manufacturer's instructions and using a flow cytometer (Becton Dickinson FACSArray Bioanalyzer).

2.9. Statistics

Experiments were performed routinely with four or more rats per group, as specified. All analyses were replicated with representative qualitative visual data shown. A regression analysis was performed to compare growth curves in the different experimental groups. Factorial analysis of variance (ANOVA) was performed to assess main the effects of treatment, time and time–treatment interaction. Where relevant, Bonferroni *post hoc* tests were performed, except for cytokine data where Fishers *post hoc* tests were used. An unpaired, two-tailed Student's *t* test for comparison of ORAC data between the control (uninjured) groups was used. All statistical analyses were done using the computer software Statistica version 8 (StatSoft Software). The accepted level of significance was $P<.05$.

3. Results

We present results that illustrate, in rats treated with PCO for 2 weeks prior to experimental contusion-induced damage, (1) increased antioxidant capacity both in circulation and in muscle tissue, (2) earlier elevations in satellite cell number, (3) altered neutrophil and macrophage infiltration patterns and (4) faster appearance of regenerating muscle fibers.

3.1. Contusion-induced damage included significant fiber disruption

Macroscopic assessment indicated that approximately a third of the muscle cross-section was damaged directly underneath the probe impact on the muscle belly, while the rest of the muscle cross-section appeared to have intact fibers. Histological comparison of PLA and PCO muscle tissue sections pre-contusion (Fig. 1A and H) and at different time points after contusion (Fig. 1B–G and I–N) indicated that the major visible effects at 4 h post-contusion were large areas of fiber disruption and significant vascular damage leading to red blood cell accumulation in the interstitial space (Fig. 1B and I), which did not appear markedly different between the two groups. In contrast, already at Day 1 (Fig. 1C and J), and also at Day 3 (Fig. 1D and K) post-contusion, the considerable inflammatory cell infiltration was greater in PLA when compared to PCO. At Day 5 post-contusion (Fig. 1E and L), inflammation was almost completely resolved only in PCO, where evidence of structural re-organisation is also present. By Day 7, it was clearly evident that fiber regeneration had progressed well in PCO, but not in PLA, where central nucleation was still evident, along with

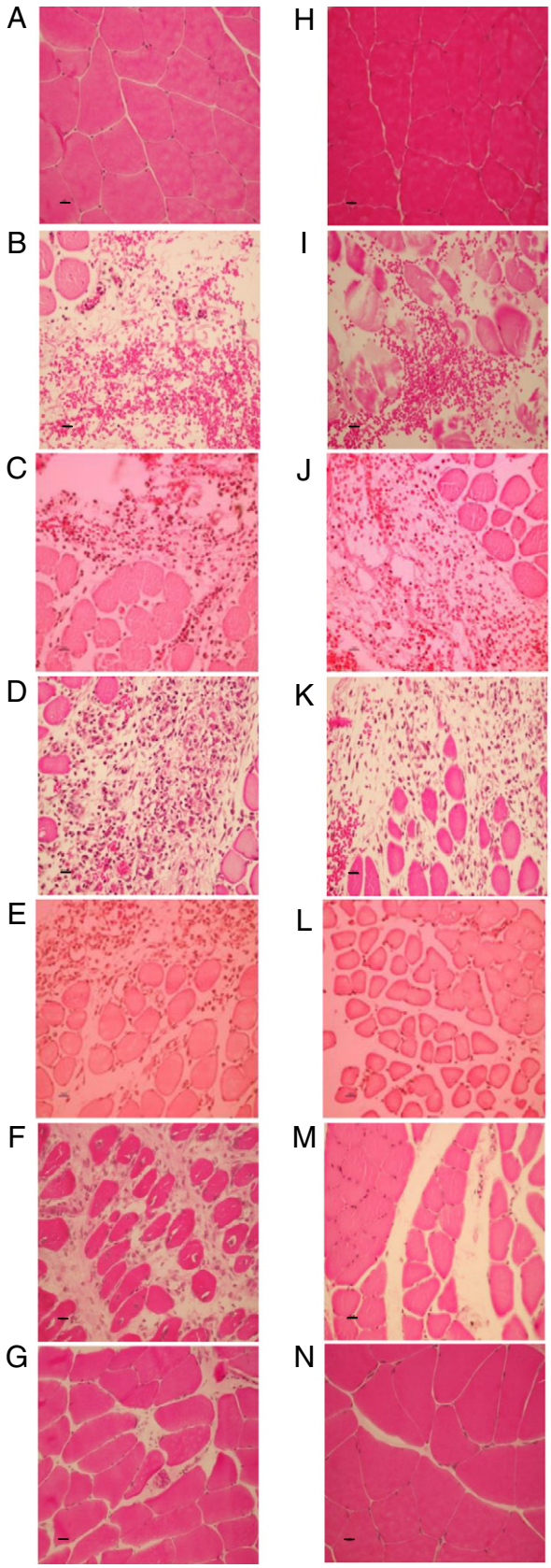

Fig. 1. Representative (H&E stained) histology of gastrocnemius muscle in uninjured muscle and after contusion in PLA- and PCO-treated Wistar rats. PLA rat histology is presented in the left column for time points of uninjured control: 4 h, 1 day, 3 days, 5 days, 7 days and 14 days post-contusion (A–G), with corresponding time points for PCO in the right column (H–N). Scale bars represent 10 mm.

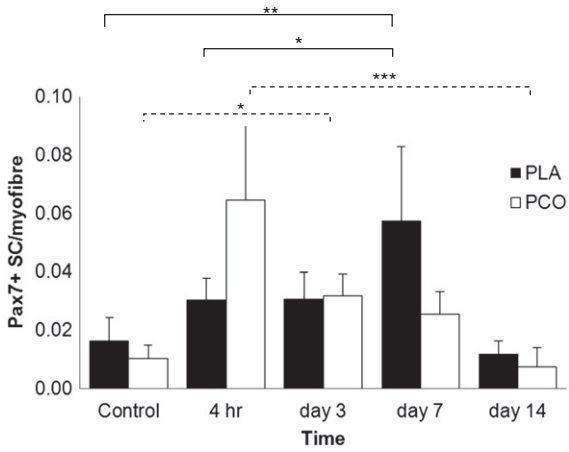

Fig. 2. Pax7 expression in satellite cells over time in contusion-injured gastrocnemius muscle. Values are means±S.D. (n=4). Statistics: ANOVA indicated main effects for time, treatment and an interaction of time and treatment (all P<.001). Asterisks on thew graph indicate results of *post hoc* analysis. Brackets indicating time differences have solid lines for PLA and broken lines for PCO. Significance: *P<.05, **P<.01, ***P<.001.

continued immune infiltration which prevented basal lamina adhesion between adjacent fibers (Fig. 1F and M). These differences on Days 3 and 7 resulted in complete regeneration in PCO, but not in PLA, as visualised on Day 14 post-contusion (Fig. 1G and N).

3.2. PCO administration increased and accelerated satellite cell response

For the purpose of satellite cell (SC) quantification, only border zone areas were assessed; since the basal lamina could not be clearly identified in muscle fibers scattered in the injured area itself, satellite cell identity could not be confirmed according to its position here. In the border zone, for the time points assessed, the number of Pax7$^+$ satellite cells (expressed as number of satellite cells per myofiber) peaked at 4 h post-contusion in PCO (0.064±0.028 SC/myofiber), but only at 7 days in PLA (0.057±0.026 SC/myofiber) (Fig. 2). With the use of CD56 as satellite cell marker, the same conclusion could be drawn for PCO, with a peak at 4 h (0.233±0.035 SC/myofiber), while PLA exhibited a much less pronounced peak on Days 3 (0.102±0.006 SC/myofiber) and 7 (0.101±0.006 SC/myofiber) (Fig. 3). CD34 is not satellite cell specific but can be used for satellite cell identification in combination with anatomical positioning. For this marker, numbers for the two groups peaked at time points similar to that of Pax7 (Fig. 4). Quantitatively, mean CD34$^+$ satellite cells per myofiber peaked at 4 h (0.140±0.025 SC/myofiber) for PCO and at 7 days (0.118±0.009 SC/myofiber) for PLA.

3.3. PCO treatment decreased the neutrophil and accelerated the macrophage response to contusion

Clear differences between PCO and PLA were evident in terms of neutrophil and macrophage infiltration into both the injured area and the border zone. No statistically significant increases in neutrophil count in response to contusion were evident for PCO, while PLA exhibited large elevations in neutrophil numbers, peaking on Day 1 in both areas (Fig. 5A and B). Despite this difference in the magnitude of the response, neutrophil counts had returned to baseline by Day 3 in both groups.

The expected elevation in macrophage count on Days 3 and 5 post-contusion, followed by normalisation by Day 7 in PLA, was not matched by PCO. Rather, PCO macrophage counts peaked on Day 1

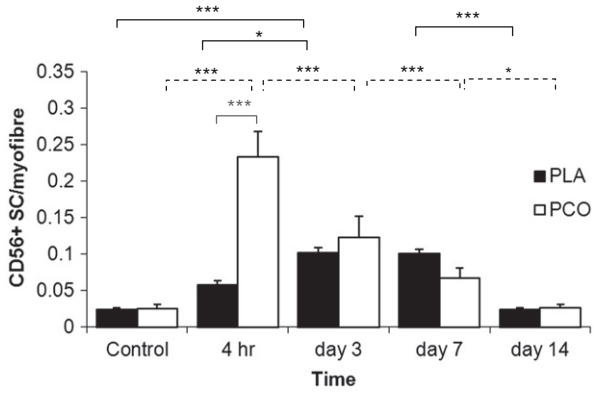

Fig. 3. CD56$^+$ satellite cell expression in gastrocnemius muscle in response to contusion-induced damage. Values represent means±S.D. (n=4). Statistics: ANOVA indicated main effects for both time and treatment (both P<.001). Asterisks on the graph indicate results of *post hoc* analysis. Differences over time are indicated by brackets in solid black lines for PLA and in broken black lines for PCO, while group differences are indicated using brackets in solid grey lines. Significance: *P<.05, ***P<.001.

and were still elevated on Day 3, but had returned to pre-damage levels already by Day 5 (Fig. 6A and B).

3.4. Cytokine elevation was limited in extent and/or shortened in duration with PCO

The major TNF-α response in the tissue occurred on Day 3 in the injured area, with significantly higher elevation in PLA compared to PCO, although values for both groups were significantly higher than before contusion (Fig. 7A). For the border zone, the two groups once again differed in the extent of TNF-α accumulation. Although both groups peaked on Day 1, elevated TNF-α concentrations were sustained only in PLA (Fig. 7B).

While TNF-α concentrations for both groups returned to baseline in the same time frame in the injured area, this was not the case in the border zone. In contrast to the injured area, TNF-α remained significantly elevated in the border zone of PLA, but not of PCO. The time course of change in IL-6 concentrations in the injured area was similar for the two groups with concentrations peaking on Day 3 (Fig. 7C), whereas in the border zone, this pattern was only evident in PLA, while PCO peaked on Day 1 and declined significantly by Day 3, compared to both PLA on Day 3 and PCO at 4 h postinjury (Fig. 7D).

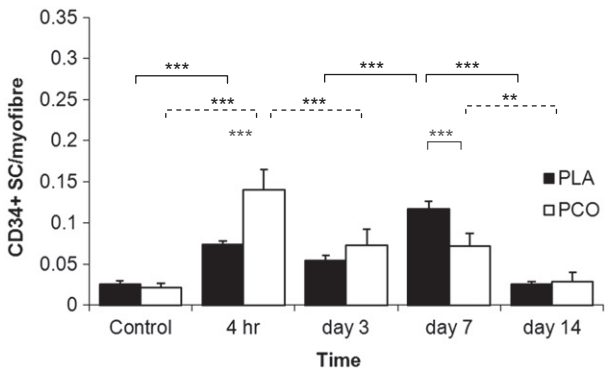

Fig. 4. Gastrocnemius muscle CD34$^+$ satellite cell expression as a result of contusion damage in PLA and PCO. Values represent means±S.D. (n=4). Statistics: ANOVA indicated a main effect of both time and treatment (both P<.001). Asterisks on the graph indicate results of *post hoc* analysis. Differences over time are indicated by brackets in solid black lines for PLA and in broken black lines for PCO, while group differences are indicated using brackets in solid grey lines. Significance: **P<.01, ***P<.001.

Circulating concentrations of pro-inflammatory cytokines (TNF-α and IL-6) remained unchanged, with the exception of a significant increase from baseline in PLA on Day 7 (Supplementary Data, Table 1, found on the journal's website at www.jnutbio.com). In contrast, the anti-inflammatory cytokine IL-10 was unchanged at all time points in PLA, but differed between groups on Day 3, with anti-inflammatory cytokine concentrations significantly higher in PCO.

3.5. Free radical quenching capacity increases with PCO supplementation in both plasma and muscle tissue

Plasma ORAC differed significantly between the two groups, also on Day 3, and, similar to the result for anti-inflammatory cytokines, was significantly higher in PCO (Fig. 8). Although absolute values of ORAC were approximately fivefold lower in muscle compared to plasma, the same significant difference was observed in tissue between the groups on Day 3 (Fig. 8).

3.6. Accelerated fetal myosin heavy chain expression

The discordant time course of events between the two groups in the inflammatory response to the contusion and the satellite cell behavior was finally also evident in a quantifiable marker of muscle fiber regeneration, namely, the number of centrally nucleated and fetal myosin heavy chain-positive myofibers (expressed as a percentage of total myofibers) (Fig. 9). PCO was significantly elevated at 4 h postinjury (approximately 8% of myofibers), peaking on Day 3

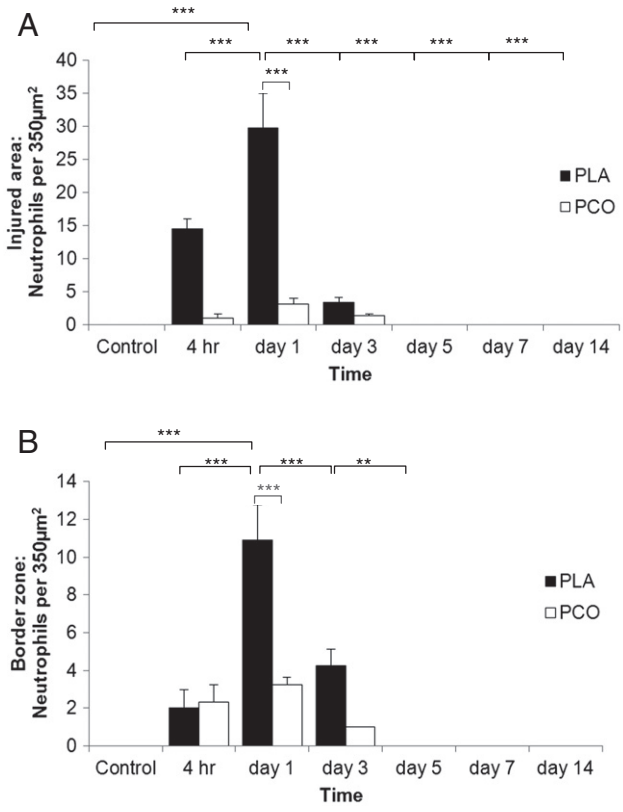

Fig. 5. Effect of prior PCO treatment on the magnitude of neutrophil infiltration in both the injured (A) and border zone (B) areas of contusion-injured muscle. Values represent means±S.E.M. (n=4). Statistics: ANOVA indicated main effects of time, treatment and time–treatment interaction (all P<.001) in the injured area, as well as main effects of time (P<.01) and time–treatment interaction (P<.05) in the border zone area. Asterisks on the graph indicate results of *post hoc* analysis. Differences over time are indicated by brackets in solid black lines for PLA, while group differences are indicated using brackets in solid grey lines. Significance: **P<.01, ***P<.001.

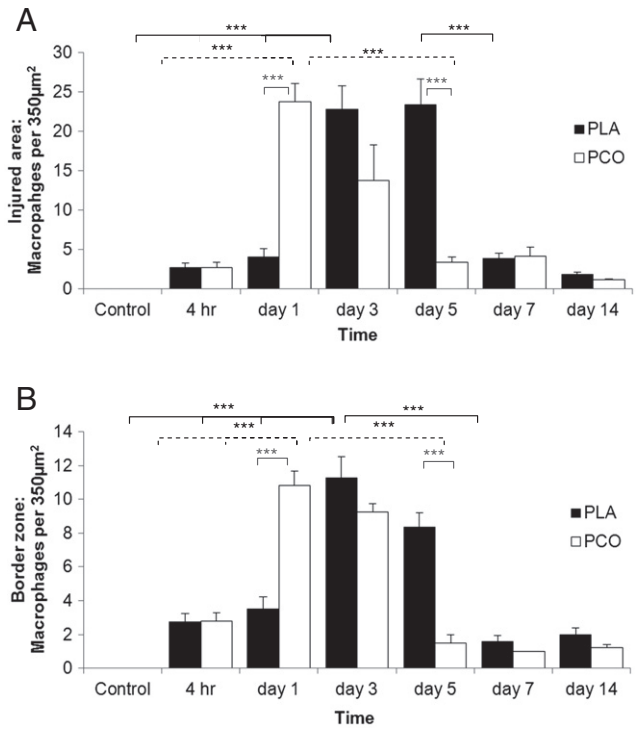

Fig. 6. Effect of 2 weeks of PCO supplementation on the macrophage infiltration pattern after contusion in rat gastrocnemius muscle in both the injured (A) and border zone (B) areas. Values represent means±S.E.M. (n=4). Statistics: ANOVA indicated main effects of treatment, time and time–treatment interaction (all P<.001). Asterisks on the graph indicate results of *post hoc* analysis. Differences over time are indicated by brackets in solid black lines for PLA and in broken black lines for PCO, while group differences are indicated using brackets in solid grey lines. Significance: ***P<.001.

(approx. 14%). Although significant, PLA, in contrast, was only elevated by approx. 4% on Day 3, with the major elevation only on Day 7 (approx. 12%). At this point, the percentage of regenerating cells in PCO had already returned to approx. 4%. These quantitative data confirm the qualitative observations of the H&E-stained sections presented earlier (Fig. 1).

4. Discussion

The role of grape-derived products as preventative complementary medicine was first highlighted by the 1979 article on the "French Paradox" [46]. Since then, the effects of grape seed extract (PCO) have been tested in a variety of experiments using rodents, *ex vivo* organs or cell culture. Specifically, the effects of PCO have been studied in models of clinical conditions such as pulmonary edema [28], intestinal damage [47] and arthritis [48]. PCO prevented or reduced the effects of the disease-promoting intervention when administered for 3, 4 or 19 days, respectively. In the current study, PCO was administered for 2 weeks prior to a blunt, noninvasive contusion injury.

In uninjured animals, increased antioxidant capacity was evident not only in the circulation but also in muscle tissue. Previous studies have shown the bioavailability of PCO or other flavonoids by monitoring their appearance in plasma [49], but this is the first study to indicate that skeletal muscle tissue antioxidant status is also enhanced. Although there is a paucity of information, there is a precedent for polyphenol supplementation to increase tissue antioxidant capacity in liver [50] and kidney [51]. Therefore, the localized response of the injured tissue may be influenced by PCO, in addition to any effect on circulating parameters.

Indeed, we present compelling evidence for an effect of PCO at the tissue level in the early phase response to injury. Firstly, PCO had a

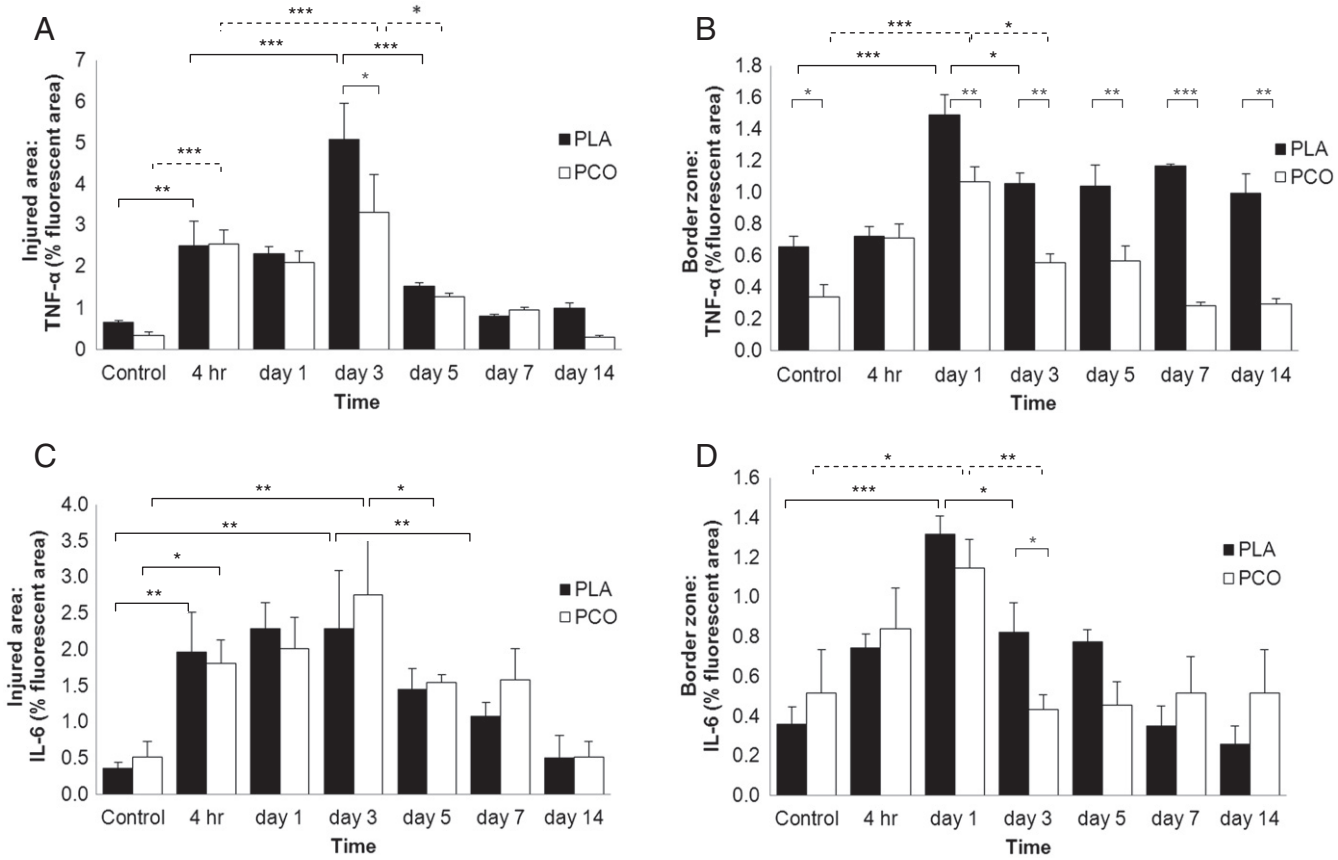

Fig. 7. Inflammatory cytokine response to contusion in gastrocnemius muscle, as assessed by immunohistochemistry in the injured and border zone for TNF-a (A and B) and IL-6 (C and D). Values represent means±S.E.M. (n=4). Statistics: ANOVA indicated main effects of treatment (P<.05 for the injured area and P<.001 for the border zone) for TNF-α only, as well as a main effect of time (P<.001 for both injured and border zone areas) for both TNF-α and IL-6. Asterisks on the graph indicate results of *post hoc* analysis. Differences over time are indicated by brackets in solid black lines for PLA and in broken black lines for PCO, while group differences are indicated using brackets in solid grey lines. Significance: *P<.05, **P<.01, ***P<.001.

profound effect on the extent of neutrophil presence in the injured area. The small elevations in neutrophil numbers in PCO were not statistically significant at any time point in either the injured area or the border zone. Secondly, there was a more robust increase in the presence of satellite cells, in particular those expressing CD56 on the cell surface. This response was present within hours of contusion. Later differences between PCO and placebo may not have been direct effects, but rather consequences of these two early differences. Low neutrophil presence may have allowed for a much earlier invasion of macrophages as well as earlier resolution of their presence.

Earlier satellite cell proliferation was associated with much earlier expression of the fetal isoform of myosin heavy chain (MHC_f). Upon histological examination, regeneration appeared to be complete at Day 14 post-contusion, but only in PCO. Khanna et al. [52], investigating skin wound healing, also indicated that the major mechanism for improved healing was an acceleration of many of the processes activated by the wound.

It is well known that an early response to muscle damage is the recruitment of neutrophils to the site of injury [53]. PCO reduced neutrophil infiltration 10-fold in the current study. Relevant to the current study are previous findings that neutrophil attraction, adhesion and migration are influenced by an increase in reactive oxygen species generation [21,54]. For example, neutrophil adhesion is increased by H_2O_2 [55], and blocking of xanthine oxidase results in decreased neutrophil infiltration [56]. On the other hand, there may be alternative mechanisms of action for PCO that could affect neutrophils. In an *in vitro* model using human umbilical vein endothelial cells (HUVEC), TNF-α-stimulated VCAM-1 expression on HUVEC was reduced by grape seed proanthocyanidins, and, furthermore, this reduced T-cell adhesion to endothelial cells in co-culture [57]. Proanthocyanidins extracted from blackcurrant leaves have also reduced neutrophil infiltration, albeit in a different model of inflammation [58]. A similar effect of grape seed-derived PCO on neutrophil-endothelial cell interaction may reduce extravasation, despite skeletal muscle injury.

Despite their known phagocytic function, macrophages are major contributors to muscle regeneration [13,17]. Of the cytokines assessed in the current study, TNF-α is associated with classical activation, while IL-10 is associated with alternative activation of macrophages [18]. TNF-α seemed to peak later in PLA (Day 7 vs. Day 3 in PCO; see Supplementary Data, found on the journal's website at www.jnutbio.com), while IL-10 was elevated only in PCO. IL-10 was elevated on Day 3 post-contusion, at a time point when macrophage presence in the injured muscle of the PCO group was already resolving, suggesting that any conversion of sub-type was completed. There is no previous evidence that macrophages are directly affected by PCO in any other model of inflammation or disease.

Ultimately, satellite cells contribute to muscle healing. Satellite cell markers cannot be rigidly defined as either quiescent or activated *in vivo*, but Pax7 is a marker specific to SCs and CD56 is usually expressed in a proportion of quiescent SCs and most activated SCs [59]. Peak $Pax7^+$ satellite cell number occurred much earlier in PCO- vs. PLA-injured muscle, while $CD56^+$ satellite cell number peaked early in PCO (more than 10-fold higher than pre-contusion) with no similar peak at any of the measured time points in PLA (highest elevation of 4-fold).

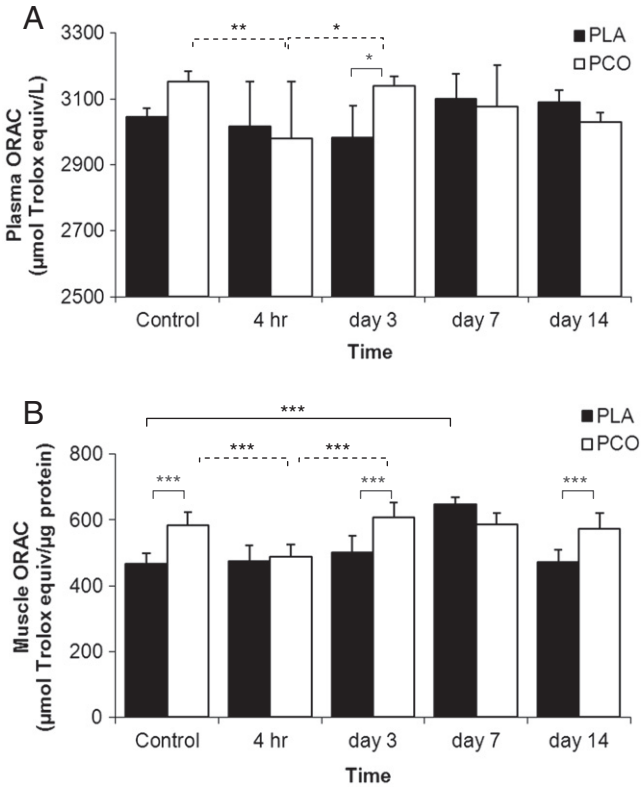

Fig. 8. Changes in ORAC over time after skeletal muscle contusion in PLA and PCO, assessed in plasma (A) and gastrocnemius muscle (B).Values are means±S.D. (n=8). Statistics: ANOVA indicated main effects of time in both plasma and muscle (both P<.001), as well as for time–treatment interaction (P<.05 and P<.001, respectively). Asterisks on the graph indicate results of *post hoc* analysis. Differences over time are indicated by brackets in solid black lines for PLA and in broken black lines for PCO, while group differences are indicated using brackets in solid grey lines. Significance: *P<.05, **P<.01, ***P<.001.

The early increase in SC numbers in the PCO-injured group occurred by 4 h post-contusion, an observation which suggests significant recruitment to the injured area. SC time courses did not follow the pattern of neutrophil or macrophage infiltration or of cytokines present in the injured area or border zone. Thus, factors influencing SCs during the early phase after damage in the current study seem to be related to factors other than the local inflammatory response. Disruption of the basal lamina [34] with release of hepatocyte growth factor [60] allows for significant satellite cell migration. A recent study has shown that experimental depletion of neutrophils spares the muscle membrane damage induced in excised atrophied muscle in the presence of lipopolysaccharide [61]. Together, these data allow for the following possible explanation: the low neutrophil infiltration in the border zone of the injured PCO muscle reduced sarcolemma damage allowing for SC migration despite basal lamina damage.

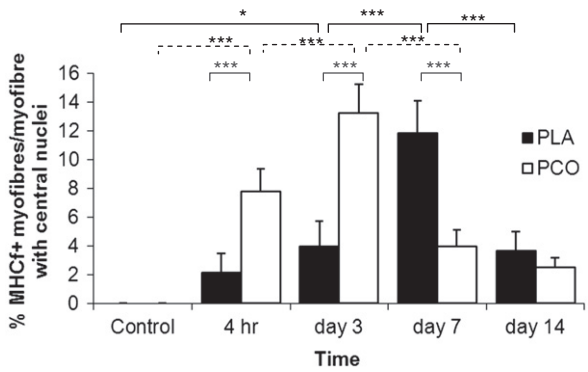

Fig. 9. Percentage fetal myosin heavy chain (MHC_f) positive myofibers. Values represent means±S.D. (n=4). Statistics: ANOVA indicated main effects of both time and treatment (P<.001). Asterisks on the graph indicate results of *post hoc* analysis. Differences over time are indicated by brackets in solid black lines for PLA and in broken black lines for PCO, while group differences are indicated using brackets in solid grey lines. Significance: *P<.05, ***P<.001.

Arnold et al. [62] have demonstrated *in vitro* that co-culture of myoblasts with the anti-inflammatory macrophage phenotype enhanced differentiation and fusion. The finding of the current study that PCO treatment accelerated and enhanced the appearance of central nuclei and the synthesis of fetal MHC suggests that this effect was influenced by the early peak in macrophage infiltration by Day 1 after contusion as well as by the associated anti-inflammatory cytokine profile.

A limitation of the current study is that we did not measure decrease in muscle function or recovery of function over time, rather relying on the appearance of central nuclei and fetal myosin heavy chain and the increased tight adhesion between fibers to indicate enhanced regeneration. Furthermore, although the current data clearly demonstrate a benefit of pretreatment in the postinjury period, it is not clear whether PCO treatment starting just after injury would have the same or less dramatic effects. In an arthritis model, PCO administration starting 14 days *after* initiation of arthritic development indicated efficacy as a remedial treatment. Lastly, we acknowledge that PCO may have exerted modifying effects on gene expression; this aspect was not within the scope of the current study and remains to be elucidated. Future studies should elucidate more clearly any direct effects of PCO on neutrophils and on subtypes of macrophages.

The limitations do not discount the fact that this study had several advantages. The damage was induced without any incision, and the inflammatory response was therefore specific to the muscle damage. The antioxidant was delivered by oral gavage resulting in slow absorption and distribution. It was taken up by the muscle compartment, while the circulatory antioxidant capacity was also chronically elevated. Regeneration requires substantial integration of cellular role players from different physiological systems and we followed an integrative approach.

In conclusion, this study has provided consistent evidence of enhanced muscle regeneration following a skeletal muscle contusion in rats administered a polyphenol, specifically proanthocyanidolic oligomers extracted from grape seeds. No other studies have focused on the influence of PCO on tissue regeneration. We also provide evidence for cell-specific mechanisms that explain the more rapid regeneration. We suggest that proanthocyanidolic oligomers have wide reaching physiological effects that are more complex than quenching circulating free radicals.

Supplementary data

Supplementary materials related to this article can be found online at doi:10.1016/j.jnutbio.2011.05.014.

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