Insulin Modulates Myogenesis and Muscle Atrophy Resulting From Skin Scald Burn in Young Male Rats

Hananiah Tardivo Quintana, PhD, Vivianne Izabelle de Araújo Baptista, MD, Mariana Cruz Lazzarin, MD, Hanna Karen Moreira Antunes, PhD, Luciana Le Sueur-Maluf, PhD, Camila Aparecida Machado de Oliveira, PhD, and Flavia de Oliveira, PhD*

Departamento de Biocieências, Universidade Federal de São Paulo, Santos, SP, Brazil

**A R T I C L E   I N F O**

Article history:
Received 19 October 2018
Received in revised form 18 June 2020
Accepted 7 July 2020
Available online xxx

Keywords:
Burn injury
Insulin
Skeletal muscle
Myogenesis
Atrophy

**A B S T R A C T**

Background: Burn injuries (BIs) due to scalding are one of the most common accidents among children. BIs greater than 40% of total body surface area are considered extensive and result in local and systemic response. We sought to assess morphological and myogenic mechanisms through both short- and long-term intensive insulin therapies that affect the skeletal muscle after extensive skin BI in young rats.

Materials and methods: Wistar rats aged 21 d were distributed into four groups: control (C), control with insulin (C + I), scald burn injury (SI), and SI with insulin (SI + I). The SI groups were submitted to a 45% total body surface area burn, and the C + I and SI + I groups received insulin (5 UI/Kg/d) for 4 or 14 d. Glucose tolerance and the homeostatic model assessment of insulin resistance index were determined. Gastrocnemius muscles were analyzed for histopathological, morphometric, and immunohistochemical myogenic parameters (Pax7, MyoD, and MyoG); in addition, the expression of genes related to muscle atrophy (MuRF1 and MAFbx) and its regulation (IGF-1) were also assessed.

Results: Short-term treatment with insulin favored muscle regeneration by primary myogenesis and decreased muscle atrophy in animals with BIs, whereas the long-term treatment modulated myogenesis by increasing the MyoD protein. Both treatments improved histopathological parameters and secondary myogenesis by increasing the MyoG protein.

Conclusions: Treatment with insulin benefits myogenic parameters during regeneration and modulates MuRF1, an important mediator of muscle atrophy.

Published by Elsevier Inc.

**Introduction**

Burn injuries (BIs) due to scalding are one of the most common accidents among children, usually occurring in a domestic environment.\(^1\) In Brazil, 70% of thermal injuries occur at home and 50% of victims are children.\(^3\) BIs greater than 40% of total body surface area (TBSA) are considered extensive and result in local and systemic responses.\(^5\) The hyperglycemic state in the acute phase of the response to extensive BI is followed by a late phase, in which protein hypermetabolism
and an increased muscular proteolysis are predominant. After extensive BIs, the systemic effects are immediately initiated, leading thus to inflammatory processes and enhancement of inflammatory cytokines, which, consequently, cause a systemic inflammatory response syndrome that affects all systems of the organism, including the skeletal muscle. Skeletal muscle mass is maintained by a dynamic balance between synthesis and degradation. All the events involved in muscular synthesis are coordinated by the expression of myogenic regulatory factors (MRFs): MyoD, Myf5, Mrf6, and MyoG. At the beginning of myogenesis, activated satellite cells express markers such as paired-box protein 7 (Pax7), whereas active cells express helix-loophelix transcription factors such as MyoD in the early stages of myogenesis. The MyoD expression starts in primary myogenesis, from the activation of satellite cells and myoblast proliferation to the differentiation of these cells, whereas the MyoG expression, on the other hand, is involved in secondary myogenesis, from the myoblast differentiation. Conversely, the most notable mechanism in states of accelerated muscle degradation is the ubiquitin-proteasome system. The proteolytic activity of ubiquitin-proteasome system involves three enzymes: ubiquitin-activating enzyme (E1), ubiquitin-conjugating enzyme (E2), and enzyme ubiquitin ligase (E3). The latter transmits specific substrate to gene expression for two muscle-specific enzymes of muscular atrophy: muscle RING finger 1 (MuRF1) and muscle atrophy F-box (MAFbx, or atrogin-1). MuRF1 preferentially interacts with structural muscle proteins and promotes the degradation of sarcomere proteins, whereas MAFbx interacts with MRFs, interfering with the protein synthesis and muscle growth.

The increase in anabolic hormones such as insulin acts on the MuRF1 and MAFbx regulatory mechanism, decreasing their expression levels and, consequently, the muscular atrophy. Recently, therapeutic approaches to extensive BIs have aimed to reverse hypermetabolic responses and catabolic states. To do so, investments must be made in anabolic agents, such as growth hormones, insulin, IGF-1, testosterone, and oxandrolone and anticatabolic agents such as adrenergic antagonists (propranolol or metoprolol), which reduce supraphysiological thermogenesis, cardiac work, resting energy expenditure, peripheral lipolysis, and efficiency of muscle protein synthesis. The use of such hormones maintains or increases muscle mass, in addition to accelerating the healing of wounds through anabolic and anticaabolic activities. Therefore, we investigated the effects of short- and long-term intensive insulin therapy on BIs in the skeletal muscle after extensive skin burn in young rats, through morphological aspects, MRFs, and expression of genes related to muscle atrophy.

**Materials and methods**

**Animals and study design**

Male prepubescent Wistar rats, aged 21 d (n = 64), were kept in individual cages and distributed into four groups: control (C), control + insulin (C + I), submitted to scalding injury (SI), and scalding injury + insulin (SI + I). Mixed-sex animals were not included because of hormonal variability in the female group and “reduction”, from animal ethics principles. Each group was further subdivided into different euthanasia periods (4 and 14 d, n = 8). The temperature of the room was controlled (22 °C) with regular 12-h light–dark cycle; water and food were provided ad libitum. This study was approved by the Research Ethics Committee of the Federal University of São Paulo (protocol no. 4857080514). All experiments complied with the ARRIVE guidelines and were performed in accordance with Brazil’s National Council for the Control of Animal Experimentation (CONCEA).

**Scald burn injury and insulin treatment**

Animals were anesthetized with intraperitoneal (IP) injection of ketamine (50 mg/mL) and xylazine (10 mg/mL), and their dorsal and ventral hairs were removed on the sixth day of adaptation in individual cages. SI and SI + I groups were submitted to nonlethal scald burn injury in 45% of TBSA, 30% in the dorsal and 15% in the ventral body, distant from joints, as described by Quintana et al. C and C + I groups were submitted to sham or scald burn injury. Rats of all groups previously received analgesic morphine (10 mg/kg) subcutaneously administered for sham or scald burn injury. Morphine was orally administered 24 h later (10 mg/kg) and when the animals showed signs of discomfort or pain. Immediately after thermal injury or the sham procedure, C + I and SI + I groups received subcutaneous administration (5 IU/kg/d) of slow-acting insulin glargine Lantus (Sanofi-Aventis Farmacêutica Ltda., São Paulo, Brazil), whereas C and SI groups received saline solution. The insulin dosage used was chosen based on studies whose authors administered insulin treatment in euglycemic murine models. Insulin-treated rats received 5% sucrose in drinking water to maintain euglycemia. The weight and water and food intake of all animals were recorded every day since weaning, which included the days of adaptation in individual cages.

**Glucose homeostasis: glucose tolerance test (GTT) and HOMA-IR index**

Glucose homeostasis was evaluated in all animals through glycemia, GTT, plasma insulin concentration, and homeostasis model assessment of insulin resistance (HOMA-IR) index. After 3 and 13 d of insulin treatment, glycemia was checked using the Accu-Check Active glucometer (Roche Diagnóstica Brasil Ltda., São Paulo, Brazil) 1 and 8 h after treatment through blood collection from the animals’ tail vein. The next day, 24 h after the last insulin administration, animals were submitted to 8 h of fasting. Then blood was collected from the rats’ tail vein to determine fasting glycemia and insulinemia (I). After finishing this process, a 40% (4 g/10 mL to 5 mL/kg) glucose solution was administered via IP to verify glucose concentration in blood after 15, 30, 60, and 120 min.

Thereafter, the fasting blood sample (25 μL) was centrifuged, and the plasma was collected using the Rat/Mouse Insulin ELISA kit (EZRMI-13K code, Merck Millipore, Darmstadt, Germany) to measure plasma insulin concentration. The fasting glycemia (FG) and I values were used to calculate
insulin sensitivity using the HOMA-IR index. This index is represented by the product of FG and I divided by a constant, in accordance with the following formula: HOMA-IR = (FGxI)/2.430. Finally, all animals were euthanized with a lethal IP injection of ketamine (150 mg/kg) and xylazine (30 mg/kg).

Morphological parameters

The medial part of each gastrocnemius muscle, which is distant from the scald burn lesion, was dissected and transversely sectioned into proximal, middle, and distal parts. The middle part of gastrocnemius was immediately flash-frozen with liquid nitrogen and covered by inclusion for freezing Tissue-Tek biological tissues (Sakura Finetek, California, USA), whereas the right side of the muscle was separated for subsequent real-time polymerase chain reaction (PCR) analyses. Both specimens were stored in a biofreezer at –80°C.

Left-side specimens were cut into transversal sections in a cryostat (10 μm). Slides were stained with hematoxylin and eosin for histopathological and morphometric analysis. Computerized image equipment (Axio Vision 4.5, Carl Zeiss, Oberkochen, Germany), attached to a binocular microscope (Axio Observer D1, Carl Zeiss), was used for histopathological analysis. For morphometric analysis, a cut of each animal was randomly chosen from four sections stained with hematoxylin and eosin, and three fields were photographed with a 40x objective. A frame of an area of 22,500 μm² (150 μm × 150 μm), with two continuous lines (considered “prohibited lines”) and two dotted lines (considered “permissible lines”), was used to profile area (μm²) and count cell density (number of fibers/mm²). Frequency distribution was obtained from the profile areas, and the values were further distributed into an area frequency histogram.

Myogenic regulatory factors: Pax7, MyoD, and MyoG immunohistochemical analysis

Slides of serial muscle sections were fixed with 4% paraformaldehyde, washed in Tris-buffered saline (TBS) with 0.1% Tween-20 (TBST), and the endogenous peroxidase was blocked with 1% hydrogen peroxide. After washes with 10% TBST, proteins blocked with 1% bovine serum albumin (BSA) were diluted in 10% TBST and incubated overnight at 4°C with primary antibodies in 1% TBST-BSA. The polyclonal primary antibodies used were anti-Pax7 (1:350, AB34360, Dako, California, USA), anti-MyoD (1:100, SC-32758), and anti-MyoG (1:100, SC-52903); the last two were purchased from Santa Cruz Biotechnology (Texas, USA). After the TBST wash, slides were incubated with an anti-rabbit IgG secondary antibody, biotin conjugate (Vector Laboratories, California, USA) at a concentration of 1:200 in PBS for 30 min, washed with 10% TBST, followed by the application of the avidin–biotin complex method conjugated to peroxidase (Vector Laboratories) for 30 min, washed with 10% TBST. Then, a 0.05% solution of 3-3-diaminobenzidine was added and, the sections, contrasted with Harris’ hematoxylin (Merck, Darmstadt, Germany). The Pax7, MyoD, and MyoG immunoreactive nucleus density (number of immunoreactive nuclei/mm²) was determined by using three cuts randomly chosen, and three photomicrographs were obtained (totaling nine photomicrographs per animal, 100x objective) with the aid of the computerized imaging system (Axio Vision 4.5, Carl Zeiss) attached to a binocular light microscope (Axio Observer D1, Carl Zeiss).

Genes related to muscle atrophy (MuRF1, MAFbx) and IGF-1: real-time polymerase chain reaction (qPCR real-time)

Right-side specimens of the gastrocnemius muscle were homogenized with 1 mL TRIzol (Invitrogen, California, USA); chloroform, isopropanol, and ethanol 75% were added and centrifuged. The cell pellet was resuspended in 40 μL of diethylpyrocarbonate (DEPC) treated water. RNA purity and integrity were observed by optical density (260/280 nm ratio >1.9; NanoDrop 2000c, Thermo Scientific, California, USA). Then the samples were kept at –80°C and treated with Deoxyribonuclease I, Amplification Grade (DNase I, Amp Grade, Invitrogen) as fixed by the producer. The total RNA extraction followed the protocol adopted from Chomczynski and Sacchi. Total RNA was treated with DNase, and cDNA was built by reverse transcriptase PCR, with the High-capacity cDNA Reverse Transcription kit (Applied Biosystems, California, USA). Primers previously designed for genes of interest and endogenous control (GAPDH) were used to analyze gene expression, and amplification was detected by intercalating DNA (Sybr Green, Applied Biosystems). The following primer sequences were used: GAPDH forward 5’-GCTCTCTGCTCCTCCTGTC-3’, reverse 3’-GACCTGGGACTTGCAAA-5’; IGF-1 forward 5’-GCATTTGGAATTAGTGTTCG-3’, reverse 3’-GCTCCCTCCATCTTCTGTA-5’; MuRF1 forward 5’-CTATGGAGAACCTGGAGA-3’, reverse 3’-CCTGAAGATGTCTTGG-5’; MAFbx forward 5’-TCTATCTCACATTCTCTGA-3’, reverse 3’-CTCGAAGATGTCTTGG-5’; MU01 forward 5’-TGCTTA-CAACTGAACATC-3’, reverse 3’-TCTATCTCCTTCCAAATC-5’. Samples in duplicate were pipetted on the 7500 Fast Real-Time PCR equipment (Applied Biosystems), and the subsequent cycling program was selected: holding stage at 95°C for 10 min, 40 cycles of 15s and 1 min at 95°C and 60°C, respectively, finishing with the melting curve: 95°C for 15s, 60°C for 1 min, and 95°C for 15s. Results were obtained by relative quantification (2-ΔΔCt method).

Statistics treatment

Statistical analysis was performed by analysis of variance with two fixed factors (scaldburn injury and intensive insulin treatment), followed by Tukey’s test for multiple comparisons, whenever necessary. Results are expressed as mean and standard deviation, and P < 0.05 was considered statistically significant.

Results

Effects of insulin on glucose homeostasis

The GTT determined the glucose metabolism in the established periods (0, 15, 30, 60, and 120 min) after insulin treatment in the fourth (Fig. 1A) and 14th (Fig. 1B) days. Figure 1C shows the area under the blood curve and indicates there were no differences in blood glucose between the groups at day 4. However, in the 14th day, SI + I showed the areas under
the blood curve was lower than C+I as well as SI was lower than C.

The HOMA-IR index was lower in groups that received insulin during 4 d (Fig. 1D), showing higher insulin sensibility. However, after 14 d, HOMA-IR was higher in C+I when compared with C, even though SI+I was lower than C+I, showing that long-term intensive insulin therapy caused insulin resistance.

**Effects of insulin on muscle morphology**

In the 4th and 14th days, the C group presented normal fibers with homogeneous size, polygonal format, and peripheral nuclei, arranged in equidistant fascicles independent of the experimental days. C+I showed the same characteristics of the C group with the addition of fibers with centralized nuclei in the fourth day (Fig. 2, first and second columns). When compared with control groups, SI presented smaller fibers. Fiber disorganization was observed in fascicles in the 4th and 14th days, as well as fibers with centralized nuclei. There are muscle fibers in the splitting process and fibrosis sites in both periods analyzed, as well as smaller fibers in the degenerating process. Insulin treatment (SI+I) presented characteristics similar to SI, except for centralized nuclei. Moreover, SI+I showed big rounded fibers in the 4th and 14th day (Fig. 2, third and fourth columns).

Regarding frequency distribution of cell profile areas, this morphometric analysis showed there were higher cell profile area peaks in C and C+I groups between 800 and 1.200 mm² intervals in the fourth day. In SI and SI+I groups, the cell profile area peaks presented 600 and 1.600 mm² intervals, showing that scald injury promotes higher muscle fiber area heterogeneity (Fig. 3A). In the 14th day, the highest value frequency for the C group was 1.800 mm² and, with insulin treatment (C+I), the interval was between 900 and 2.100 mm². SI presented cell profile area peak between 900 and 1.800 mm², and in the postinsulin treatment (SI+I), the cell profile area peak ranged between 1.200 and 2.100 mm² (Fig. 3B).

**Effects of insulin on myogenic regulatory factors: Pax7, MyoD, and MyoG**

Pax7 immunolabeling was detected in the muscle fiber nuclei. C and C+I groups presented weak immunolabeling in the 4th and 14th days; however, a strong reaction was detected in SI and SI+I groups (Fig. 4A). The analysis of the immunoreactive nucleus density showed that the number of immunoreactive nuclei/mm² in scalded injured animals (SI and SI+I) was statistically higher than control (C and C+I) groups, in both investigated periods (Fig. 4B).

MyoD immunolabeling was weak in the C and C+I groups. Conversely, strong immunolabeling occurred in the SI and SI+I
Fig. 2 – Muscle morphology. Transverse muscle sections of gastrocnemius obtained from rats after burn injury and insulin treatment for 4 and 14 d. In C and C + I, normal fibers with homogeneous size, polygonal format, and peripheral nuclei and arranged in equidistant fascicles. Note that C + I presented some fibers with centralized nuclei (thick arrows) 4 d after insulin treatment. SI and SI + I, in the fourth day showed difference in the distance between fibers and fascicles and sites of fibrosis (#) and fibers in the degeneration process (thin arrows). Note the splitting process (arrowhead) in SI after 4 and 14 d and in SI + I (in the 14th day). In addition, big rounded fibers in SI + I in both experimental days. HE staining; scale bar = 50 μm. (Color version of figure is available online.)

Fig. 3 – Frequency distribution of cell areas. Histogram of gastrocnemius cell profile area in the fourth (A) and 14th (B) days. In (A), C and C + I groups had highlighted area peaks between 800 and 1,200 μm², and in SI and SI + I groups, area peaks were between 600 and 1,600 μm². In (B), the C group presented highlighted area peak of 1,800 μm², and in C + I, the interval ranged between 900 and 2,100 μm². SI shows area peak between 900 and 1,800 μm², and SI + I between 1,200 and 2,100 μm².
groups, evaluated in the 4th and 14th days (Fig. 5A). MyoD immunoreactive nucleus density was higher in injured groups (SI and SI + I) than in controls (C and C + I), in both experimental days. Furthermore, in the 14th day, insulin-treated groups (C + I and SI + I) showed higher MyoD immunoreactive nucleus density than noninsulin-treated groups (C and SI) (Fig. 5B).

Regarding the MyoG protein, we observed the C + I group presented MyoG extravasation into the cytoplasm in the fourth day, forming sarcoplasmic vacuoles with this protein. Overall, SI, in the fourth day, and SI and SI + I, in the 14th day, showed stronger reaction than control groups (Fig. 6A). MyoG immunoreactive nucleus density was higher in SI and SI + I groups than controls (C and C + I) in the 4th and 14th days. Insulin-treated groups (C + I and SI + I) showed higher MyoG immunoreactive nucleus density than the respective controls (C and SI) in the fourth day. In the 14th day, only the density of the insulin-treated scalded injury group (SI + I) was higher than the respectively control, SI (Fig. 6B).

**Effects of insulin on genes related to muscle atrophy (MuRF1, MAFbx) and IGF-I**

In the analysis of atrophy-related genes, the MuRF1 gene expression in SI was higher than in C, and the insulin-treated
animals (SI + I) presented lower MuRF1 than SI in the fourth day. In the 14th day, scalded injured animals (SI + I and SI) showed higher values than C + I and C (Fig. 7A). For the MAFbx gene expression, we verified that the SI group presented a mean gene expression higher than the C group, and the C + I group had a mean gene expression higher than the C group in the fourth day. No difference was found in the MAFbx gene expression in the 14th day (Fig. 7B). In the analysis of IGF-1, scalded injured groups (SI + I and SI) showed higher gene expression when compared with the respective controls (C + I and C) in the fourth day. In the 14th day, SI and C + I groups presented higher gene expression than the C group (Fig. 7C).

Discussion

BIs represent an epidemiological problem for children worldwide. To ensure a proper care to patients who suffer from
extensive BIs, a good understanding of systemic responses is necessary, such as prolonged hypermetabolism, protein catabolism, metabolic and hormonal changes, among other consequences. Authors of recent clinical studies have been using treatments with anabolic hormones in the intensive care of these patients. Our research group found improvement in elastic-collagen rearrangement and reepithelization in short-term insulin treatment when comparing short- and long-term insulin treatments in burn-injured skin. However, in this study, we investigated the effects of insulin treatment on a muscle distant from extensive burn lesion. Regarding experimental studies on extensive BIs and intensive insulin therapy, most authors studied short-term therapy acting in the acute postburn phase, ranging from 1 to 5 d. Few

Fig. 6 – Effects of burn injury and insulin treatment on the expression of myogenic protein MyoG (image) and MyoG immunoreactive nucleus density (graph) in the 4th and 14th days. Note, in A, the weak MyoG immunohistochemistry reaction to C and C + I and a strong reaction to the SI and SI + I groups. In B, the MyoG immunoreactive nucleus density (immunoreactive nuclei/mm²) showed that scalded injured groups (SI and SI + I) were statistically higher than control groups (C and C + I groups) in the 4th and 14th days (* = P < 0.05). In the fourth day, insulin-treated animals (C + I and SI + I) showed density of MyoG immunoreactive nuclei was higher than their respective controls (C and SI). In the 14th day, only SI + I was higher than SI (# = P < 0.05). Arrows = MyoG immunostaining; asterisk = MyoG extravasation into the citoplasm; scale bar = 20 μm. (Color version of figure is available online.)
studies address long-term therapies, for 7,31,32,12,37, and 15 d.38 The results of these studies were based on serology blood tests. However, little is known about the anabolic effect of insulin on skeletal muscle after SI. Therefore, we evaluated the effects of short- and long-term intensive insulin therapy after extensive BIs, specifically on the skeletal muscle of young Wistar rats, by morphological, myogenic, and atrophy parameters.

In accordance with the main results, short-term insulin therapy (SI1) in the fourth day) increases insulin sensitivity in the acute phase after injury, favoring muscle regeneration by the appearance of myotubes and reduction in the profile areas of fiber and improvement in muscle atrophy due to decreased MuRF1 expression. Long-term insulin therapy (SI1 in the 14th day) improved myogenesis, as shown by the increased MyoD and MyoG proteins. In addition, insulin improved muscle growth, as observed by the increase in the peaks of profile areas of treated animals and of the IGF-1 expression. Both periods of insulin therapy showed improvement in histopathologic parameters, increased the formation of myotubes, and favored the secondary myogenesis, improving thus the acute period after burn, seen by the increase in MyoG.

Interestingly, after long-term treatment, the C+I group presented the highest mean glucose clearance time in the GTT analysis, in addition to having insulin resistance assessed by HOMA-IR. In this last analysis, this group also presented the highest value among all groups. An acute increase in the insulin level is stimulatory, but persistently high or long-lasting insulin levels desensitize the target cells through multiple mechanisms, including the decreased number of insulin receptors39 and the alteration or malfunction of the insulin postreceptor level, thus leading to deficit in glucose transport.40 Hence, the long-term insulin treatment for healthy uninjured animals seems to have a negative impact on insulin activity.

Regarding morphological parameters, the control group featured normal fibers with homogeneous size, polygonal format, and peripheral nuclei, arranged in equidistant fascicles. In studies on SI 45% TBSA in rats, injured animals presented rounded fibers with irregular contours and several sizes, with greater distance between degenerating fibers23,41 and inflammatory cell from transversally sectioned vessels with vascular congestion.42 Although the control groups had morphological characteristics of an uninjured tissue, the insulin treatment promoted the appearance of rounded myotubes in the cell profile. This finding shows the anabolic role of insulin in promoting the growth of new myofibers.

In the SI1 groups, the morphological evaluation showed more histopathological alterations in response to muscle remodeling because of anabolism provided by insulin therapy, in addition to the protein catabolism resulting from SI. This fact was more evident in short-term treatment, which promoted statistical difference in the reduction in cell profile. Corroborating our findings, homogeneity was observed between 4-d groups for the profile area frequency. However, this
was not observed in the 14-d groups, which presented smaller frequency peaks for C + I and SI groups than those of SI + I and C groups. Such difference can be justified by the presence of myotubes, observed in the histopathologic evaluation of C + I, and by fibers of several sizes in SI, in the 14th day. Interestingly, intensive insulin therapy was efficient, approaching the peaks of SI + I to C after 14 d.

In our study, we verified an increased immunoexpression of Pax7 and MyoD—regulators of primary myogenesis—in SI when compared with control groups, in both experimental periods. The insulin treatment did not influence the activation of satellite cells. However, long-term insulin therapy was sufficient to maintain the MyoD expression in the late period of muscle regeneration, thus showing its anabolic capacity. Insulin-treated groups (SI + I and C + I) had increased MyoD immunostaining when compared with noninsulin-treated groups, although the expression peaks occurred near the fourth day. According to Zammit et al.,43 MyoD regulates the activation of satellite cells that, when activated, leave the quiescent state cell to express the Pax7 protein. Then the coexpression of Pax7 and MyoD occurs in the nuclei; subsequently, the satellite cell proceeds to proliferation by Pax7 regulation. In our study, we observed that short- or long-term insulin therapy had a beneficial effect on the MyoG expression, increasing its immunolabeling in treated animals. Long-term insulin therapy kept the MyoG expression increased in the SI + I group, especially when compared with the SI group. MyoG acts based on the differentiation in myogenesis; hence, the greater expression occurs in late moments of muscle repair. Although characteristically expressed in the nuclei, the MyoG immunoexpression was observed in the cell cytoplasm of the control group with short-term insulin therapy (C + I in the fourth day). This extravasation usually occurs in atrophic fibers, much smaller than the others, forming vacuoles in the cytoplasm, which may show the anabolic influence of insulin on the increase in MyoG expression. When analyzing the muscle biopsy of adult patients with myositis, researchers observed the formation of sarcoplasmic vacuoles when the MyoG expression occurred in large quantities. This process usually occurs in areas with active regenerating fibers, fibrosis, and atrophic or hypertrophic fibers.12

Regarding IGF-1, we observed the injury has increased the expression of this hormone in both experimental periods (SI and SI in the 4th and 14th days). In individuals with severe BIs, there is an increased expression of this hormone because IGF-1 is recognized as a factor that coordinates muscle growth (promoting muscle hypertrophy) and improves the muscular regenerative capacity.45 In addition, muscles under protein degradation also show insulin-receptor substrate degradation, interfering in the intracellular signaling of insulin and IGF-1.46 Therefore, in muscles in which protein degradation, atrophy, IR, and consequent alterations in insulin or IGF-1 signaling occur, an increase in the expression of this hormone is justifiable. Consequently, long-term insulin for C + I increased the IGF-1 expression in comparison with control in the 14th day. Concomitantly, the C + I group also presented

---

**Fig. 8** – Differential response of muscle injury as a consequence of an extensive burn injury. Without insulin therapy parameters such as hyperglycemia, insulin resistance, morphological alterations and muscle atrophy markers (Murf1 and MAFbx) are elevated. In addition, primary and secondary myogenesis are reduced, and all these events result in muscle atrophy. Conversely, short- or long-term insulin therapy ameliorates the most part of these parameters. (Color version of figure is available online.)
insulin resistance because it showed the highest I value compared with all groups in this study.

In our study, SI leads to increased MuRF1 expression in the acute and late phase after injury. The MAFbx expression increased only in the acute postinjury phase, 4 d. The increased MuRF1 and MAFbx expression is related to muscle atrophy, that is, these proteins are responsible for changes in the protein balance of synthesis and degradation, thus inducing the loss of muscle mass.47 Short-term insulin therapy decreased the MuRF1 expression in injured animals (SI + I) compared with the injured and untreated ones (SI), ameliorating muscle atrophy. However, the treatment did not ameliorate the MAFbx expression, neither in the short-term (fourth day) nor in the long-term (14th day). Because the short-term insulin therapy was beneficial to the increase in MyoD expression in both periods, we can infer that the nonstatistical significance found in the MAFbx expression was influenced by the insulin therapy, as it was already promoting an increase in MRF, preventing its inhibition by MAFbx. According to Folleta et al.,48 the MAFbx atrophy mediator interacts with MRF, inhibiting MyoD and MyoG activity and consequently preventing the differentiation and degradation of myotubes. Authors of a study using ghrelin instead of insulin treatment for 24 h after BIs above 30% TBSA in rats16 verified that ghrelin inhibits burn-induced muscle protein breakdown via MuRF1 suppression, similar to our study in short-term insulin therapy. However, conversely, ghrelin was also able to suppress MAFbx. These data show the importance of developing novel and alternative therapies to control these proteins related to muscle atrophy in BIs.

Interestingly, the insulin therapy leads to the increase in the MAFbx expression for C + I when compared with C in the fourth day. In addition, it promoted an increment of Pax7 and MyoG and significantly increased MyoD. Because C + I in the 4-d group were in physiological state, without injury, they did not require a large increase in MRF. Perhaps, the increase in the MAFbx expression occurred to avoid intense MRF activity in a tissue that, even when growing, was not injured. In addition, MAFbx in interaction with MRF induces the negative regulation of muscle fiber size.49 Furthermore, we also observed in the C + I group, after 4 d, the presence of several myotubes, with small cell profile area, and in the long-term, peak frequency was smaller than in the other groups. It is important to highlight that all results of this study are applicable to male animals, despite clinical studies demonstrate sex differences in BI response. A study investigated the determinants of skeletal muscle mitochondrial respiratory capacity and functions in burned children and verified that girls exhibited a 23-30% lower capacity for respiration-coupled ATP production than boys.50 Another study, with adult burned patients, concluded that females have a significantly higher risk of 60-d mortality.51 Considering sex as an important biological variable to BI response, we would recommend mixed-sex animals studies to verifying possible differences in drug effects.

In spite of anabolic pharmacological therapies that have been used to attenuate hypermetabolism and protein catabolism, further studies are needed to explore the time and amount of insulin administered to prevent the pathophysiological effects of extensive BI. Henceforth, we can conclude that short-term insulin therapy promoted myocyte appearance, assessed by small area profile fibers, and decreased the MuRF1 expression, an important mediator of muscle atrophy. In the long-term, it maintained the primary myogenesis for a longer period, which was verified by the increased MyoD immunoexpression. In addition, both therapy periods improved secondary myogenesis and promoted the formation of myotubes (Fig. 8). Finally, each therapy period has its own benefits; however, if maintained for a long period, it exerts more positive effects on muscle tissue. Considering the mechanisms elucidated here, we would recommend that future studies incorporate an anticalcorticoid drug into insulin, to evaluate muscle parameters after an extensive BI.

Acknowledgment

This study was supported by FAPESP, Brazil (Fundação de Amparo à Pesquisa do Estado de São Paulo), process number 2014/11458-0.

Authors’ contributions: H.T.Q. and F.O. participated in performance of the research, data analysis, and writing the article. V.I.A.B. and M.C.L. contributed to performance of data analysis. H.K.M.A., L.L.S.M., and C.A.M.O. contributed to reviewing the article.

Disclosure

The authors reported no proprietary or commercial interest.

REFERENCES


