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ORIGINAL ARTICLE • LIMBS - TRAUMAS

Fast and slow myosin as markers of muscle regeneration in mangled extremities: a pilot study

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Abstract
Mangled extremities were classically managed by amputation. But over the past few decades, with the advancement in surgical techniques, an increased number of limb salvages have been possible. As muscles usually get damaged in such grievous injuries, a thorough understanding of muscle regeneration may give a better insight into muscle healing in these injuries. Muscles are composed of slow and fast fibers which can be represented by slow and fast myosin, respectively. There are some animal studies which reported differential regeneration of slow and fast muscle fibers during muscle healing. We conducted this pilot study to find out whether the same holds true for muscle healing in mangled extremities also. This pilot study is designed in 15 patients with lower limb mangled extremities presenting to trauma center of PGIMER, Chandigarh, who were operated within 24 h of injury to see whether muscle healing in mangled extremities follows the same pattern. Biopsies were taken during initial surgery conducted within 24 h of injury and on the 7th day of injury when patient was posted again for secondary wound closure procedure or revision amputation. The biopsy samples were subjected to histopathological and immunohistochemistry examination using antibodies against fast and slow myosin. We found that the regenerating muscle fibers in the biopsy sample taken on the 7th day of injury showed only slow muscle fibers with the absence of fast muscle fibers when compared with the initial biopsy results showing differential regeneration of slow muscle fibers.

Keywords Mangled extremity · Slow myosin · Fast myosin · Immunohistochemistry · Antibodies · Differential regeneration

Introduction

A mangled extremity is by definition an extremity with a significant injury to at least three out of five major tissue groups, i.e., skin, soft tissue, nerves, vessels and bone. In the past, mangled extremities were associated with high rates of amputation. With the recent advances in the management of these groups of patients, there is a decreasing trend in amputation and limb salvage approach can be adopted more often [1]. The critical decision depends on correctly identifying when to conduct amputation or limb salvage, as many patients whose limbs were salvaged primarily may undergo secondary amputation which causes great physical, psychological, financial and social distress to the patient. Again even after amputation, many patients require debridement or revision amputation or may have problems in healing of amputation stump in postoperative period. A thorough understanding of the healing process in these injuries is needed. As muscles form the major bulk of the limbs and usually get damaged and crushed in such injuries, the potential and pattern of healing of these injured muscles become an important predictor for overall wound healing and postoperative course. Therefore, there is a need to better accustom with the muscle healing pattern and its outcome after such injuries. A lot of research work is going at cellular level dealing with different aspects of muscle healing. In this study, we have attempted to find out whether there is any differential regeneration of fast and slow muscle fibers in mangled extremities following amputation.

Skeletal muscles constitute slow (type I) and fast (type II) fibers. The proportion of these fibers is different for the variety of muscle and even depending on the part of a muscle [2]. Depending on the specific fiber type, the contractile

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proteins have different isoforms. Among these proteins, myosin is the most essential contractile protein. Thick filaments are primarily made up of myosin, and according to the fiber type, i.e., fast or slow, the myosin molecule is made up of two heavy chains and four light chains [3].

There are some studies in published English literature that has shown differential regeneration of muscle fibers following injury in animals and following exercise-induced injury in humans (Table 1). But none of these studies have been conducted in humans in the setting of mangled extremity. This study has been designed with the aim to understand the muscle healing pattern after injury in mangled extremities in humans.

Aims and objectives

To study the expressions of fast myosin and slow myosin along with proliferative marker Ki 67 in mangled extremities up to 24 h and at 7th day after the initial injury so as to assess the status of regenerative activity of the muscles.

Materials and methods

Study setting

This is a prospective study which included 15 patients who had presented with mangled extremities within 24 h of injury and who required subsequent debridement or revision amputation at 7th day of the first surgery, at Level I trauma center in North India from January 2017 to December 2017.

Inclusion criteria

1. All patients from 18 years and above with lower limb mangled extremity.
2. Patients who are not a known/detected case of diabetes and hypertension.
3. Patients presenting with isolated/combined mangled lower limb.
4. Those patients who required subsequent surgery for wound debridement or revision amputation on the 7th day of injury.

Exclusion criteria

1. Patients who are morbidly obese
2. Patients suffering from any immunodeficiency disorder
3. Malnourished individuals
4. Patients suffering from autoimmune disorders and vasculitis
5. Known case of any peripheral vascular disease
6. Patients who are unwilling to be a part of the study.

Plan of study

As soon as the patient with mangled extremity arrived at trauma center, the patients were resuscitated and stabilized. Thorough wound wash with an adequate amount of normal saline was given, and wound was dressed and splinted. MESS scoring was done. Only those patients who had presented within 24 h of injury and fulfilling the above criteria were selected in this study and shifted to Operation Theater for appropriate management as per the MESS score.

Sample collection

First sample

Under appropriate anesthesia and antibiotic cover, the level of amputation was decided based on surgeons experience and clinical parameters like color, consistency, contractility, and bleeding of the soft tissues. Intraoperatively, tissue sample from viable skeletal muscle was taken from the most healthy muscle compartment.

Second sample

Out of these patients who were selected initially, only those who require subsequent debridement with secondary wound closure procedure or revision amputation at 7th day of injury were selected. Patients with grossly infected wounds were excluded. Muscle sample was taken, under proper anesthesia and antibiotic cover, from the healthy and viable muscle compartment intraoperatively after debridement or revision amputation.

Sample processing and examination

All the biopsy samples from both groups were subjected to routine fixation using 10% buffered formalin and were examined in hematoxylin and eosin (H&E)-stained sections for the extent of histopathological changes, i.e., extent of muscle necrosis, inflammation granulation tissue formation, fibrosis and muscle regeneration if any.

Immunohistochemistry (IHC) was carried out by peroxidase–anti-peroxidase (PAP) method, and 2–4-μm-thick paraffin sections were stained with primary antibodies targeted against the following novel markers:

1. Fast myosin (in dilution 1:10 with overnight staining, rabbit monoclonal antibody made by Abcam company, Cambridge, UK).

Table 1 List of studies providing showing differential regeneration of fast and slow myosins following muscle injury

References	Journal	Country	Species	Study	Conclusion
Bigard et al. [21]	Journal of applied physiology	France	Wistar rats	Endurance training affects myosin heavy chain phenotype in regenerating fast-twitch muscle	There are increased concentrations of type I, IIa, and IIx myosin heavy chain (MHC) isoforms with the decrease in the relative concentration of type IIb MHC
Salvani et al. [14]	The Anatomical Record	Brazil	Mice	Long-term regeneration of fast and slow murine skeletal muscles after induced injury by ACL myotoxin isolated from Agkistrodon contortrix laticinctus (broad-banded copperhead) venom	There is an increase in expression of type I muscle fibers, while that of type II fibers decreased
Harber et al. [19]	International Journal of Sports Medicine	USA	Human	Myosin heavy chain composition of single muscle fibers in male distance runners	Increase in MHC I expression was found in distance runners, while MHC IIa was elevated in training for mid-distance events
Rodríguez et al. [20]	American Journal of Physical Medicine & Rehabilitation	Spain	Human	Effects of training status on fibers of the musculus vastus lateralis in professional road cyclists	Increased percentage of type I and IIC fibers and decreased percentage of type IIA and IIB fibers, an increased cross-sectional area of all fiber types after 3-year training except IIB fibers, an increased mitochondrial volume in all fiber types except type IIA fibers, and an increased capillary density
Russell et al. [18]	Diabetes	Switzerland	Human	Endurance training in humans leads to fiber type-specific increases in levels of peroxisome proliferator-activated receptor-gamma coactivator-1 (PGC-1) and peroxisome proliferator-activated receptor-alpha (PPAR-α) in skeletal muscle	Increase in PGC-1 and PPAR-α mRNA and protein content in type I, IIa, and IIx muscle fibers with increase of type I muscle fiber
Tetsuya et al. [13]	Journal of Orthopaedic Research	USA	Mouse	Skeletal muscle fiber type conversion during the repair of mouse soleus: potential implications for muscle healing after injury	I in the expression of slow-type myofibers increased, while that of fast-type myofibers decreased during muscle repair
Zimowska et al. [12]	Muscle Nerve	Poland	Wistar rats	Inflammatory response during slow- and fast-twitch muscle regeneration	The efficiency of muscle repair differs by muscle fiber type and is affected by the inflammatory response

2. Slow myosin (in dilution 1:20 with overnight staining, mouse monoclonal antibody made by Abcam company, Cambridge, UK).
3. Ki 67 (in dilution 1:300 with overnight staining, made by Dako, Denmark).

Ki 67 is a marker of cell proliferation. All samples were subjected to histopathological examination for the presence of regenerating muscle fibers and those samples in which regenerating fibers were seen were stained with Ki 67 for confirmation of proliferative activity.

Interpretation

It was done in context to normal muscle by identifying muscle fibers which failed to stain by either or both antibodies detecting fast and slow muscle fibers in both the groups. Ki-67 positivity was specifically looked into the skeletal muscle fibers in the second group of biopsies.

Observation and results

Histopathology findings of biopsy samples

The histopathological findings of the study can be summarized in Figs. 1, 2, 3:

1. Patchy necrotic muscle fibers were seen as eosinophilic cells with loss of nuclei and few cells having pyknotic nuclei in 46.67% of the samples taken within 24 h. In the second biopsy samples taken on the 7th day of injury, 66.67% showed patchy necrosis. Necrotic fibers had been mainly replaced by granulation tissue and proliferating fibroblasts.
2. In samples taken within 24 h of injury, 46.67% showed inflammatory changes. Neutrophils were predominantly seen. Inflammation was seen in 93.33% of the second biopsy samples which was mainly composed of macrophages, lymphocytes and polymorphs infiltrating the skeletal muscle fibers.

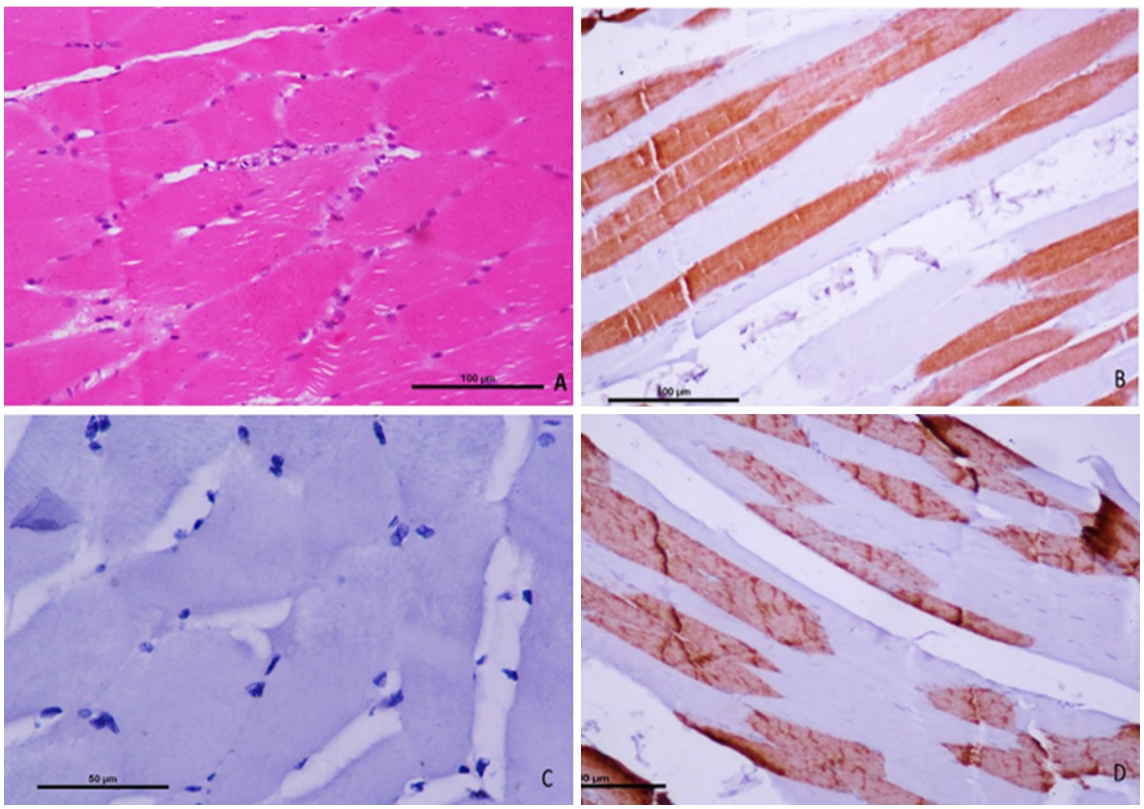

Fig. 1 A panel of photomicrographs to show normal muscle. **a** Hematoxylin and eosin-stained section showing uniformly stained regularly arranged compact muscle fibers with regularly placed small hyperchromatic nuclei at the periphery of each fiber (H&E, ×300); **b** immunohistochemistry staining showing positively stained slow muscle fibers (peroxidase–anti-peroxidase, ×400); **c** immunohistochemistry staining showing no positively stained muscle fiber nuclei for Ki67 (peroxidase–anti-peroxidase, ×400); **d** immunohistochemistry staining showing positively stained fast muscle fibers (peroxidase–anti-peroxidase, x400)

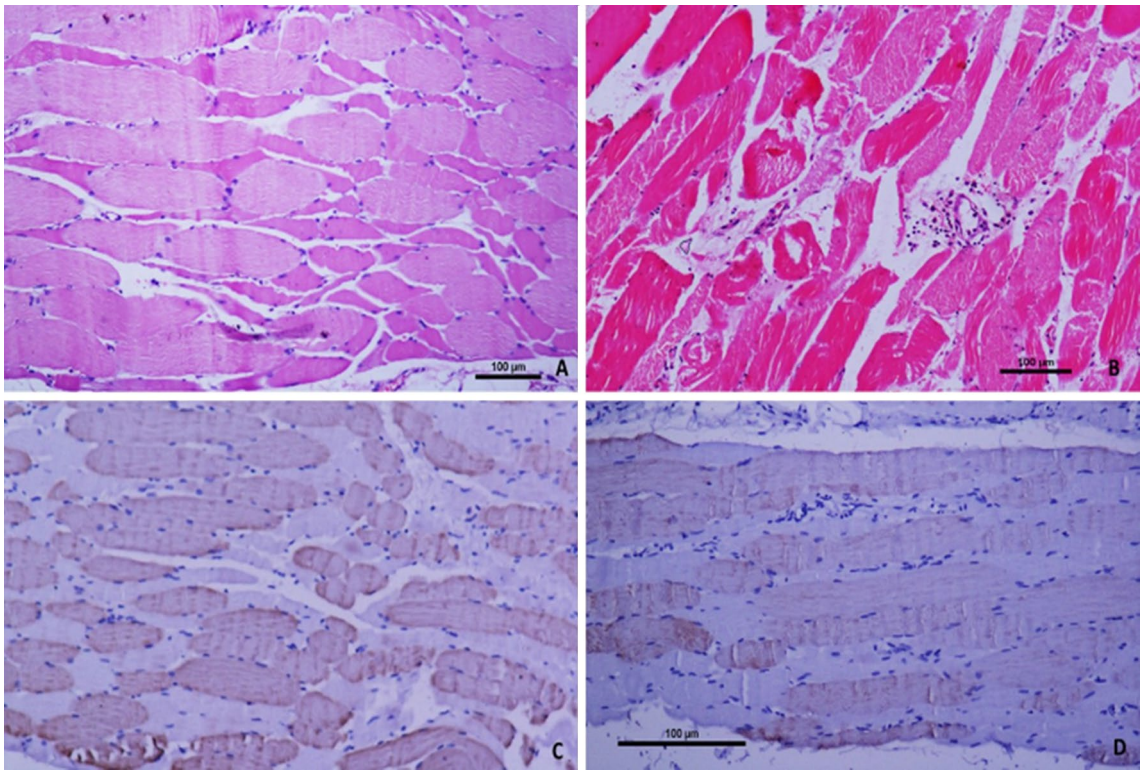

Fig. 2 Panel of photomicrographs showing representative histomorphological changes in biopsies from patients who presented within 24 h of injury. **a**, **b** Photomicrographs of hematoxylin and eosin-stained sections showing variation in staining intensity, characters, sizes and loss of cellular details of individual muscle fiber with intercellular; **b** in addition shows a small focus of early inflammatory cell collection in the intercellular perivascular focus. (H&E, ×300); **c**, **d** photomicrographs of immunohistochemistry-stained sections showing loss of immunoreactivity both for slow and fast myosin proteins (peroxidase–anti-peroxidase, ×400)

3. All samples showed viable muscle fibers. The second biopsy samples also had few atrophic and regenerating fibers along with fibrotic changes in some areas.
4. Regenerating muscle fibers appear basophilic with centrally localized single or multiple large nuclei following H&E staining. No regenerating fibers were seen in any sample taken within first 24 h. But, all samples taken on the 7th day of injury were found to have variable degree of regenerating fibers.

Both groups of biopsies were stained for fast and slow myosin proteins and also for Ki-67 in the second group with respective antibodies, by peroxidase–anti-peroxidase technique using normal muscle as positive controls. The observations were interpreted subjectively by studying the regeneration as indicated by centrally placed single or multiple large nuclei with prominent nucleoli with basophilic cytoplasm amidst the granulation tissue (Fig. 4).

Corresponding to these areas of muscle fiber necrosis with the absence of regenerating muscle fibers, the first group of biopsy samples showed mixed positive expressions for both slow and fast myosin proteins. However, in the second group of biopsy samples, where the necrotic fibers have been replaced by granulation tissue and proliferating fibroblasts, the regenerating muscle fibers stained positive only for the slow myosin proteins. Fast myosin proteins were not expressed in these regenerating muscle fibers. And though proliferative activity as indicated by Ki-67 was seen in second group, it was very minimal.

Statistical analysis (Table 2)

Data collected were checked for consistency and completeness. Then it was entered in database software. Comparison of first and second biopsy samples was carried out using McNemar test to see the significant difference between the histopathology and immunohistochemistry findings of the two biopsy samples. All statistical tests were two-sided and performed at a significance level of $\alpha = 0.05$.

Fast myosin protein was found to be present in all samples of the first group of biopsy. But it was not detected in the regenerating muscle fibers in the second group of biopsy samples, and it was found statistically significant with p value of < 0.001.

Slow myosin protein was seen in all the biopsy samples of either group under study including the regenerating fibers

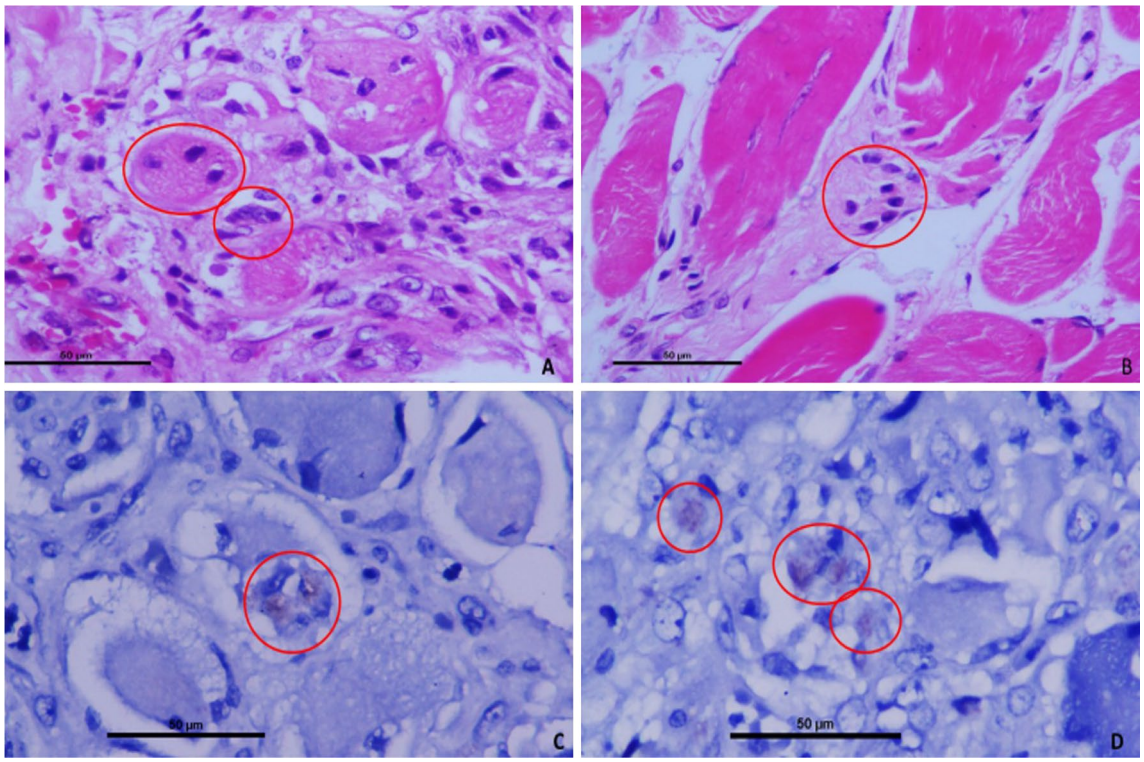

Fig. 3 Panel of photomicrographs showing representative histomorphological changes in biopsies from patients who underwent the second surgery on the 7th day of the first surgery. **a**, **b** Photomicrographs of hematoxylin and eosin-stained sections showing degenerating and regenerating (highlighted in red circles) muscle fibers with inflammatory granulation tissue in A and early collagenization in B (H&E, ×300); **c**, **d** photomicrographs of immunohistochemistry-stained sections highlighting nuclei positivity for Ki67 (in red circles) (peroxidase–anti-peroxidase, ×400) (color figure online)

in the second biopsy sample, and it was not statistically significant. Also Ki-67 representing proliferating tissue was present in some of the regenerating muscle cells in all the biopsy samples of the second group.

Discussion

Since the muscles contribute to the major bulk of the limbs, they get injured invariably in any trauma. The process of muscle repair after injury consists of the phases of degeneration and regeneration. The process of muscle healing is complex and not fully understood. The outcome of this process is dependent on multiple factors like the extent of injury and effect of inflammation on the muscle fibers [4, 5]. During the degeneration phase, necrotic changes in the muscle fibers are seen at first with damage to the sarcolemma of the muscle fibers which causes an increase in permeability of the fiber [6–8]. This initial phase of injury is associated with inflammatory cell infiltration of the injured muscle fibers. Neutrophils are the first inflammatory infiltrates followed by mononuclear cell infiltration consisting of macrophages and lymphocytes after 48 h [7]. As a response to injury, regenerating muscle fibers start appearing gradually, thereby replacing the necrotic and degenerated muscle fibers. These regenerated fibers appear basophilic on H&E staining with central localization of the nuclei, and there is focal fusion of cells along with splitting of the fibers [9–11].

Not all aspects of muscle healing are well understood at present, and even on extensive PubMed search, only few English literatures could be traced that have tried to delineate the variable muscle healing pattern and different factors and pathways that control muscle healing (Tables 1, 3). Most of these studies were conducted in animals and have shown differential regeneration of muscle fibers during muscle healing. Few studies had also reproduced the same results in humans but in non-traumatic scenario.

Zimowska et al. [12] had observed that the efficacy of muscle regeneration is different depending on the type of affected muscle fiber. Tetsuya et al. [13] used cardiotoxin in mouse to cause muscle injury and examined the conversion of fiber type during muscle repair. They examined the mice at 2, 4, 8, and 12 weeks after injury and assessed soleus muscle repair. They found that the amount of slow fibers increased during muscle regeneration with the decrease in fast fibers, whereas hybrid fibers are found

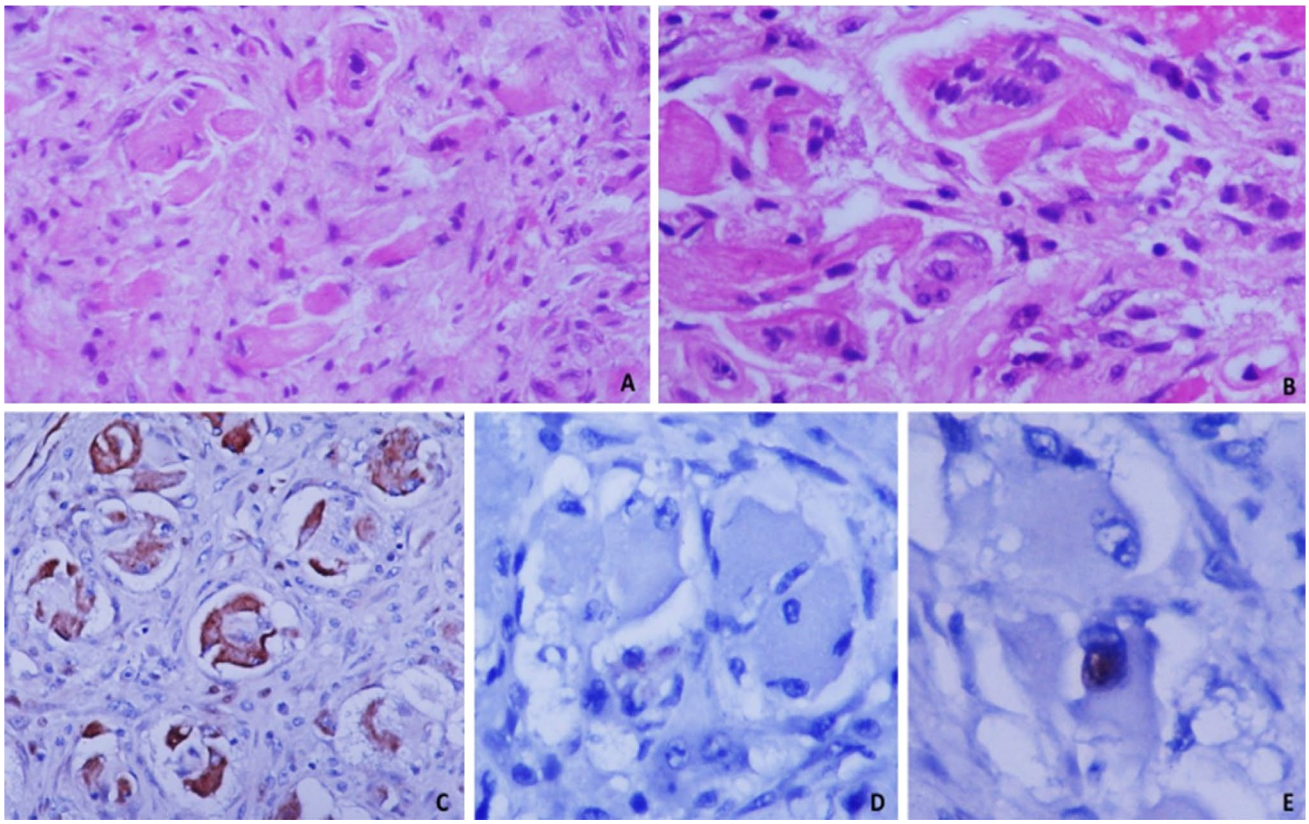

Fig. 4 Panel of photomicrographs to show representative histomorphological changes in biopsies from patients who underwent the second surgery on the 7th day of the first surgery. **a**, **b** Photomicrographs of hematoxylin and eosin-stained sections showing many degenerating as well as many regenerating muscle fibers with inflammatory granulation tissue (H&E, ×300); **c**, **d** photomicrographs of immunohistochemistry-stained sections for slow and fast myosins where only the slow myosin staining is positively expressed by the regenerating muscle fibers and the same regenerating muscle fibers failed to express the fast myosin (peroxidase–anti-peroxidase, ×400). **e** Photomicrograph of immunohistochemistry-stained section highlighting nuclei positivity for Ki67 (peroxidase–anti-peroxidase, ×400)

Table 2 Statistical analysis of immunohistochemistry results

Tissue markers	First biopsy (%)	Second biopsy (%)	*p* value
Fast myosin	100	0	< 0.001
Slow myosin	100	100	1.00
Ki-67	–	100	–

Table 3 Database search methodology

Database	Results
Pub med (1950—01/08/2018)	
1. Fast myosin	4054 articles
2. Slow myosin	3743 articles
3. Muscle regeneration	26,546 articles
4. Differential muscle regeneration	491 articles
5. Differential regeneration of fast and slow muscle fibers	13 articles
6. Differential regeneration of fast and slow muscle fibers in humans	No hits

to be raised at 2 weeks after injury and then decreased gradually by 6 weeks.

Likewise, Salvini et al. [14] observed the regeneration of gastrocnemius muscle of mice. They used myotoxin to injure the muscle and evaluated after 8 months. They found that following myotoxin injury there is a conversion of fast muscle fiber to slow muscle fibers.

Lagord et al. [15] conducted an in vitro study to see the proliferative and differentiating abilities of satellite cells present in extensor digitorum longus (a fast muscle) and soleus (a slow muscle) in rats. They found that the properties of satellite cells from extensor digitorum longus are different from those of soleus. This difference in properties in satellite cells may lead to differential regeneration of fast and slow fibers.

The present study is a prospective pilot study as it has been conducted in human volunteers in the setting of acute trauma. No such kind of study could be traced in any English literature to the best of our knowledge till the present point of time. This study was designed with the aim of

exploring the pattern of muscle healing in humans who sustained severe crush injury of lower limb. In the present study, on histopathological examination, regenerating muscle fibers were seen only in the second group of biopsy samples which was further confirmed by staining with Ki-67 antibody, which is a marker of cell proliferation [16, 17]. As no regenerating fibers were seen in histopathological examination in the first group, these samples were stained with fast and slow myosin antibodies only. Both fast and slow myosin proteins were positively stained in degenerated as well as viable muscle fibers indicating the presence of both proteins in the first biopsy samples. It was also important to note that in the second group of biopsy samples, only regenerating muscle fibers were subjected to examination with these fast and slow myosin antibodies. It was observed that these regenerating fibers showed positive expression for slow myosin protein only.

Likewise, there were also some studies that have shown differential expression of slow and fast muscle fibers following exercise-induced injury. Russell et al. [18], Harber et al. [19] and Rodríguez et al. [20] studied the muscles of humans taking part in an endurance sports. They found that there is an increase in slow muscle fibers with concomitant decrease in fast muscle fibers. Bigard et al. [21] studied the effect of endurance training on regenerating fast muscle fibers in rats. They observed a significant difference in MHC isoforms of fast myosin protein in the regenerating muscles when compared to the control with higher expression of types I, IIa, and IIx isoforms in the regenerating muscle fibers.

This knowledge of differential expression of only slow myosin fibers in regenerating muscle fibers may, directly or indirectly, affect postoperative muscle bulk and strength thereby possibly influencing our management of the reconstructed limbs or amputation stumps. It may also influence the early functional recovery of these patients. It may also affect the rehabilitation period of such patients with less wound complications. The utility of these findings is yet to be explored and requires more evidences as this study was conducted in small sample size with limited resources and lack of long-term follow-up. Thus, this study is just a baby step and requires large multicentric trials with long-term follow-up to further explore the possible opportunities in future.

Conclusion

After analyzing the findings of the study, we can conclude that there is differential regeneration of slow muscle fibers in humans following injury in mangled extremities. No regenerating fast muscle fibers were seen. Though the significance of these findings could not be well understood at present, it may influence the postoperative outcomes and functional recovery of the patient. This study is, merely, a food for thought to further evaluate and understand the muscle healing pattern and its implications and thus requires large multicentric trials, better resources, and long-term follow-up.

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Compliance with ethical standards

Conflict of interest There is no conflict of interest from any of the authors.

Ethical standard The study was approved by the Institute's Ethical Committee and was performed in accordance with the ethical standards laid down in the 1964 Declaration of Helsinki and its later amendments.

Informed consent Informed consent was taken from the patients at the time of enrollment, the study procedure was explained in detail to all the enrolled patients, and they were made to understand that they could withdraw from the study at any point of the study period. The study has not altered any of the management protocols of these patients. Identity of the participants was kept confidential.

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