

Original Article

Protective role of melatonin on skeletal muscle injury in rats

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Received August 22, 2016; Accepted November 10, 2016; Epub January 15, 2017; Published January 30, 2017

Abstract: *Background:* The functional restoration of skeletal muscle after injury is highly affected by its regenerative response that requires the coordinated regulation of inflammation, energy metabolism, and growth. *Aim:* The aim of the present study was to investigate the possible protective role of melatonin on cellular functions and tissue homeostasis during muscular regeneration after skeletal muscle injury in rats. *Materials and Methods:* The study was conducted on 80 male Wister rats with an open blunt injury of the left soleus muscle. Rats were divided into 2 groups; Group I (melatonin treated group), 40 rats with left soleus muscle injury and received intra peritoneal (i.p) injection of 10 mg/kg body weight/ day of melatonin. Group II (untreated group) 40 rats with soleus muscle injury and received equivalent volumes of 4.5% ethanol i.p. 3.3 mL/kg/day. Subsequent observations were performed at day 1, 4, 7, and 14 after injury, including muscle strength assessment, melatonin receptor 1a expression, bcl-2-associated-X protein (Bax) level and histological imaging. *Results:* Melatonin treatment significantly increased the twitch force of the injured muscle at day 1, 4, 7, and 14. MT1a receptor mRNA in the injured muscle showed a significant up-regulation in the melatonin treated group at day 4, 7 and 14. Intra peritoneal daily injection of melatonin for 4, 7, and 14 days resulted in significant decrease in Bax level in the injured soleus muscle in comparison to the corresponding injured muscles of untreated group. Histological examination further elucidated the anti-apoptotic and the anti-inflammatory action of melatonin. *Conclusions:* These data supported the hypothesis that melatonin supports muscle restoration after muscle injury, via its anti-inflammatory and anti apoptotic effects and thus might represent an attractive adjuvant therapy to optimize muscle healing after injury.

Keywords: Skeletal muscle injury, melatonin, skeletal muscle regeneration

Introduction

Musculoskeletal trauma is one of the most common causes of pain and impaired function in sports worldwide [1]. Injuries to the skeletal muscle occur during contact sports, prolonged exercise, or even high-velocity crashes [2].

It is estimated that muscle injuries represent approximately 30-67% of athletic injuries [1]. Depending on the injury mechanism, muscle trauma can be subdivided into strain injuries, contusions, or lacerations [3]. Muscle injury represents a serious factor limiting the therapeutic outcome in the fields of trauma surgery, plastic surgery and orthopedics [4]. Injuries to the musculoskeletal apparatus are difficult to treat and often associated with pain, discomfort, reduced mobility, and limited quality of life.

Thus, muscle injuries do have a high impact on the medical system and need to be analyzed [5]. According to current therapeutic strategies and daily clinical practice, muscle injuries predominantly heal with residual deficits since till now no causal treatment options exist [3]. As a result, complications such as enduring pain, impaired functionality and atrophy are often encountered. A crucial requirement for the development of novel therapeutic strategies after muscle injuries is to understand how muscle regeneration is regulated and how muscle loss occurs [4]. Therapeutic approaches to enhance recovery from muscle injury focus on strategies to limit necrosis, reduce fibrosis, and suppress excessive inflammation of the injured tissue. Therefore, non-steroidal anti-inflammatory drugs are widely used in daily clinical practice and are combined with rest and cooling

therapies. However, dependent on the severity of trauma, these therapeutic options cannot fully restore muscle function [2]. Several groups have attempted to improve muscle recovery using a variety of drugs, applying cytokines, transplanting cells or even modifying genes in the muscle [6].

Skeletal muscle has a great plasticity and unique ability to reconstruct in response to injury [7].

Once trauma of the contractile apparatus happens, ultra structural changes in the tissue and myofiber disruption take place, which induce a local inflammatory response [8]. The basic sequence of cellular events during regeneration after major injury is associated with a primary inflammation phase that is followed by cell proliferation and apoptosis, and is finally completed with scar tissue formation [6]. Consequently, the extent of regeneration is reflected at later points in time by the functional restoration of the muscle as well as by the muscle force production [5].

The skeletal muscle regenerative response requires the coordinated regulation of inflammation, energy metabolism, and myofiber growth [9]. The myofibers are physiologically crucial cells in the muscle tissue and are essential for the restoration of the injured tissue together with the satellite cells [10]. Under normal conditions, tissue repair is mediated by a variety of growth factors and cytokines and can be boosted by numerous anabolic substances and endogenous polypeptides [5].

The regeneration occurs in two overlapping stages: myolysis and reconstruction. Myolysis phase encompasses active muscle degeneration and inflammation processes that occur in the first few days post-injury. Numerous macrophages and leukocytes gather at the site of injury and phagocytose fragments of necrotic cells [10, 11]. The reconstruction of muscle relies on the pool of tissue undifferentiated myogenic precursor cells and satellite cells (mononuclear myoblasts) [12]. In the intact muscle, satellite cells remain mitotically quiescent, but become activated in response to muscle injury, denervation, stretching or exercise. Upon activation, the satellite cells res-

ume proliferation. After several cell cycles the majority of these cells start to differentiate and fuse to form new myofibers or to repair damaged ones [13].

Melatonin (N-acetyl-5-methoxytryptamine) is an indolamine originally isolated from bovine pineal tissue. However, this melatonin is also produced in multiple, perhaps all, cells and organs in all organisms of the plant [14] and animal kingdoms as well as in humans [15].

Many biological functions of melatonin have been identified. It influences circadian rhythms, sleep-wake cycle, tumor growth inhibition, and immune function and provides antioxidant protection and redox homeostasis in tissues [16]. Ubiquitously distributed melatonin receptors, MT1 and MT2, belong to the family of G-protein-coupled receptors [17] and their binding sites (putative receptors) have also been found in the nuclei of many cell types [18].

Melatonin also binds to calmodulin, which assists in regulating intracellular events such as nitric oxide synthase (NOS) activity [19]. Although the beneficial effects of melatonin can be traced back to antioxidative properties, improved microcirculation, and amplified tissue protective effects [5], little is known about the action of melatonin on the injured skeletal muscle.

Because of the poor study results and application, the field of muscle injury and regeneration requires further investigations. Until now, many scientific efforts have focused on the identification of new substances, which can significantly improve muscle regeneration.

Therefore the present study aimed to investigate the possible protective role of melatonin injection on cellular functions and tissue homeostasis during muscular regeneration after open blunt injury of soleus muscle in rats.

Material and methods

Animals and experimental groups

The study was carried out on 80 male Sprague Dawley rats with body weight of 250-300 g. Animals were housed under the same environmental conditions with 12-h light-dark cycles, at $22 \pm 2^\circ\text{C}$ with free access to standard rat

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chow and water. All experimental procedures were carried out based on the ethical guidelines for care and use of laboratory animals of Alexandria University.

Melatonin preparation

Melatonin (M5250; Sigma-Aldrich) was dissolved firstly in pure ethanol and later diluted with isotonic sodium chloride (0.9% NaCl) with a final concentration of 4.5% [5].

Anesthesia and induction of injury

Rats were anesthetized with ketamine hydrochloride (30 mg/kg IM) and xylazine hydrochloride (5 mg/kg IM), and then the left lower limb was shaved and disinfected with povidone-iodine. The soleus muscle was mobilized through a 2-cm posterolateral longitudinal incision of the skin and the underlying fascia from the lateral gastrocnemius head to the Achilles tendon. A blunt injury was induced on the left soleus muscle via an instrumented clamp (area of contact between muscle tissue and clamp: 10 mm²), which allowed a standardized force application of 25 N for 10 s. The muscle was manually clamped seven times throughout its complete length (total crushed area: 70 mm²) with the exception of the entrance of the neurovascular structures that are located near the midpoint of the medial gastrocnemius [20]. After induction of the blunt injuries, the superficial muscles and skin were closed. Rats were divided into 2 groups, each of 40 rats: Group I (n = 40): Animals underwent crush injury in the left soleus muscle but not in the right soleus muscle, and received i.p. injection of melatonin (10 mg/kg body weight/day) immediately after injury. Group II (n = 40): Animals underwent crush injury in the left soleus muscle, but not in the right soleus muscle, and received equivalent volumes of 4.5% ethanol i.p. (3.3 mL/kg body weight/day) immediately after injury.

All animals tolerated the experimental procedure well. The usage of the hind limb was reduced in the first day but started to get better in the 2nd or the 3rd day after injury. No other manifest signs of pain or discomfort. Subsequent observations were performed at day 1, 4, 7, and 14 after injury. Analysis was performed on the injured left soleus muscle as well as on the healthy right soleus muscle.

Assessments

Muscle strength assessment

For *in vivo* assessment of muscle strength, animals were re-anesthetized at day 1, 4, 7, and 14 post-injury (10 rats for each time point). After bilateral exposure of the sciatic nerve and the soleus muscle, the Achilles tendon was cut and the lower extremity was fixed into the muscle force-measuring device. The sciatic nerve was subsequently stimulated with 9 mA/75 Hz [5].

Contraction forces under fast twitch were analyzed by calculating the mean of the maximal values from first ten consecutive contractions and given as percentage of the corresponding values of the contralateral non-injured right muscle.

After completion of muscle strength measurements, experiments were terminated by dissection of the injured left soleus muscle for subsequent histological and biochemical studies.

Histological assessment

The left soleus muscles were dissected from rats of all groups. All samples were fixed in 10% formal saline. Each specimen was then processed to get 6 µm thick paraffin longitudinal sections to be stained with Haematoxylin & Eosin (H&E) stain for light microscopic examination [21].

Biochemical tests

Melatonin 1a receptor (MT1a) mRNA expression was detected by reverse transcriptase polymerase chain reaction (RT-PCR) [22]. About 30 mg soleus muscle tissues were stored at -80°C in lysis buffer containing guanidium-thiocyanate and β-mercaptoethanol for RNA extraction. Total RNA was extracted and purified from homogenized soleus muscle tissue using the "RNeasy Fibrous tissue mini kit" (Qiagen, Hilden, Germany) including the DNA digestion step. The concentration of extracted RNA was measured spectrophotometrically at 260 nm. Melatonin 1a receptor (MT1a) PCR synthesized only one amplification product of 66 bp length (Primer pair: forward CAGTACG-ACCCCGGATCTA, reverse GGCAATCGTGACG-CCG).

Rat BAX (BCL-2 Associated X Protein) level was measured by ELISA technique using (Elabscience Product ID: E-EL-R0098; Detection Range: 0.156~10 ng/mL; Sensitivity: 0.094 ng/mL) [23]. Levels of soleus muscle tissue Bax protein were expressed in ng/mg. The total protein concentration in all samples was measured using the Lowry method [24].

Statistical analysis

The obtained results were expressed as mean \pm standard deviation (SD). Differences between melatonin treated and vehicle group for muscle strength data were assessed using Paired t-test. Other presented results were statistically analyzed as followed: comparison between melatonin treated and untreated groups were done using Student t-test, comparisons among the different periods of the study were done using analysis of variance (ANOVA; F test) and comparisons between periods were done using adjusted Bonferroni Post Hoc Test. Values for $P \leq 0.05$ were considered statistically significant. Statistical analyses were carried out using the Statistical Package of Social Sciences (SPSS) computer program version 20.0 for Windows.

Results

Muscle strength assessment

All animals tolerated the experimental procedure well. The usage of the left hind limb was reduced only in the first day, but started to get better in the 2nd or the 3rd day after injury. No other manifest signs of pain or discomfort were recorded.

In vivo assessment of muscle contraction force by calculating the mean of the maximal values from first ten consecutive contractions, revealed that crushed injury applied to left soleus muscles resulted in significant decrease in their contraction forces compared with that obtained from right uncrushed muscles in both groups at all different time points (day 1, 4, 7, and 14 post-injury, **Table 1**).

Melatonin treatment caused no significant change on the contraction forces of the uninjured right soleus muscles in group I when compared to that obtained from group II at all different time intervals ($P > 0.05$). On the contrary,

Melatonin caused significant increase in the contraction forces of the injured left soleus muscles compared to that recorded from the injured untreated left soleus muscles among the assessed time intervals (day 1, 4, 7, and 14 post-injury) ($P < 0.001$, **Table 2**).

In Group I (injured melatonin treated group), the twitch force obtained from the injured left soleus muscle was significantly decreased 1 day after injury, which was only 20% of that obtained from the contralateral non-injured right muscle. With time, melatonin treatment resulted in significant increase in the percentage of change of the twitch force in day 4 and 7, reaching 34% and 59%, respectively. Moreover, daily treatment with melatonin for 14 days after the applied injury caused a significant improvement in contraction force forming about 74% of the contralateral muscle ($P < 0.001$, **Table 2**).

Similarly, the twitch force in the untreated group showed significant reduction when compared to that of the uninjured contralateral muscle along time, for example, at day 14, the twitch force was 35% of the uninjured contralateral muscle force, compared to 74% in the treated group. The percentage of change was shown to be significantly higher in treated groups than that in the untreated ones at day 1, 4, 7 and 14 after injury ($P < 0.001$, **Figure 1**).

Melatonin 1a receptor (MT1a)

The improvement of the force of contraction with the use of melatonin was further confirmed through the analysis of the mRNA expression of melatonin 1a receptor (MT1a) in the muscles by means of RT-PCR as shown in **Table 3**. Results revealed significant increased expression of MT1a in the melatonin treated injured muscles when compared to the receptors expressions in the non-injured contralateral muscles through all examined time intervals ($P < 0.001$).

The melatonin 1a receptors mRNA expression in the injured treated muscles was increased with time. It significantly increased at 4, 7 and 14 days after melatonin injection compared to its expression after a day of injection ($P < 0.001$). Also, the receptors expression was significantly increased after 7 and 14 days of daily treatment compared with the expression after 4

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Table 1. Comparison between the two groups according to Muscle twitches (contraction) (all results $\times 10^{-2}$)

Muscle twitches	Treated			Un Treated		
	Normal	Crushed	P	Normal	Crushed	P
Day 1	103.1 \pm 8.82	20.80 \pm 2.04	<0.001*	107.2 \pm 9.84	16.80 \pm 2.49	<0.001*
Day 4	103.1 \pm 8.33	35.50 \pm 3.81	<0.001*	105.9 \pm 10.1	21.80 \pm 2.70	<0.001*
Day 7	106.7 \pm 10.2	64.60 \pm 5.36	<0.001*	106.9 \pm 13.6	33.20 \pm 5.09	<0.001*
Day 14	108.4 \pm 12.3	80.30 \pm 10.9	<0.001*	104.1 \pm 11.6	35.50 \pm 4.77	<0.001*

P: p value for Paired t-test for comparing between treated and untreated. *: Statistically significant at $P \leq 0.05$.

Table 2. Comparison between the two groups according to Muscle twitches for calculating the percentage of change (all results $\times 10^{-2}$ except the %)

Muscle twitches	Day 1	Day 4	Day 7	Day 14	p_1
Normal					
Treated	103.10 \pm 8.82	103.10 \pm 8.33	106.70 \pm 10.18	108.40 \pm 12.29	0.610
Un Treated	107.20 \pm 9.84	105.90 \pm 10.08	106.90 \pm 13.57	104.10 \pm 11.56	0.964
P	0.340	0.507	0.971	0.431	
Crushed					
Treated	20.80 \pm 2.04	35.50 ^a \pm 3.81	64.60 ^{a,b} \pm 5.36	80.30 ^{a,b,c} \pm 10.9	<0.001*
Un Treated	16.80 \pm 2.49	21.80 ^a \pm 2.70	33.20 ^{a,b} \pm 5.09	35.50 ^{a,b} \pm 4.77	<0.001*
P	0.001*	<0.001*	<0.001*	<0.001*	
% of Change					
Treated	20.2 \pm 1.5	34.2 ^a \pm 3.0	59.5 ^{a,b} \pm 2.3	74.0 ^{a,b,c} \pm 3.6	<0.001*
Un Treated	15.6 \pm 1.3	20.6 ^a \pm 2.1	31.1 ^{a,b} \pm 2.6	34.6 ^{a,b} \pm 4.9	<0.001*
P	<0.001*	<0.001*	<0.001*	<0.001*	

P: p value for Student t-test for comparing between treated and untreated. p_1 : p-value for ANOVA with repeated measures for comparison between the different periods. Sig bet. periods was done using adjusted Bonferroni Post Hoc Test. a: Significant with Day 1. b: Significant with Day 4. c: Significant with Day 7. *: Statistically significant at $P \leq 0.05$.

days treatment. There was increased MT1a mRNA expression in the injured muscles after 14 days of treatment when compared with its expression after 7 days of treatment but this increase was not statistically significant.

Rat BAX (BCL-2 Associated X Protein)

To investigate apoptosis in the injured muscle, the pro apoptotic marker, Bax was measured by ELISA technique. Results showed a significant increase in Bax concentration in injured muscles throughout the studied time intervals; it is significantly increased in the injured untreated group in day 4, 7, and 14 compared to the detected concentration one day after the applied injury ($P < 0.001$).

Intra peritoneal daily injection of melatonin in group I for 4, 7, and 14 days resulted in significant decrease in the concentration of Bax in the injured soleus muscle as compared to its

concentration detected in the corresponding injured muscles of group II with the same time intervals ($P < 0.001$, **Table 4**).

With time, melatonin resulted in decrease Bax concentration in the injured muscles of group I. Statistical analysis confirmed significant decrease in its level at day 4 compared to day 1 and at days 7 and 14 compared to days 1 and 4 with melatonin administration. ($P < 0.001$, **Table 4**).

However, no significant difference was observed in the concentration of Bax when melatonin administered for 14 days after application of muscle injury compared to that detected in rats with 7 days treatment after application of injury (**Table 4**).

Histological assessment

To further elucidate the anti-apoptotic action of melatonin and to assess its anti-inflammatory

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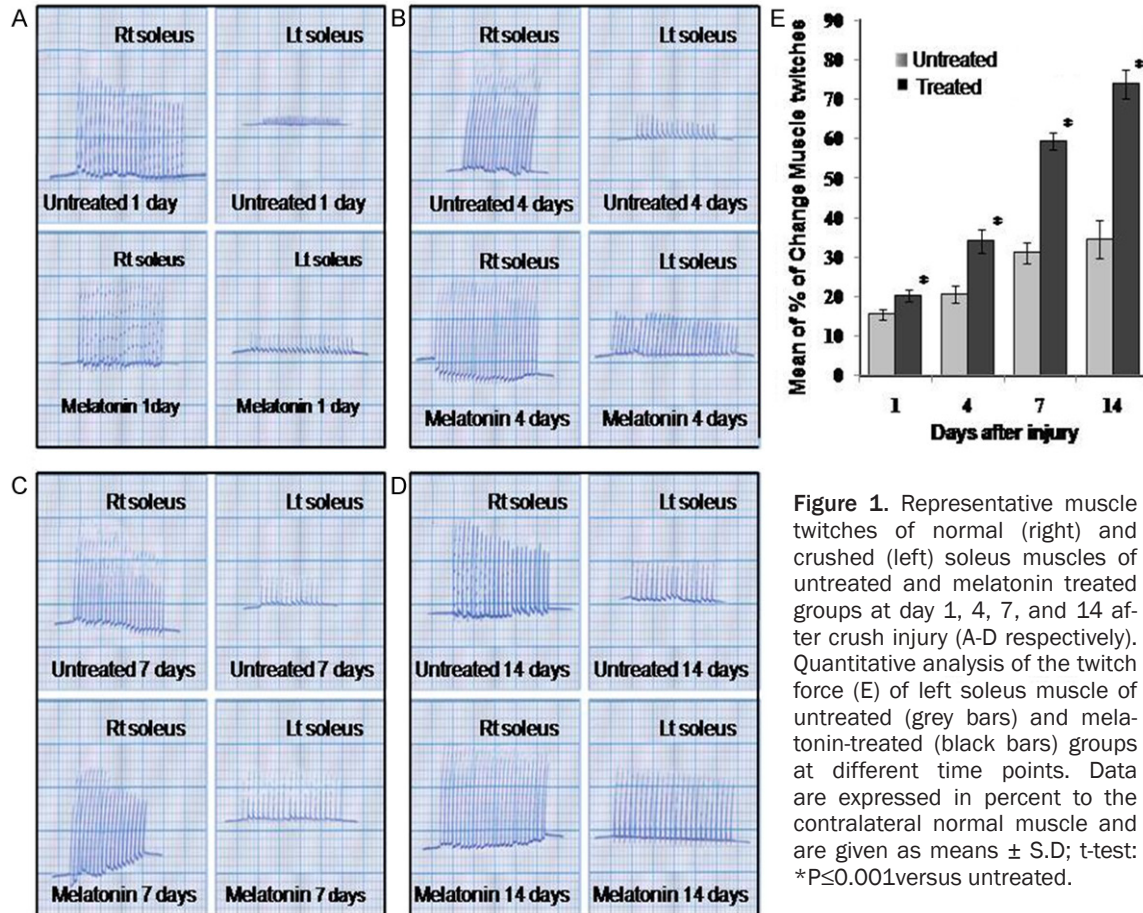


Figure 1. Representative muscle twitches of normal (right) and crushed (left) soleus muscles of untreated and melatonin treated groups at day 1, 4, 7, and 14 after crush injury (A-D respectively). Quantitative analysis of the twitch force (E) of left soleus muscle of untreated (grey bars) and melatonin-treated (black bars) groups at different time points. Data are expressed in percent to the contralateral normal muscle and are given as means \pm S.D; t-test: * $P \leq 0.001$ versus untreated.

Table 3. Comparison between the two groups according to results of melatonin receptor 1a

Melatonin rec 1a	Day 1	Day 4	Day 7	Day 14	p_1
Normal					
Treated	1.41 \pm 0.06	1.43 \pm 0.07	1.45 \pm 0.07	1.44 \pm 0.07	0.681
Un Treated	1.43 \pm 0.05	1.41 \pm 0.07	1.47 \pm 0.09	1.45 \pm 0.07	0.176
P	0.550	0.392	0.490	0.664	
Crushed					
Treated	2.43 \pm 0.08	6.89 ^a \pm 0.16	7.94 ^{a,b} \pm 0.76	8.05 ^{a,b} \pm 0.92	<0.001*
Un Treated	1.41 \pm 0.09	1.39 \pm 0.07	1.43 \pm 0.07	1.45 \pm 0.08	0.444
P	<0.001*	<0.001*	<0.001*	<0.001*	

P: *p* value for Student t-test for comparing between treated and untreated. p_1 : *p*-value for ANOVA with repeated measures for comparison between the different periods. Sig bet. periods was done using adjusted Bonferroni Post Hoc Test. a: Significant with Day 1. b: Significant with Day 4. *: Statistically significant at $P \leq 0.05$.

effect, histological examination of longitudinal sections of normal right soleus muscle of rats in both groups showed classical appearance of the muscle fibers. They appeared as long cylindrical fibers and arranged in a parallel pattern. The transversely striated appearance formed of alternating light and dark bands was evident. Also, muscle fibers showed multiple

nuclei that appeared flat and peripheral (**Figure 2**).

Examination of the H&E stained longitudinal sections after one day of application of blunt crush trauma to the left soleus muscles of rats of untreated group revealed evident histological changes. Muscle fibers appeared pale and

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Table 4. Comparison between the two groups according to results of results of Bax

Bax	Day 1	Day 4	Day 7	Day 14	p ₁
Normal					
Treated	0.67 ± 0.04	0.63 ± 0.05	0.64 ± 0.03	0.62 ± 0.06	0.122
Un Treated	0.65 ± 0.05	0.66 ± 0.05	0.67 ± 0.06	0.66 ± 0.05	0.796
P	0.370	0.171	0.250	0.119	
Crushed					
Treated	1.49 ± 0.48	0.91 ^a ± 0.11	0.71 ^{a,b} ± 0.09	0.70 ^{a,b} ± 0.07	<0.001*
Un Treated	1.64 ± 0.47	2.23 ^a ± 0.32	2.79 ^{a,b} ± 0.31	3.38 ^{a,b,c} ± 0.04	<0.001*
P	0.480	<0.001*	<0.001*	<0.001*	

P: p value for Student t-test for comparing between treated and untreated. p₁: p-value for ANOVA with repeated measures for comparison between the different periods. Sig bet. periods was done using adjusted Bonferroni Post Hoc Test. a: Significant with Day 1. b: Significant with Day 4. c: Significant with Day 7. *: Statistically significant at P≤0.05.

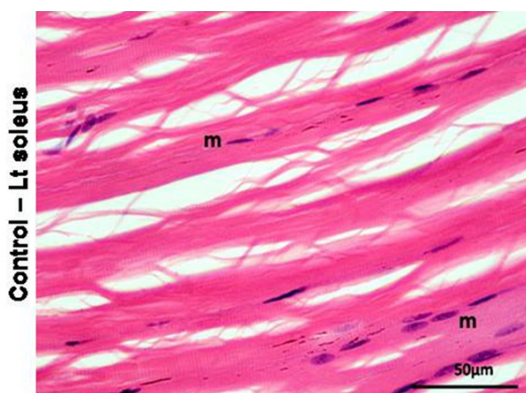


Figure 2. Light microscopic picture of L.S of skeletal muscle fiber of uncrushed right soleus (control) showing well-organized parallel muscle fibers (m) with evident transversely striated pattern. Mic Magx400A.

swollen with loss of striation. Evident vascular congestion together with inflammatory cellular infiltration was seen in between muscle fibers. Also, disrupted irregular fibers were noticed (**Figure 3A**). In Melatonin treated group for one day after injury, variable degrees of histological changes were shown. Some of the muscle fibers revealed almost classical histological appearance of straight, regular and parallel arrangement, some other appeared severely disrupted. Limited inflammatory cellular infiltration was also seen (**Figure 3B**).

Four days after trauma the longitudinal sections in soleus muscle fibers of untreated group showed evident histological changes in most of the muscle fibers. Many muscle fibers appeared disrupted and irregularly arranged. Myoblasts with single central nucleus were seen. Moreover, widening of the interstitial spaces was evident. Also, apparent vascular conges-

tion and limited inflammatory cellular infiltration were noticed (**Figure 3C**). On the other hand, evident amelioration of histological changes was noticed after the same interval of melatonin treatment, where many areas showed well arranged muscle fibers with apparent striations. Also, a minimal area of inflammatory cellular infiltration and vascular congestion was noticed (**Figure 3D**).

In Untreated group, 7 days following the injury of left soleus muscles, histological examination of their longitudinal sections showed irregular arrangement of muscle fibers. A myoblast with single central nucleus was seen. Minimal areas of inflammatory cellular infiltration and vascular congestion were noticed (**Figure 4A**). The histological changes were again improved 7 days after the daily melatonin injections from the blunt trauma, where muscle fibers appeared mostly regularly arranged with limited areas of irregular appearance (**Figure 4B**).

On day 14 histological examination of longitudinal sections in skeletal muscle fibers of the untreated group did not show more changes compared to that appeared on day 7. It showed some areas with markedly irregular muscle fibers. Also, some vascular congestion was noticed (**Figure 4C**). On contrary, melatonin treated group revealed muscle fibers with almost control pattern and minimal lateral branches after 14 days (**Figure 4D**).

Discussion

The skeletal muscles have the power to recover from injury in all living organisms [25]. The reduction of contractile elements with subse-

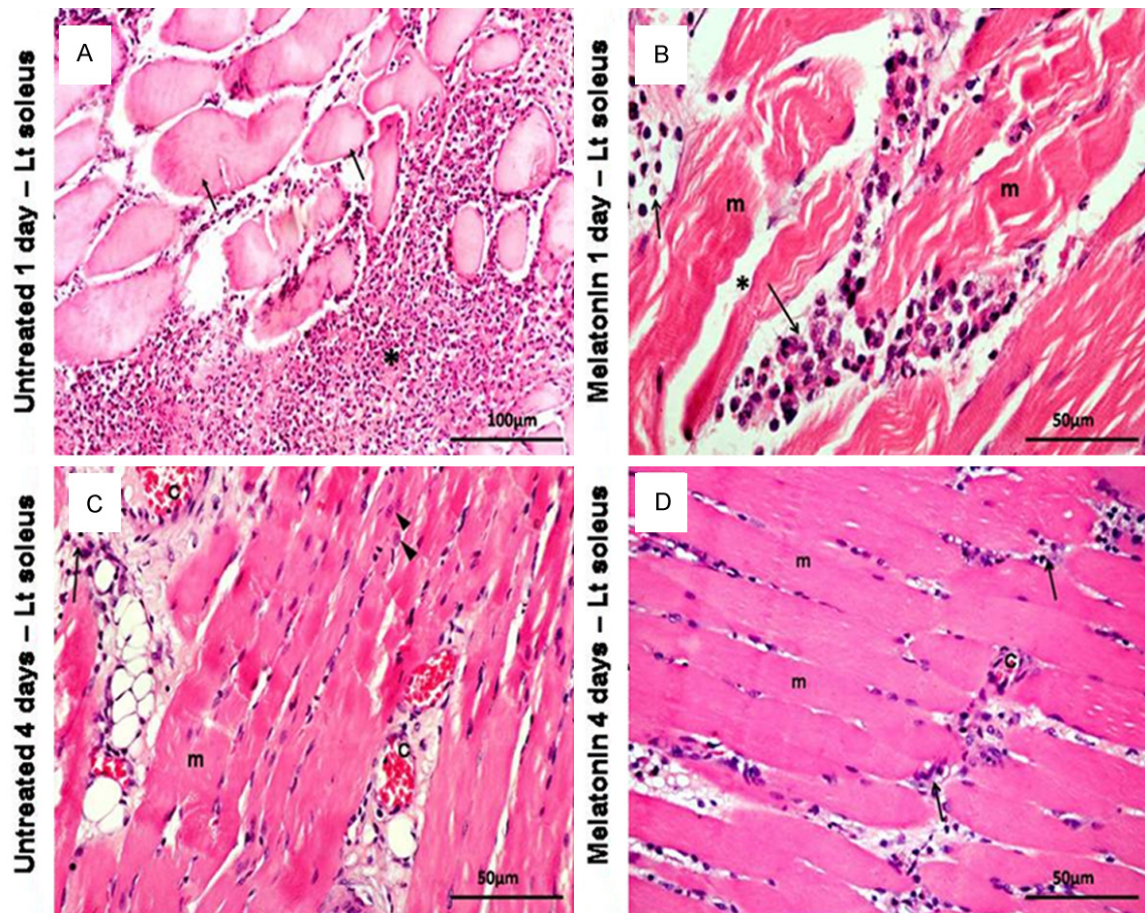


Figure 3. Light microscopic picture of L.S of skeletal muscle fiber (A) untreated group 1d, showing swollen and pale muscle fibers (arrows) with loss of the transversely striated appearance. Massive inflammatory cellular infiltration (astrix) is also noticed. (B) Melatonin treated group 1d, revealing disrupted muscle fibers (m). Widening of interstitium is noticed (astrix), together with inflammatory cellular infiltration (arrows). (C) Untreated group 4d group, revealing evident vascular congestion (c). Limited inflammatory cellular infiltration (arrows) is noticed. Muscle fibers (m) show loss of the striated appearance. (D) Melatonin treated group 4d, showing regularly arranged muscle fibers (m) with no apparent striation. Minimal inflammatory cellular infiltration (arrows) is apparent. (c) Vascular congestion. Mic. Mag. X400.

quent scar formation, and fatty muscle degeneration are the challenges in regaining functional recovery following muscle injury. Also, the change in muscle fibers arrangement contributes to limited muscle function. So, regeneration aims at restoring both, structural and functional alteration [26].

The treatment of muscle injuries after trauma, surgery and in sports medicine remains an unsolved problem. So, there is a need to discover new successful therapeutic strategies for skeletal muscle regeneration based on a better understanding of the mechanisms of muscle injury.

The primary objective of this study was to provide evidence that melatonin improves mu-

scle healing following blunt skeletal muscle injury, by using the open crush injury of the soleus muscle while preserving the central neurovascular structure. This allows new blood vessels formation after injury and prevents ischemic necrosis of the traumatized muscle. Also, the muscle fibers and interstitial tissue were selectively affected without harming the main innervations. This would avoid an initial complete denervation, which could make it impossible to differentiate between the consequences of denervation and myofiber trauma.

Melatonin is known to have good pharmacokinetic properties with low toxicity. It can be used as a nontoxic therapeutic agent and a safe molecule for systemic application [27]. This study demonstrated that treatment with melatonin

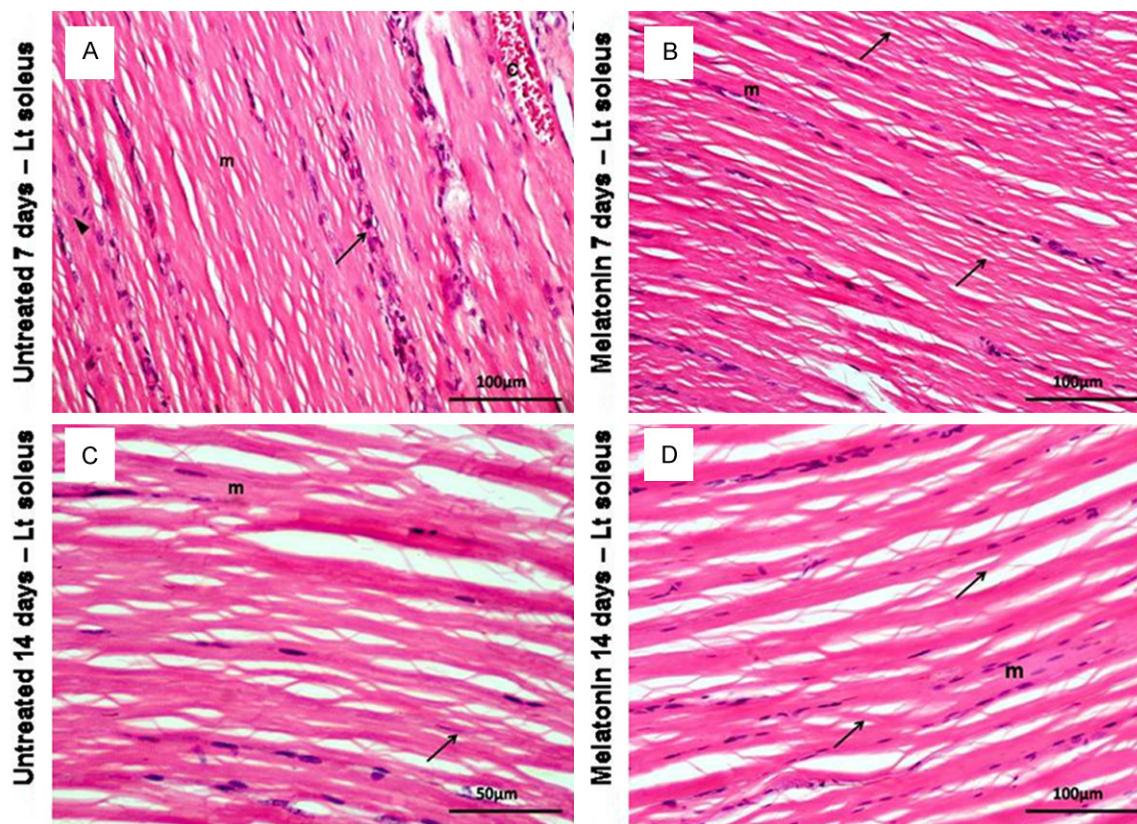


Figure 4. Light microscopic picture of L.S of skeletal muscle fibers (A) untreated group 7d, showing muscle fibers (m) with irregular arrangement. Myoblast (arrowhead) is seen with a single central nucleus. Minimal inflammatory cellular infiltration (arrows) is apparent, (c) vascular congestion. (B) Melatonin treated group 7d, showing well organized muscle fibers (m). Disorganized muscle fibers (arrows) are also noticed. (C) Untreated group 14d, revealing straight parallel fibers (m). Laterally branching fibers (arrows) are noticed. D. Melatonin treated group 14d, revealing some muscle fibers with regular arrangement and evident striation (m). Mic. Mag. X400.

enhances the biomechanical functions of the injured soleus muscle by improving the force of contraction after the applied crush injury that had greatest functional impact on the muscle contraction. These findings were associated with improvement of histological arrangement of myofibers, resolution of vascular congestion, reduction of the leukocyte infiltration and signs of inflammation together with the appearance of myoblasts in the muscles.

It is known that, besides a wide range of functions, melatonin has a beneficial effect on the oxidative stress, by acting directly as a free radical scavenger and, indirectly, by enhancing the production of antioxidant enzymes and the efficiency of mitochondrial electron transport chain [28, 29].

In accordance with our findings, variety of studies showed the protective effects of melatonin

on the inflammatory responses of different organs. Veneroso C *et al* [30] reported that melatonin decreases cardiac inflammatory injury following acute exercise through downregulation of the NF-kappaB signal transduction pathway and impaired production of pro-inflammatory mediators. In addition, its therapeutic potential in sepsis was investigated by Wu JY *et al* [31] who concluded that melatonin prevents organ failure during sepsis by its anti-oxidative and anti-inflammatory properties. These involve the inhibition of pro-inflammatory cytokines, the reduction in free radicals as well as the decrease in tissue-infiltrating leukocytes. Furthermore, melatonin treatment was shown to normalize plasma pro-inflammatory cytokines and markers of muscle injury in patients suffering from Duchenne muscular dystrophy reducing subsequently the muscle degenerative process [32]. On the other hand, Beck WR *et al* [33] reported that melatonin increased the perfor-

mance but also the inflammation and damage to skeletal muscle tissue with exhaustive swimming exercise in adult rats. This suggests that the high ergogenic effect of melatonin presented by longer periods of muscle contraction is significantly stronger than its protective effect with respect to long duration exercise such as marathon competitions.

Application of the blunt injury to the rat soleus muscles resulted in the increased expression of the pro-apoptotic protein Bax that signs for starting of muscle apoptosis. Melatonin resulted in significant decrease in Bax after 1 day of treatment, and continued to decrease its expression with continued treatment for 7 days. However, 14 days of continued treatment did not affect the level of bax any more. This confirms the anti-apoptotic action of melatonin which is initiated early after injury.

The anti-apoptotic effect of melatonin is consistent with the observations demonstrating that melatonin prevents cardiac [34] and skeletal muscle [35] apoptosis after ischemia/reperfusion injury. We further identified the mRNA of MT1a receptor in the injured muscle and showed an up-regulation of the MT1a mRNA after melatonin application at day 4 and continued with treatment. The presence of the melatonin receptor in injured muscle suggests that melatonin can act directly on cell populations in the injured muscles besides its systemic effects that may indirectly support the restoration of injured muscle. Statros *et al* [5] demonstrated increase mRNA of MT1a receptor in the injured muscle following blunt injury. This supports its role in mediating melatonin effect in restoration of the contractile function and regenerative capacity of injured muscle most probably via modulation of apoptosis-associated signaling pathways.

Experiments and measurements of cell lines and organs proved that melatonin reduces cell apoptosis by activation of the serine/threonine protein kinase (Akt/PKB) pathway and the phosphorylation of the glycogen synthase kinase 3 α/β (GSK3 α/β) in human neuroblastoma cells [36]. Furthermore, activation of the survival-promoting pathway of extracellular signal-regulated kinase, mitogen-activated protein kinase (ERK MAPK) mediates the anti-apoptotic signaling of melatonin in human leukocytes irradiated with UVB light as a model of stress-

induced apoptosis [37]. These pathways are all present in skeletal muscle and play a major role in muscle physiology and muscle regeneration [38, 39].

Current data support the hypothesis that melatonin hormone exerts regenerative capacity in injured muscle most probably via its anti-inflammatory and anti apoptotic effects and thus, overall, melatonin might represent an attractive adjuvant therapy to optimize muscle healing after injury.

Acknowledgements

The authors wish to thank the University of Alexandria for the unlimited support. The study was formally approved by the Ethical Committee of the Faculty of Medicine, Alexandria University.

Disclosure of conflict of interest

None.

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