

Treatment of Muscle Injuries by Local Administration of Autologous Conditioned Serum: Animal Experiments Using a Muscle Contusion Model

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Abstract

Muscle contusions represent a major part of sports injuries. The suggested treatments are generally sufficient to support muscle healing, but require a relatively long period of time. Given that autologous blood products are safe treatments, we have used a technique which stimulates the release of certain growth factors in the autologous conditioned serum (ACS). Those growth factors are known to improve the proliferative activity of myogenic precursor cells. Mice were subjected to an experimental contusion injury to their gastrocnemius muscle; one group received local injections of ACS at 2 hrs, 24 hrs, and 48 hrs after injury, a control group received saline injections. The histology results showed that satellite cell activation at 30/48 hrs post injury was acceler-

ated and the diameter of the regenerating myofibers was increased compared to the controls within the first week after injury. ELISA results on the ACS have shown that the elevations in FGF-2 (460%) and TGF- β 1 (82%) could be partly responsible for the accelerating effects on regeneration due to proliferative and chemotactic properties. We conclude that ACS injection is a promising approach to reduce the time of recovery from muscle injury. In terms of clinical targets, this new approach could be used in the treatment of sports injuries and may also be interesting in postoperative situations.

Key words

Growth factors · muscle injury · regeneration · satellite cell

Abbreviations

ACS	autologous conditioned serum
FGF-2	basic fibroblast growth factor (also bFGF)
HGF	hepatocyte growth factor
IGF-1	insulin-like growth factor-1
IL-1Ra	interleukin 1 receptor antagonist
NGF	nerve growth factor
PDGF	platelet derived growth factor
TGF- β 1	transforming growth factor beta 1

Introduction

Muscle injuries account for up to 30% of the injuries sustained in sports events [8]. More than 90% of these muscle injuries are caused either by contusion or by excessive strain of the muscle [7,9]. In professional sport, any of these injuries can lead to significant pain and disability causing loss of training and competition time.

The healing process of damaged muscle tissue is characterised by three phases: (1) the initial destruction phase with concomitant haematoma formation, myofiber necrosis, and inflammation; (2)

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the repair phase, consisting of phagocytosis of the necrotised tissue and regeneration of the myofibers by activated and proliferating satellite cells; (3) the remodelling phase, where the regenerated myofibers mature and the function of the repaired muscle is restored [14].

A large body of evidence suggests that individual growth factors such as IGF-1, PDGF, FGF-2, HGF, NGF, and TGF- β 1 play a specific role during muscle regeneration [1,13,15]. Of these trophic substances, FGF-2, HGF, and TGF- β 1 are thought to be key regulators in the chemotaxis and activation of satellite cells [6,18,25,29]. *In vivo* studies in mice have shown that FGF-2 enhances muscle regeneration after injury [15,16]. Lefaucheur et al. reported that following denervation and devascularization injuries, the injection of neutralizing antibodies against FGF-2 into the muscle reduced the number and diameter of regenerating myofibers, suggesting a delay in proliferation and/or fusion of activated satellite cells [18]. HGF has the capacity to activate quiescent skeletal muscle satellite cells *in vitro* [3]. Furthermore, HGF also has chemotactic activity on satellite cells [6] and it enhances the effect of FGF-2 on satellite cell proliferation by stimulating the expression of one of the FGF receptors [24]. TGF- β 1 supports the action of other growth factors, namely the production of PDGF which is known to have a highly stimulating effect on the proliferation of satellite cells [13,21,23]. However, concerning the satellite cells, *in vitro* studies have shown that TGF- β 1 leads to a slight suppression of proliferation and to an inhibition of differentiation [1,22].

Thus, inducing higher levels of certain growth factors at the site of injury may be used as an approach to treat muscle injuries. Contusion injuries, on which we have focused here, are the result of the impact of a non-penetrating object and are common in all contact sports [5]. In this paper, we use a newly elucidated method of physically and chemically stimulating whole blood under specific conditions [19] so that the isolated serum contains a mixture of cytokines and growth factors [27]. We used this autologous conditioned serum (ACS) in mice to treat a contusion injury and to monitor in detail its effects on muscle regeneration at the histological level.

Materials and Methods

Mice

A total of 108 mice (syngenic C57 Bl/6 mice purchased from Iffa Credo, France; age 13–18 wk; weight 20–25 g) were used. The Regional Ethics Committee on Animal Experimentation (Comité-Ile-de-France, Paris n° 5) approved the experimental protocols. Animals were housed and handled according to recommended guidelines [26]. All procedures were performed under anaesthesia with ketamine (100 mg/kg; Ketalar, Panpharma, France) and xylazine (40 mg/kg; Rompun, Bayer, France) and at the end of the experiments the mice were humanely sacrificed by CO₂ inhalation. It must be mentioned that the mice used for ACS production, for the histological evaluation, and for the immunohistochemical experiments were all different. However, the syngenic nature of the C57 Bl/6 inbred mice allows to consider that the blood from one mouse is autologous to the blood of all mice from the same strain.

Conditioned serum (ACS)

The conditioning of the serum was performed by a method originally developed for human blood [19]. Whole blood (600–800 μ l per animal) from a total of 20 mice was withdrawn via intracardiac puncture into non-heparinated 2-ml syringes containing 12 glass beads of medical grade, each with 2.5 mm in diameter and a surface area of 21 mm². After incubation for 24 hrs at 37°C it was centrifuged (15°C for 10 min at 4000 rpm). The supernatant serum was then removed under sterile conditions with care so as not to aspirate any blood cells. For the control (not-conditioned serum), blood was withdrawn from 10 animals and then centrifuged (15°C for 10 min at 4000 rpm) to obtain the serum. Both the unconditioned and the conditioned serum were separated into cryotubes and frozen at –20°C for later ELISA tests or for administration (only ACS) to the injured muscles. After injury (see below), 10 μ l of ACS were injected with a Hamilton syringe (with a 26-gauge needle) into the damaged gastrocnemius muscle (at the marked site of contusion) at 2 hrs, 24 hrs, and 48 hrs after contusion impact. The same volume of saline was injected at the mentioned time points into the injured control animals to make sure that an assumed additional muscle damage produced by injection pressure was comparable.

Contusion model

A simple and reproducible muscle contusion model in mice was used [16]. The animal's hind limb (one per animal) was positioned on a board dorsiflexing the ankle to 90° and the centre of the point of contusion was marked to help define the injection site. A 16.3-g (diameter: 15.9 mm) stainless steel ball was dropped from the height of 100 cm through a tube (interior diameter of tube: 16 mm) onto an impactor [16] resting with a surface of 26.04 mm² on mouse gastrocnemius muscle.

Regeneration quantification (satellite cells)

Muscle samples from a total of 12 mice were obtained 30 hrs and 48 hrs after the contusion, fixed in ethanol, embedded in paraffin, cut into sections (4 μ m) and then mounted on slides coated with 3-aminopropyl-triethylsilane (APES; Sigma, St. Louis, MO, USA). The sections were deparaffinized, incubated in a pH 9.0 antigen retrieval buffer (Micom, France), and then placed in a microwave pressure cooker (900 W for 13 min and then at 150 W for 20 min). For immunoperoxidase, primary monoclonal Ki-67 antibody (rat antimouse clone TEC-3, Dako, Glostrup, Denmark) diluted 1:50 in Optimax wash Buffer (BioGenec, San Ramon, CA, USA) was applied overnight, followed by a 30-min incubation with biotin-conjugated goat anti-rat Ig specific polyclonal antibody (PharMingen, San Diego, CA, USA) at 1:50 dilution. Finally the bound biotin was detected with streptavidine peroxidase diluted to 1:100 (Dako). The colour was developed with DAB (Diamino-benzidine, Microm). Sections were lightly counterstained in Mayer's hematoxylin and mounted according to standard procedures. The negative control involved substitution of the primary antibody by Optimax wash Buffer (BioGenec). For the positive control, we used sections from the gastrocnemius muscle of mdx (C57 Bl/10 ScSn) mice exhibiting a strong Ki-67 positive labelling in regenerative fibers. This procedure allows for the detection of activated satellite cells that become conspicuous by dark brown staining [10]. Ki-67 is a good marker of proliferation but is not selective for satellite cells only. The positively stained nuclei outside the basal membrane (BM) corresponded

to proliferating fibroblasts, inflammatory cells, or endothelial cells. The percentage of Ki-67-positive satellite cell nuclei was estimated by counting only the positive nuclei which were clearly located beneath the BM of the muscle fibers. Counting of activated satellite cells was done in the area of largest and severest injury at 30 and 48 hrs after injury, on a total of 12 mice. For these counts a light Leitz microscope model Laborlux 12 (Germany) was used. At magnification $\times 400$, 4 to 5 microscope fields of 0.25 mm^2 were counted.

Regeneration quantification (centronucleated myofibers)

Three ACS treated and 3 untreated animals were sacrificed at specific intervals postinjury (0, 2, 4, 6, 7, 8, 14, 21, 28, and 35 days). The experiment was repeated for day 7 to strengthen the results; resulting in a total of 66 animals for histological evaluation. Upon sacrifice, the gastrocnemius muscle was dissected and fixed for 24 hrs in Glyo-Fixx (Shandon, Pittsburgh, USA). After paraffin embedding, muscle histological sections ($4 \mu\text{m}$) were stained with hematoxylin eosin safranin (HES). The section with the largest injury in terms of size and severity was used for further analysis. Slides were analysed both qualitatively and quantitatively by determination of the percentage of small regenerating centronucleated (CN) cells versus large CN cells. We determined that the size of the healthy non-damaged muscle fibers varies between $60\text{--}100 \mu\text{m}$ and that of the regenerating CN cells between $10\text{--}60 \mu\text{m}$. Given the large range in size of the CN fibers we decided to make a distinction between "small" (indicative for little maturation) and "large" (indicative for an advanced growing/maturation stage) CN fibers, and we established a cutoff diameter of $25 \mu\text{m}$ above which a CN fiber was counted as large.

ELISA tests

Growth factor concentrations in the ACS were measured by ELISA kits purchased from R&D Systems (Minneapolis, USA). TGF- $\beta 1$ and FGF-2 were assayed respectively with human TGF- $\beta 1$ ELISA

kit and FGF basic High Sensitivity ELISA kit (DFB 100 and DFB50 Quantikine Immunoassays, respectively). According to the manufacturer's instructions the murine FGF-2 and TGF- $\beta 1$ can be accurately measured using these human kits.

Statistical analysis

All data are given as means \pm SEM. Statistical comparisons between groups were carried out using the unpaired Student's *t*-test and $p < 0.05$ was set to indicate statistical significance.

Results

Satellite cells

Paraffin sections of mouse gastrocnemius muscle were successfully stained with the Ki-67 antibody reacting selectively with nuclei of proliferating cells (Fig. 1). The percentage of positive satellite cells was estimated as their total number divided by the total number of myofibers in the evaluated fields. At 30 hr after injury the percentage of activated satellite cells (Ki-67-positive nuclei) was significantly larger in the ACS-treated muscles ($6.7\% \pm 0.6$) than in the respective controls ($3.6\% \pm 0.4$). Similarly, at 48 hr after injury the percentage of satellite cells was also significantly larger in the treated muscles ($10.0\% \pm 1.0$) than in the controls ($5.4\% \pm 1.0$). Thus the treatment increased satellite cell activation by 84% at 30 and 48 hrs after muscle injury (Fig. 2).

Regenerating myofibers

The histological finding over time coincided with those phases described for muscle regeneration after contusion injuries [16] and need not be described in detail here. It became, however, evident that the ACS treatment had boosted up the process of regeneration with the final result of acceleration and maturation of muscle healing. A noticeable difference was observed between the treated and non-treated muscles from the 6th to the 8th day

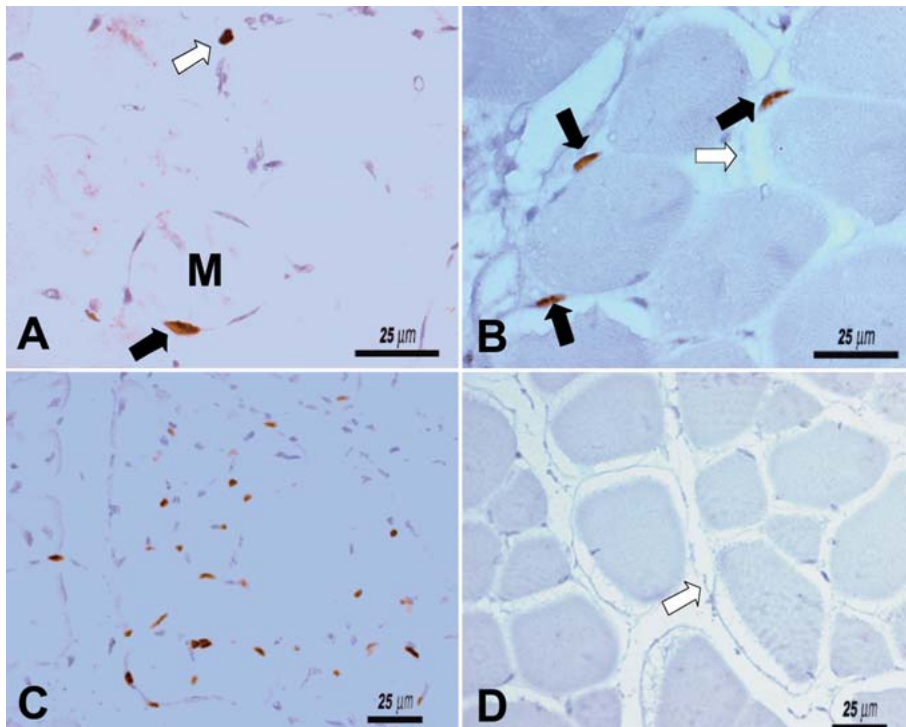


Fig. 1 **A to D** Light micrographs of mouse gastrocnemius muscle immunohistochemically stained with monoclonal Ki-67 antibody. **A** At 30 hrs post contusion injury and treatment with autologous conditioned serum (ACS), a brown coloured Ki-67-positive satellite cell nucleus is seen (closed arrow) located within the basal lamina of the muscle fiber (M). Ki-67 positive nucleus outside the basal lamina (open arrow) represents extramuscular proliferation of fibroblasts, inflammatory cells, or endothelial cells and not of satellite cells (not counted). **B** Satellite cells at 48 hrs post injury and treatment with ACS. Basal lamina is well detected (arrowhead) and satellite cell nuclei (closed arrows) are clearly distinguishable. **C** Positive control of satellite cell activation with mdx (C57 BL/10 ScSn) mice injected with a recombinant adenovirus encoding basic FGF (Ad-RSVbFGF). **D** Negative control with basal lamina (arrowhead) clearly distinguishable.

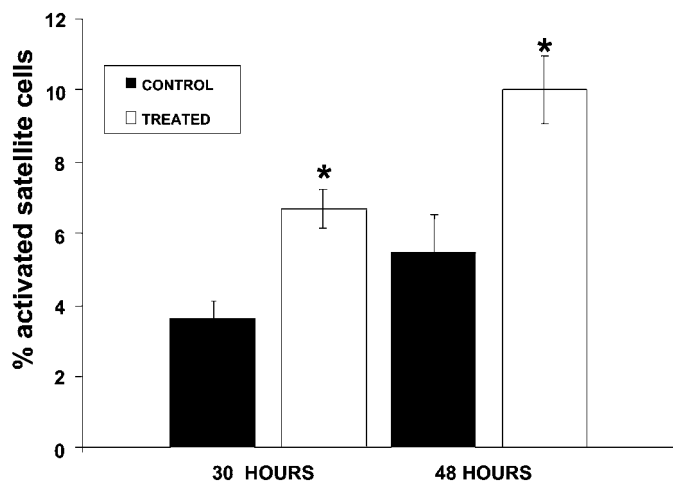


Fig. 2 Comparison of the percentage of activated satellite cells (Ki-67 positive nuclei) per number of myofibers at 30 and 48 hrs after injury in the control and the ACS-treated mouse gastrocnemius muscles. Mean \pm SEM, $n = 12$; * $p < 0.05$ control vs. treated.

after injury. This was most conspicuous around day 7 after injury, when many newly regenerated myofibers (centronucleated or CN cells) were found together with persisting necrosis at the injury site. Especially on day 7 after injury it was noted that the ACS-treated animals had less necrosis and less granulomatous tissue (Fig. 3). Furthermore, from day 6 to 8 after injury the CN cells in the treated mice showed larger diameters than the fibers of the mice that had not received the ACS (Figs. 3 and 4). The quantification of this difference showed that the percentage of large diameter fibers ($> 25 \mu\text{m}$) is higher in the treated ($87.42\% \pm 0.93$) than in the non-treated cases ($60.30\% \pm 5.22$). This was found 1 week after injury (pooled data for days 6, 7, and 8) (Fig. 4). By day 14 there was no difference anymore between control and treated mice in terms of the regenerating myofibers' size. By days 14, 21, and 35 necrosis was not present anymore but a considerable number of large CN cells was still observed in both groups, decreasing over time.

ELISA results

The results of the ELISA tests (Fig. 5) performed on the mouse conditioned serum showed significant increases in the FGF-2 (460% increase) and TGF- β (82% increase) concentrations after the 24-h incubation process with the glass beads.

Discussion

In this study, the aim was to test the effect of the ACS on the muscle regeneration process. We have used an animal model of muscle contusion injury which was previously developed by Kassemkijwattana et al. [16]. Our histological observations regarding the course of spontaneous recovery from injury were similar to those already published [16]. Indeed, after an initial haematoma and granulation tissue at the injury site, the muscle starts to undergo massive regeneration through activation of the satellite cells, a pool of undifferentiated mononuclear cells normally located between the basal membrane (BM) and plasma membrane of the muscle fiber [11,12]. The activation of the satellite cells, observed at 30/48 hrs after injury by Ki-67 antigen

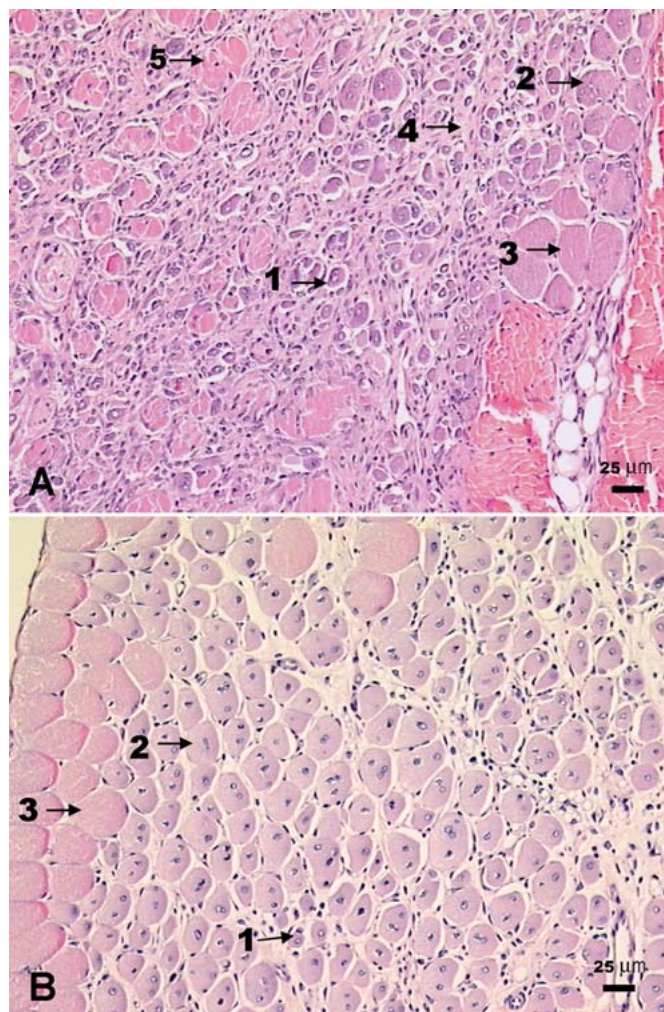


Fig. 3 **A** and **B** Histological characterisation of the regeneration process in the mouse gastrocnemius muscle at 7 days post injury (HES staining). (1) small diameter regenerating CN myofibers; (2) large diameter regenerating CN myofibers: the percentage of large diameter ($> 25 \mu\text{m}$) regenerating CN myofibers was greater in the ACS-treated (**B**) than in the control (**A**) cases (87.42% vs. 60.30% , $p < 0.05$); (3) normal healthy muscle fibers; (4) granulomatous tissue; (5) necrotic muscular tissue.

immunohistostaining, leads to their proliferation and differentiation into myoblasts and subsequently into centronucleated immature myotubes. These centronucleated (CN) myotubes (diameter between 10 and 60 μm) then grow and differentiate into mature myofibers with peripheral nuclei.

Given that CN cellular muscular structures grow in diameter as a function of maturation, the discrimination between small (diameter $< 25 \mu\text{m}$) and large (diameter $> 25 \mu\text{m}$) appears as a justified means of analyzing the progress of regeneration. The histological findings show that at the end of the first week after injury, the ACS-treated muscles have a significantly increased percentage of large regenerating myofibers (Figs. 3 and 4). This suggests that the treatment is accelerating the healing process so that the regenerating myofibers are comparatively more mature. By day 14 there was no difference anymore between the control and the treated animals suggesting that at some point in the second week after injury the regenerating process of the non treated muscles caught up with that of the ACS-treated muscles.

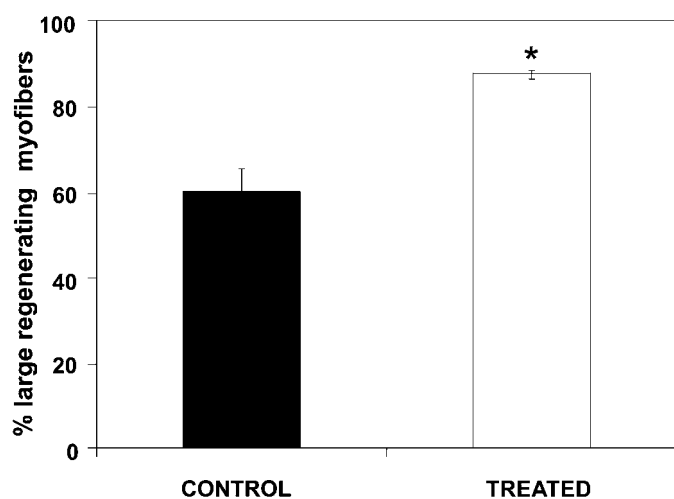


Fig. 4 Percentage of large diameter (> 25 μm) CN cells in the control (n=8) and the ACS-treated mouse gastrocnemius muscles (n=9) at one week after injury (pooled data for days 6, 7, and 8). Mean \pm SEM; * p < 0.05 control vs. treated.

The accelerated regeneration in the treated muscles has to be the result of an increased proliferation rate of satellite cells. These muscle precursor cells have been looked at after Ki-67 immunostaining [10,17]. Between 30 and 48 hrs after injury, the number of activated satellite cells increased (Fig. 2) speaking in favour of a progressively intensified induction of the regenerative mechanisms. The fact that the percentage of activated satellite cells in the ACS-treated muscles almost doubled that of the controls, should allow for the conclusion that either the activation was initiated earlier by the ACS or a larger pool of satellite cells was recruited, or both mechanisms go hand in hand. Whatever of these mechanisms may be present, they result in an earlier completion of regeneration induced by the administration of ACS.

Indeed, the beneficial effects of the ACS are substantiated by the ELISA tests. Here we have seen higher concentrations of two growth factors which are of interest in the natural regeneration process, namely FGF-2 (5.6-fold) and TGF- β 1 (1.8-fold). Because of the largely demonstrated role of FGF-2 in muscle regeneration [2, 4, 13, 15, 16, 18, 20, 24, 28], the important increase in FGF-2 is thought to explain a large part of the efficacy of the ACS. Extended ELISA tests for growth factors and cytokines have been conducted on human ACS [27]. In this case the most important increases have been seen in the FGF-2 (7.5-fold) and the IL-1 Ra (6-fold) concentrations but slight increases in HGF and TGF- β 1 have also been seen.

The present set of animal experimentation has clearly demonstrated that ACS treatment appears to be a powerful tool for the treatment of muscle contusion injuries. The time necessary for recovery from various injuries (e.g., also muscle strains) can probably be substantially reduced by conditioning the cellular systems and mechanisms responsible for regeneration and repair. This may receive clinical importance in general, but especially in those athletes who compete at top levels and for whom a shortened recovery time is not only important for their career but may also have economic benefits. Given the autologous nature of the treatment, no side effects have to be expected, and fu-

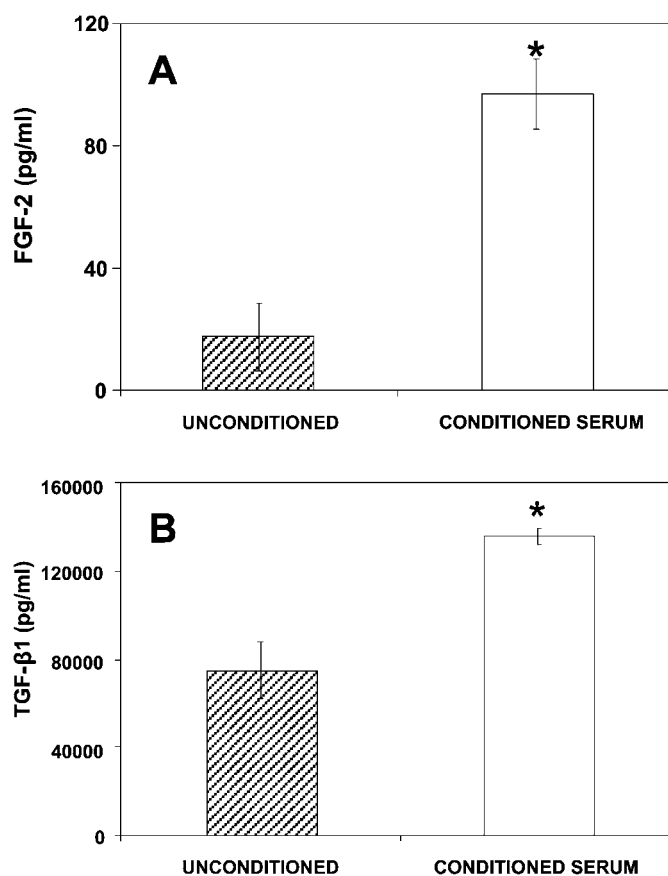


Fig. 5A and B Levels of FGF-2 (panel A) and TGF- β 1 (panel B) in mouse conditioned serum. Mouse serum levels measured (n=2 for unconditioned controls and FGF-2 conditioned serum; n=3 for TGF- β 1 conditioned serum) at time 0 (unconditioned) and after 24 hrs of incubation in the conditioned serum system are reported as means \pm SEM. * p < 0.05 unconditioned control vs. conditioned serum.

ture clinical studies appear promising to improve the standards of muscle lesion treatment [10].

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