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Skeletal muscle repair in a rat muscle injury model: the role of growth hormone (GH) injection

M. CIANFORLINI[1], M. GRASSI[2], V. COPPA[2], S. MANZOTTI[2], F. ORLANDO[3], M. MATTIOLI-BELMONTE[2], A. GIGANTE[2]

[1]Orthopedic Division, Ospedale Carlo Urbani Jesi (AN), Italy
[2]Department of Clinical and Molecular Sciences, Università Politecnica delle Marche, Ancona, Italy
[3]Centro di Tecnologie Avanzate nell'Invecchiamento, IRCCS-INRCA, Ancona, Italy

Abstract. – OBJECTIVE: Muscle injury tends to heal with incomplete functional recovery. Among the growth factors released in the physio-pathological response of muscle lesion, the Insulin-like Growth-Factor-1 (IGF-1) results in an engine factor of the reparation program. The therapeutic use of growth factors has been exploited to improve healing. As IGF-1 is a primary mediator of the effects of growth hormone (GH), we exploited its systemic administration to muscle recovery in a rat model of muscle injury.

MATERIALS AND METHODS: Monolateral lesion of the *longissimus dorsi muscle* of rats was performed. Animals were divided into 5 groups: four groups for histological studies and serum hormone dosage, whilst the fifth group represented the uninjured control. Rat GH was intraperitoneally administered after 24h from the surgical lesion at three different concentrations (0.1, 0.2, 0.4 mg/kg). At 3 days from surgery, immunohistochemical and histological analyses evaluated the expression of MyoD and Myogenin, and the presence of neovascularization and inflammation, respectively. After 2 months, we analyzed the presence of muscle regeneration and fibrosis.

RESULTS: The treatment with GH resulted in a significant increase in neovascularization and Myogenin expression at 24h from injury in comparison with the control. This suggested speed up biological recovery times. After two-months, a dose-dependent increase of the connective component was observed.

CONCLUSIONS: The potential effect of GH on muscle repair and regeneration, through the activation of satellite cells already demonstrated *in vitro*, was confirmed in this *in vivo* experimental approach. This study sheds light on the role of growth factors in damage repair mechanisms to find an appropriate biological treatment for muscle injury.

Key Words:
Muscle regeneration, Muscle injury, GH, IGF-1, Satellite cells.

Introduction

Muscle injury is very common, representing one-third of all injuries in sports. Today, not many well-established treatments for muscle damages are present, and most of them are performed conservatively. The return of the players to training and matches in the shortest time possible is an occurrence that might sometimes cause a re-injury. Moreover, current standard treatments for muscle injury are unsatisfactory, and complications such as muscle atrophy, contracture, and fibrotic scar formation at the site of wound may lead to suboptimal clinical outcomes. In this respect, regenerative medicine approaches have the potential to play a major role in muscle rehabilitation, enhancing the healing process with growth factors[1]. Platelet-Rich Plasma (PRP) is a pool of growth factor that include: platelet-derived growth factor (PDGF), epidermal growth factor (EGF), insulin-like growth factor-1 (IGF-I), transforming growth factor-beta (TGF β-I), vascular endothelial growth factor (VEGF), hepatocyte growth factor (HGF), fibroblast growth factor (FGF), stromal-derived growth factor-alpha (SDF-1α), tumor necrosis factor-alpha (TNF α) and others[2]. At present, the treatment of the patients and athletes with platelet-based applications is permitted and regulated by the Food and Drug Administration (FDA) and the World Anti-Doping Agency (WADA), and from 2011 the use of autologous PRP is also allowed in competitive sports[3]. Despite, PRP treatment has been studied and used in various musculoskeletal disorders[2,4-7], two systematic reviews[8,9] showed uncertainty about the real effectiveness of PRP injections in musculoskeletal injuries. Moreover, two clinical studies evidenced the lack of PRP efficacy after in-

Corresponding Author: Marco Grassi, MD; e-mail: marco.grassi7@gmail.com

jections in patients with acute hamstring muscle injuries[10,11]. The concentration of growth factors in PRP varies according to individual variability, method of preparations, and storage; otherwise, these bias affects clinical outcomes. The knowledge of the individual effect of growth factors could enable the development of the best clinical treatment. Indeed, the growth factors contained in PRP have all been well characterized in terms of inhibitory or acceleratory differentiation parameters using C2C12 murine myoblast cell line *in vitro* experimental investigations. As far as IGF-1 is concerned, it has been demonstrated its capability to stimulate C2C12 murine myoblasts proliferative response in the first 24-36 h of treatment, followed by an increase in myogenic differentiation[12]. Moreover, IGF-1 overexpression was shown to induce a significant increase in mouse muscle mass and enhance its potential regenerative acting on satellite cells[13]. IGF-1 binds to its muscular receptor (IGF-1-R) activating several intracellular pathways (CaMK, PI3K, mitogen-activated protein kinase) and transcription factors among which the muscle-specific MyoD and Myogenin. These signalings produce the proliferation and differentiation of satellite cells and "muscle-derived stem cell-like population" a new cellular line different from the myogenic and mesenchymal line, expressing marker for the hematopoietic lineage such as CD34 and Sca-1, recently re-named telocytes[14,15]. These cells are located near capillaries and are activated in case of muscle damage. In muscle tissue regeneration, in addition to the local myogenic stem cells, circulating cells (Sca-1+) that arise from the bone marrow are also involved[16]. The latter are recruited through local signals (chemokine) whose production is induced by IGF-1.

The clinical use of a single growth factor/cytokine is often expensive, it can be difficult to replicate in physiologically relevant quantities and it needs to be approved by the FDA. For these reasons, in this experimental study, we used a systemic administration of Growth Hormone (GH) in a rat model of muscle injury, to evaluate the potential effect of IGF-1 on the activation of satellite cells for muscle repair and regeneration[17,18]. GH induces the synthesis of IGF-1 in the liver generating a systemic hormone (cIGF-1), and in other tissues, including muscle (mIGF-1)[16]. The relevance of GH on muscle biology is evidenced by the comparison of its role in two opposite pathological conditions like GH-deficiency and acromegaly. GH hypertrophic induction in GH-deficiency, or in situations where the stimulus is temporally reduced, has a positive effect on the skeletal muscle, while protracted high GH blood level (i.e., acromegaly) shows a pathological role inducing myopathy[19]. Therapeutic use of GH in humans is contraindicated in the case of neoplastic pathologies, renal failure or hypersensitivity reactions. The most common side effects to treatment are myalgia, arthralgia, widespread paraesthesia and injection site problems.

The purpose of this *in vivo* experimental study was the assessment of the GH effects on muscle injury in an animal model. Morphological, immunohistochemical, and histomorphometric analyses were performed to test the efficacy and safety of this therapeutic approach and to evaluate a possible relationship between GH concentrations and muscle regeneration.

Materials and methods

Animal Model

The study was performed in 34 male Wistar rats (340 ± 40 g/BW) between 6 and 8 weeks of age. The rats were inbred; therefore, they could be considered genetically identical. Rats were housed at 21-24°C and maintained on a 12-h light/dark cycle. Water and food were given ad libitum during the experiments. The policies and procedures of their use and maintenance were in accordance with those detailed by the directive no. 86/609/CEE regarding animal care and experimental usage. The rats were randomly assigned to 5 groups according to the treatment (Table I). All groups except the "uninjured group" (E) sustained a unilateral lesion of *longissimus dorsi* muscle in a controlled manner[5,20], followed by their designated treatments. Animals were maintained with a normal diet and no forced exercise was induced. Rat GH (rGH) used for treatment (rGH B-9; biopotency, 1.9 IU/mg) was obtained through the NIDDK Rat Pituitary Hormone Distribution Program (Rockville, MD, USA). GH was administered intraperitoneally subsequently to disinfection of the injection site at 24h from surgical lesion.

Groups A, B, C, D were used for the histomorphological evaluation (both time points) and dosage of the GH serum level (48h). Groups E underwent only histological analyses.

Four animals for each group (A, B, C, D) were euthanized by intraperitoneal injection of an overdose of ketamine (75 mg/kg) and xylazine (10 mg/kg) at 2 and 60 days from surgery, respectively.

Table I. Experimental planning

GROUPS	Lesion	rGH	No. of animals	Sacrifice from surgery	
				2 days	60 days
A	Yes	No treatment	8	4	4
B	Yes	0.1 mg/kg	8	4	4
C	Yes	0.2 mg/kg	8	4	4
D	Yes	0.4 mg/kg	8	4	4
E	No	No treatment	2	2	/

GH serum level was evaluated by E-EL-R0029 (Labome® Princeton, NJ, USA; range 0.313-200 ng/ml, sensibility 0.18 ng/ml) at 24 h from the injection, through intracardiac blood sampling after euthanasia. To overcome possible experimental limitations, we used inbred animals that lived in the same environmental conditions, and blood sampling was performed at the same time to respect the circadian rhythm of the hormone.

Surgical Procedure of Skeletal Muscle Injury

Rats were anesthetized by an intramuscular injection of a mixture of ketamine (40 mg/kg, i.m.) and xylazine (5 mg/kg, i.m.) before surgical procedure and placed in ventral decubitus on a warm pad (38.5°C) by the fixation of tail and extremities with adhesive strips. The electric scissors were used to shave off the hair on the back surface of mice. Unilateral cutaneous incision (3 cm in length) was performed in the paravertebral region. Then, muscular tear lesion (0.7x 0.3 cm) was performed on the *longissimus dorsi muscle* using a standard pincer technique, in a controlled manner[5,20]. This muscle was chosen because its position prevents rats from interfering (i.e., biting or scratching) with the surgical treatment (these factors could interfere with the biological response through an increase in inflammation).

Histological Analyses

The experimental sites were dissected and the collected samples were fixed in formaldehyde 4%, embedded in paraffin, sectioned using a Cryotome (5 μm) to perform histological and immunohistochemical analysis. For light microscopy, sections were stained with Hematoxylin-Eosin (E.E) and Sirius Red staining. For immunohistochemistry slides (Menzel-Gläser, Braunschweig, Germany) were used. Dewaxing, rehydration, and antigen unmasking were performed with EnVision™ FLEX Target Retrieval Solution High pH (Dako, Carpinteria, CA, USA) by PT Module (Lab Vision Corporation, CA, USA). Endogenous peroxidase activity was quenched by incubating the sections in 3% H_2O_2 for 10' at Room Temperature (RT). Sections were then incubated with the monoclonal antibodies anti-MyoD (5.8A) and anti-Myogenin (5FD) (both from Santa Cruz Biotechnology, Santa Cruz, CA, USA) diluted 1:150 in Antibody Diluent with Background Reducing Components (Dako) for 1h at RT in a humidified atmosphere. The reaction was visualized with LSAB®Plus System-HRP DAB+ kit (Dako, Carpinteria, CA, USA). Sections were counterstained with Mayer hematoxylin (Bio-Optica SpA, Milan, Italy). Negative control was represented by primary antibody untreated sections. The reaction was examined with a light microscope (Nikon Eclipse 600). Each sample was evaluated in a blinded manner by three experienced observers (SM, MB and FO). The blinded examiner considered three fields for each section for a total of 5 sections for each lesion. All sections were evaluated at 10X magnification using a semiquantitative score, considering the following parameters: neovascularization, inflammation, fibrosis, and muscle regeneration (Table II). In the case of disagreement between observers, AG reviewed the samples and an undisputed score was made in agreement with all observers. The presence of metaplastic zones, calcifications, and heterotopic ossifications was further evaluated by histological analysis.

Histomorphometric Evaluation

Computerized morphometric analysis was performed with a Leica Q5OOMC Image Analysis System (Leica Leitz DMRBE, Cambridge, UK) and the associated software was used. The camera images were digitized and modified in a binary way to make them suitable for measurement. The considered parameters were: MyoD, Myogenin, fibrosis and muscle regeneration. After standard filtration procedures for background

Table II. Semiquantitative analysis.

Score	Neo-vascularization	Inflammation	Fibrosis	Muscle regeneration
0	no evidence	no evidence	no evidence	no evidence
1	< 25% of the fields with new vessels	< 25% of the fields filled with inflammatory cells	< 25% of the fields with fibrosis	< 25% of the fields with new muscular tissue
2	25%-50% of the fields with new vessels	25%-50% of the fields filled with inflammatory	25%-50% of the fields show fibrosis	25%-50% of the fields with new muscular tissue
3	> 50% of the fields with new vessels	> 50% of the fields filled with inflammatory cells	> 50% of the fields with fibrotic features	more than 50% of the fields with new muscular tissue

smoothing, the system identified all the regenerating muscle fibers and the other parameters according to a threshold value set by the operator. Based on a calibration factor determined by a suitable procedure in the setup menu, the system calculates the fraction area (Aa %) occupied by the selected parameters. Five fields were studied for each section. Data are expressed as mean ± standard deviation (SD). For clarity, fibrosis and muscle regeneration have been both assessed with semiquantitative and histomorphometric evaluation.

Statistical Analysis

Statistical analysis of histomorphometric data was performed with the Wilcoxon non-parametric test. The level of statistical significance was established at $p<0.05$.

Results

Macroscopically, at the time of dissection, there were no differences between the treated lesions and controls. In injured rats euthanized at 48 h, we evaluated neovascularization and inflammation (histological parameters), and MyoD and Myogenin (immunohistochemical parameters). In injured rats euthanized at 2 months, we assessed muscle regeneration and the occurrence of fibrosis.

GH Serum Level

At 24h from the injection (48h from injury), we dosed the GH serum level to understand its influence on serum baseline levels. No signs of side effects due to high doses of growth factors were detected. As expected, the basal serum level of GH (37.6±16.3 ng/ml) raised with regards to GH dosage (Table I): Group B 116.2±23.1 ng/ml, Group C 141.8±32.5 ng/ml and Group D 183.1±22.4 ng/ml, respectively.

Histological Analysis at 48h

Semiquantitative analysis performed at 48h showed that inflammation at the injury site was similar between control (Group A score: 1.2±0.1) and rats infiltrated with GH (Groups B-C-D mean score: 1.3±0.4). Otherwise, vascularization increased with increasing dosages of GH (Group A score: 1.3±0.2; Groups B-C-D scores: 1.5±0.3 - 2.1±0.3 - 2.4±0.1 respectively). MyoD and Myogenin expression at histomorphometric evaluation was different between groups, showing a direct relationship with the amount of GH injected (Table I). The percentage of cells positive for MyoD was lower compared to Myogenin expression (Figure 1).

Histological Analysis at 2 Months

At 2 months from injury, we observed that in the control group (Group A) there was good muscle regeneration and a poor fibrotic scar in the proximity of the lesion. On the contrary, the histological sections of rats treated with high doses of GH (Group D) presented an increase in fibrosis, as evidenced by Sirius Red staining, and a reduced and disarranged muscle regeneration (Figure 2). In the site of the muscular lesion, the scar tissue was directly proportional to the concentration of the administered GH. A good correspondence between semiquantitative histological and histomorphometric data was observed. No areas of metaplasia, calcification, heterotopic ossification, or other pathological variants were observed.

Discussion

In the current study, we evaluated the effects of muscle injury treatment with growth hormone in terms of the inflammatory phase, muscle regeneration, and fibrosis. We specifically assessed the existence of a possible correlation between the concentration of administered GH and tissue healing.

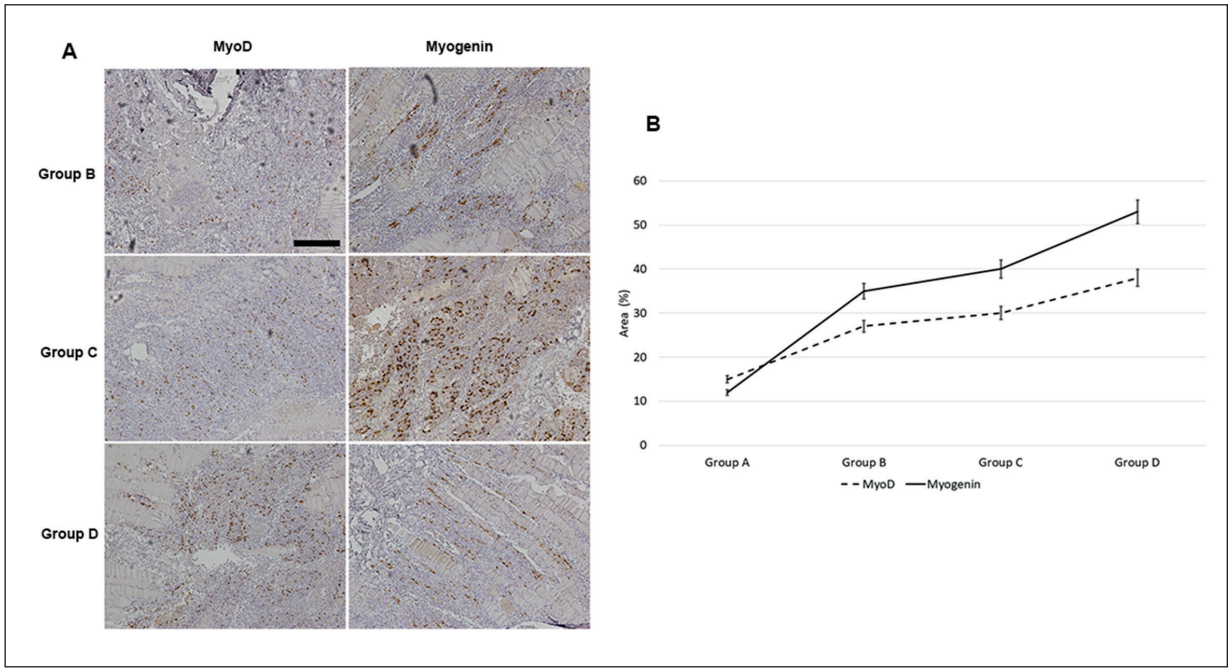

Figure 1. A, Representative images of MyoD and Myogenin immunohistochemical detection at 48h from injury in the differently treated groups (Scale bar 20 μm). **B**, Graph representing histomorphometric evaluation of the area occupied by positive cells after GH treatment.

Immunohistochemical detection of MyoD did not detect significant differences between treated groups and controls (group A). On the contrary, the immunohistochemical staining for Myogenin showed an expression increase in the active phase of muscle regeneration, which was higher in treated animals in comparison to controls (group A) and directly proportional to GH concentration. This evidence could be explained as Myogenin is expressed later than MyoD in the physiological process of muscle healing[21]. The lack of significant variations detected in Myo D

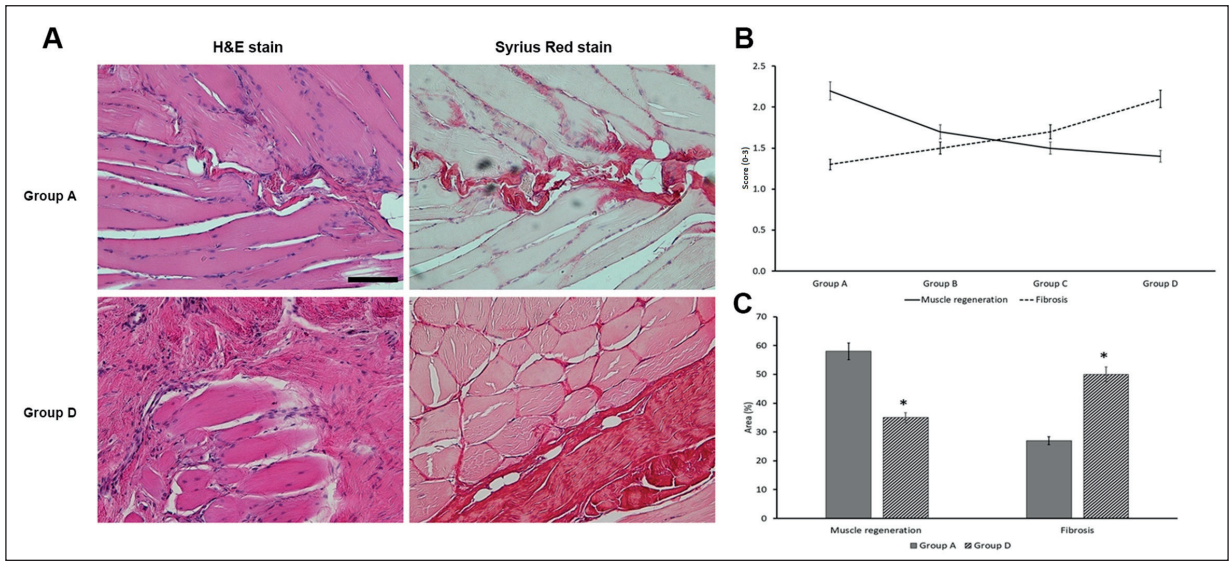

Figure 2. A, Representative histological sections of lesion after 2 month in Group A and D showing muscle regeneration (H&E) and fibrosis (Sirius Red); **B**, Graph depict semiquantitative evaluation of muscle regeneration and fibrosis 2 months from injury. (A: no treatment; B: GH 0.1 mg/kg, group C: GH 0.2 mg/kg, group D: GH 0.4 mg/kg group); **C**, Histogram of histomorphometric comparison between group A and D: *p<0.05.

expression between treated and untreated lesions could be ascribable to the fact that at our time point (i.e., 48h) the healing process of the control group is situated in the so-called ascending phase of "MyoD curve", while the treated groups are in the descending ones[21]. We can, therefore, hypothesize that GH administration fastened the muscle healing process.

Ferrari et al[22] had already demonstrated that muscle cell growth promoted by GH is mainly mediated by IGF-1: the administration of GH in wild-type rats increased muscle mass and the size of muscle fibers, while no effects were present in IGF-1-R knockouts rats. During muscle regeneration, IGF-1 supported satellite cell mobilization, function, and proliferation under pathological conditions[13]. The enhanced expression of mIGF-1, the local isoform of IGF-1, accelerates regenerative processes after skeletal muscle injury in a mouse model, creating a qualitative environment capable of efficiently support an appropriate tissue repair[23]. The role of IGF-1 in muscle regeneration was confirmed by a research performed on MDX (Duchenne muscular dystrophy model) mice. In the latter, the IGF-1 gene was over-expressed by gene modification, bringing a benefit to muscle regenerative capacity[24].

In our investigation, we found that the increase in muscle regeneration is also associated with a rise of fibrous connective tissue close to the muscle injury, as evidenced by Sirius Red staining. This could be related to the route of GH administration (i.e., through the peritoneum): since IGF-1 receptors are expressed on fibroblasts, the presence of exuberant connective tissue, directly proportional to the administered GH concentration is conceivable. Therefore, GH action is ubiquitous and increased the amount of muscle tissue as well as connective tissues of endomysium and perimysium. To our knowledge, this is the first research that considers the use of GH/IGF-1 for muscle repair and regeneration[17,18,25,26]. There are several limitations to the current study that warrant discussion. First, the surgical procedure is not universally recognized. On the other hand, this surgically-induced lesion determines with accuracy the same type of damage in all rats and could mimic the human skeletal muscle lesions. Second, to observe the effects of the IGF-1 more clearly possible, we used very high GH concentrations compared to basal serum values. Further studies should clarify the best hormone concentration.

Conclusions

The present study showed the *in vivo* potential effect of IGF-1 on muscle repair and regeneration, through the activation of satellite cells as already demonstrated *in vitro* studies. These outcomes confirm the good results already obtained by the use of PRP for the muscle injury treatment. It also revealed that muscle repair with hyperplasia of the surrounding connective tissues is dependent on GH and that GH administration fastened the muscle healing process. These findings may contribute to the development of new regenerative approaches to facilitate the healing process and to reduce scar tissue formation in muscle injury.

Statement of Interests

The Authors declare that they have no conflict of interests and none financial support

Ethical Review Committee Statement

The investigation has been performed in accordance with the policies and procedures detailed by the directive No. 86/609/CEE regarding animal care and experimental usage.

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