

Original Research Article

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## Quantitative and Morphological Evaluation of the Effect of Platelet Rich Plasma on Collagen Fibers in Experimentally Induced Skeletal Muscles Injury in Adult Male Albino Rats

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### ABSTRACT

Muscle injuries present frequently in sports medicine. Healing with conventional therapy is often inadequate, leading to incomplete functional recovery due to fibrosis. Generating substantial interest in the potential for emerging technologies such as platelet-rich plasma (PRP) to enhance soft-tissue healing and decrease time of recovery became mandatory. The aim of this work was to evaluate the role of PRP in promoting healing of experimentally induced skeletal muscle injury in adult male albino rat model. 48 adult male albino rats were used and were divided into three groups. Group I was served as control for obtaining muscle specimens from their gastrocnemius muscle and PRP. Group II was exposed to bilateral gastrocnemius muscles injury and was left without treatment. Group III were subjected to bilateral gastrocnemius injury and were immediately treated with PRP intramuscularly. Muscle specimens were excised after 1, 7 and 21 days from the onset of injury to be processed for light microscopic study after staining with Mallory trichrome stain. Assessments of the mean area percentage of collagen fibers were also statistically analyzed. Treatment with PRP resulted in enhanced regeneration of skeletal muscle injury without fibrosis. The PRP treated group demonstrated absence of fibrosis on days 14 and 21 as compared to their associates in non-treated group. Local injection of PRP into the injured gastrocnemius muscle resulted in enhanced muscle regeneration without fibrosis.

#### Keywords

Platelet rich plasma,  
Muscle injury,  
Fibrosis, Mallory  
trichrome

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### Introduction

Muscle injuries are common and may be associated with impaired functional capacity, especially among athletes. The results of healing with conventional therapy including rest, ice, compression, and elevation are often inadequate, generating substantial interest in

the potential for emerging technologies such as platelet-rich plasma (PRP) to enhance the process of soft-tissue healing and to decrease time to recovery (Mosca and Rodeo, 2015).

There are abundant evidences suggesting that growth factors (GFs) may play a key role in the healing process, especially in the early

stages of inflammation. These observations constituted the basis for the use of platelet rich plasma (PRP) as a new therapeutic tool in the field of dentistry, acute trauma, chronic tendinopathies and plastic surgery (Foster *et al.*, 2009; Kazakos *et al.*, 2009; and Kaux and Criclaard, 2013)

Nowadays there is increase of using PRP in playground injury resides in the fact that it is a simple, efficient and minimally invasive method of obtaining a natural concentration of GFs and cytokines from the  $\alpha$  granules (Menetrey *et al.*, 2000), so this work was carried out to evaluate the role of platelet rich plasma in healing of experimentally induced skeletal muscle injury in adult male albino rats.

## Materials and Methods

This study was carried out using 48 adult male albino rats of average weight 200 grams. Rats were housed in clean and properly ventilated cages under the same environmental conditions and fed on a standard laboratory diet. They were allowed a two weeks pre-experimentation period to be acclimatized to the laboratory conditions. The experiment was approved by the local ethical Committee of Faculty of Medicine, Tanta University, Egypt.

### The animals were divided into three groups

**Group I (control group):** 24 rats subdivided into three subgroups: Subgroup IA (12 rats): from which blood was collected from retro orbital plexus for platelet rich plasma preparation. Subgroup IB(4 rats): from which the gastrocnemius muscles were obtained from both lower limbs without any maneuvers after 1, 7, 14 and 21 days.

Subgroup IC (8 rats): from which the gastrocnemius muscles were obtained from both lower limbs after 1, 7, 14 and 21 days of

their injection with platelet rich plasma without injury.

**Group II (Muscle injury induced group) (12 rats):** The rats were anesthetized the skin over the gastrocnemius muscle of both lower limbs was shaved and cleaned with betadine solution. The gastrocnemius muscle was palpated guided by tendon Achillis and was crushed between the blades of the hemostat at level 3 for 2 minutes (Allbrook, 1962). The muscles of both lower limbs were injured and left without treatment. This group was subdivided into four equal subgroups: IIA, IIB IIC and IID where muscle specimens were obtained at day 1, 7, 14 and 21 after injury.

**Group III (Muscle injury and PRP treated group) (12 rats):** the gastrocnemius muscle of both lower limbs were injured as in group II and were injected intramuscularly immediately with 100  $\mu$ l (0.1 ml) of platelet rich plasma by insulin syringe within the gastrocnemius muscle. <sup>(9)</sup> This group was further subdivided into four equal subgroups: IIIA, IIIBIIC and IIID. The muscle specimens were obtained at day 1, 7, 14 and 21 after injury.

### Blood collection and preparation of platelet rich plasma

Blood was collected from donor rats (subgroup I A 3 rats for each time period) from retro-orbital plexus and occasionally by cardiac puncture after being anesthetized by ether inhalation (Kim *et al.*, 2011; and Quarteiro *et al.*, 2015). 2.5-3 ml were collected from each rat by sterile syringe containing 0.3 ml of 3.8% sodium citrate (0.5ml complete blood was taken to count number of platelets in complete blood sample by adding 10  $\mu$ l to 2.5 ml platelet counting solution using hemocytometer, it ranged from  $(355 \times 10^3 - 470 \times 10^3/\mu\text{l})$ . The collected citrated blood was then put in sterile 15ml centrifuge

(falcon) tube and was centrifuged at 3000 rpm for 7 minutes. Subsequently the supernatant (containing the buffy coat with platelets and leucocytes) was aspirated by a micropipette leaving heavy red blood cells. The supernatant was then placed in another sterile tube and centrifuged at 4000 rpm for 5 minutes. Cell pellet (platelets pellet) appeared in the bottom of the tube represented the plasma part rich in platelets (platelet rich plasma, PRP). The supernatant which represent the plasma poor in platelets (platelets poor plasma, PPP) was aspirated leaving only 1 ml to suspend the cell pellet in it.

The resuspended cell pellet was aspirated (10  $\mu$ l) were taken to hemocytometer for manual counting of platelets to be sure it was PRP as it is nearly 5 times the number in complete blood sample<sup>(11)</sup>. The number of platelets was ranging from  $1.4 \times 10^6$  -  $2.6 \times 10^6/\mu$ l.- Then PRP was activated by adding calcium chloride at ratio 10:1 (0.1 ml of calcium chloride to each 1 ml of PRP). Once PRP was activated, it should be injected rapidly within 10 minutes to avoid jellification of the plasma.

### **Tissue processing**

For histological study muscle samples were fixed in 10% formalin buffered saline and processed to get paraffin sections for Mallory Trichrome staining.

### **Morphometric study and statistical analysis**

Area percentage of collagen fibers in Mallory trichrome stained sections was assessed in 10 randomly different microscopic fields for each specimen for each rat at a magnification power of (x10). This was done using image J software (V 1.48). The data were analyzed by Kruskal-Wallis test (Non-parametric ANOVA test). Differences were regarded as significant if  $P > 0.05$ .

## **Results and Discussion**

### **Group I (Control group)**

1- Subgroup IB showed the same results for all intervals (1, 7, 14 and 21 days) in the form of normal few blue collagen fibers in epimysium and perimysium and mainly around blood vessels at intervals (Figure 1).

2- Subgroup IC (Gastrocnemius muscle was injected with platelet rich plasma without injury) (Figure 2).

After 1 and 7 days, gastrocnemius muscles depicted blue fine collagen fibers inbetween muscle fibers.

After 14 and 21 days revealed similar findings like the control group IB.

Group II (Muscle injury induced group) displayed blue collagen fibers scattered inbetween inflammatory cells after 1 day which increased after 7 days to be prominently presented around muscle fibers after 14 and 21 days (Figure 3).

Group III (Muscle injury and PRP treated group) denoted blue collagen fibers scattered inbetween inflammatory cells after 1 day which increased after 7 days to be distributed inbetween newly formed muscle fibers. After 14 and 21 days collagen fibers decreased and arranged in the endomysium and perimysium to be more or less similar to control group (Figure 4).

### **Statistical results**

Morphometric study showed insignificant difference ( $P$ - value = 0.4207) in the mean area percentage of collagen between subgroup (IB) (control group), subgroup (IC), subgroup (IIA) and subgroup (IIIA) on the 1<sup>st</sup> day. This difference became significant on the 7<sup>th</sup> day and the 14<sup>th</sup> day with ( $P$ -value = 0.0009) and

(P-value = 0.0008) in subgroups IIB and IIC respectively when compared to other groups on the same days.

Within the same group II, the mean area percentage of collagen in subgroup IID was significantly increased when compared with subgroup IIA (P-value<0.01) and insignificantly in relation to subgroups IIB and IIC (P- value >0.05 for both).

Statistical analysis also, showed significant increase in the mean area percentage of collagen on the 21<sup>th</sup> day in non-treated subgroup (IID) as compared to control, subgroup (IC), subgroup (IIID) (P-value =

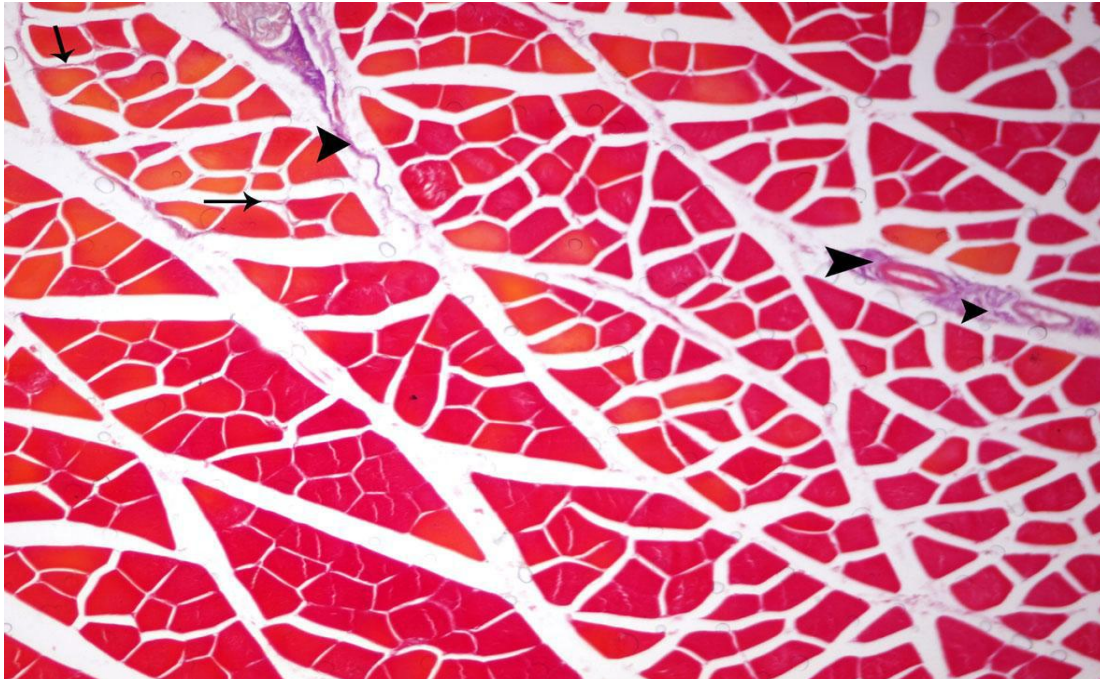
0.0025). Remarkably, group III displayed significant decrease in the mean area percentage of collagen in subgroup (IIID) when compared with subgroup IID (p=0.0025) and subgroup IIB (P-value <0.05). This difference became insignificant when compared with subgroups IIIA, IIIC, control and subgroup IC on the same day (P-value >0.05 for all).

Furthermore, the mean area percentage of collagen in subgroup IC at the four periods, exhibited insignificant difference when compared to control group (P-value = 0.6256). Table 1 and Histogram (1).

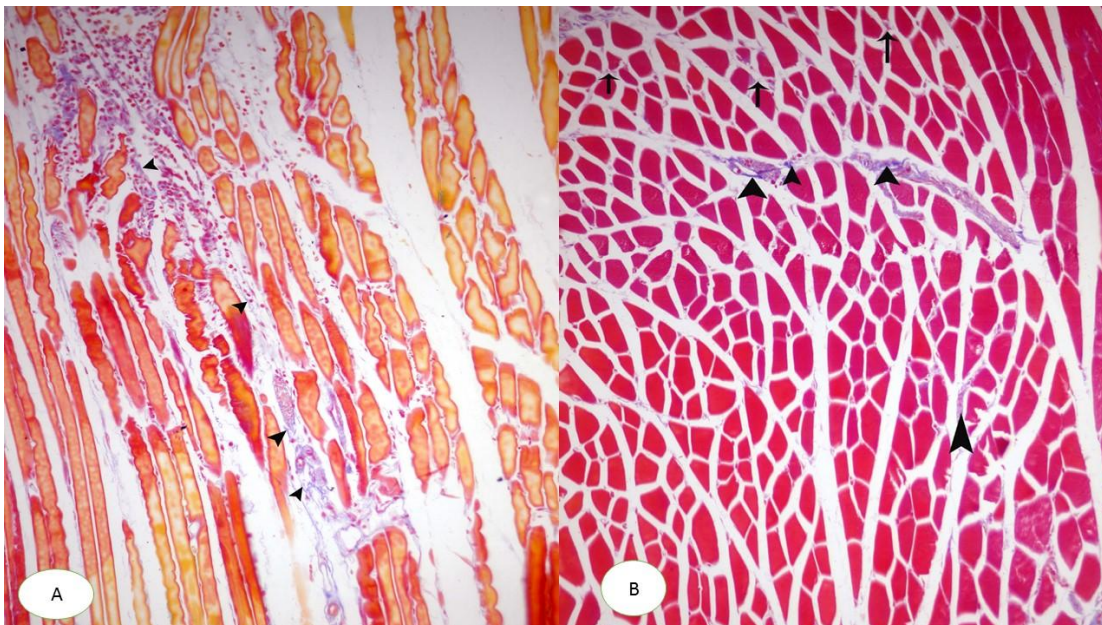
**Table.1** Comparison between the studied groups as regard mean ± SD of area percentage of collagen fibers

		Group IB	Group IC				Group II				Group III			
			1 day	7 days	14 days	21 days	II A	II B	II C	II D	III A	III B	III C	III D
			1 Day	7 days	14 days	21 days	1 Day	7 days	14 days	21 days	1 day	7 days	14 days	21 days
Area percentage of collagen fibers	<b>Mean</b>	3.07	2.88	2.21	2.43	2.38	4.12	16.37	21.27	22.04	4.07	10.25	8.40	4.93
	<b>SD</b>	0.6666	1.738	1.073	0.4809	1.115	1.557	1.443	2.694	3.082	1.984	1.786	2.550	1.648
Kruskal-Wallis test (Non parametric ANOVA test)		<b>P1</b>				0.6256				<i>Not significant</i>				
		<b>P2</b>				<b>0.0019*</b>				<i>Significant</i>				
		<b>P3</b>				<b>0.0036*</b>				<i>Significant</i>				
		<b>P4</b>				0.4207				<i>Not significant</i>				
		<b>P5</b>				<b>0.0009*</b>				<i>Significant</i>				
		<b>P6</b>				<b>0.0008*</b>				<i>Significant</i>				
		<b>P7</b>				<b>0.0025*</b>				<i>Significant</i>				
										P1 comparison between subgroups IB, IC (1 day), IC (7 days), IC (14 days) & IC (21 days)				
										P2 comparison between subgroups IIA, IIB, IIC & IID				
										P3 comparison between groups IIIA, IIIB, IIIC & IIID				
										P4 comparison between subgroups IB IC (1 day), II A & III A				
										P5 comparison between subgroups IB IC (7 days), II B & III B				
										P6 comparison between subgroups IB IC (14 days), II C & III C				
										P7 comparison between subgroups IB IC (21 days), II D & III D				

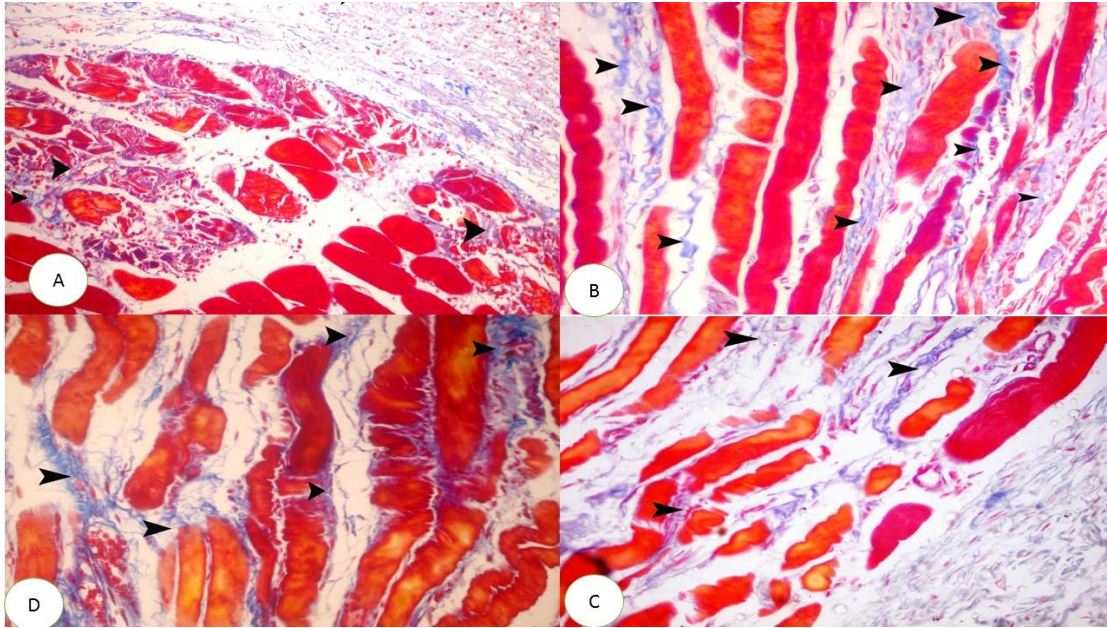
**Figure.1** A photomicrograph of a transverse section in the gastrocnemius muscle of a control rat (subgroup IB), showing blue collagen fibers in endomysium (arrow) and in perimysium around blood vessels (arrow heads). (Mallory's trichrome, x200)



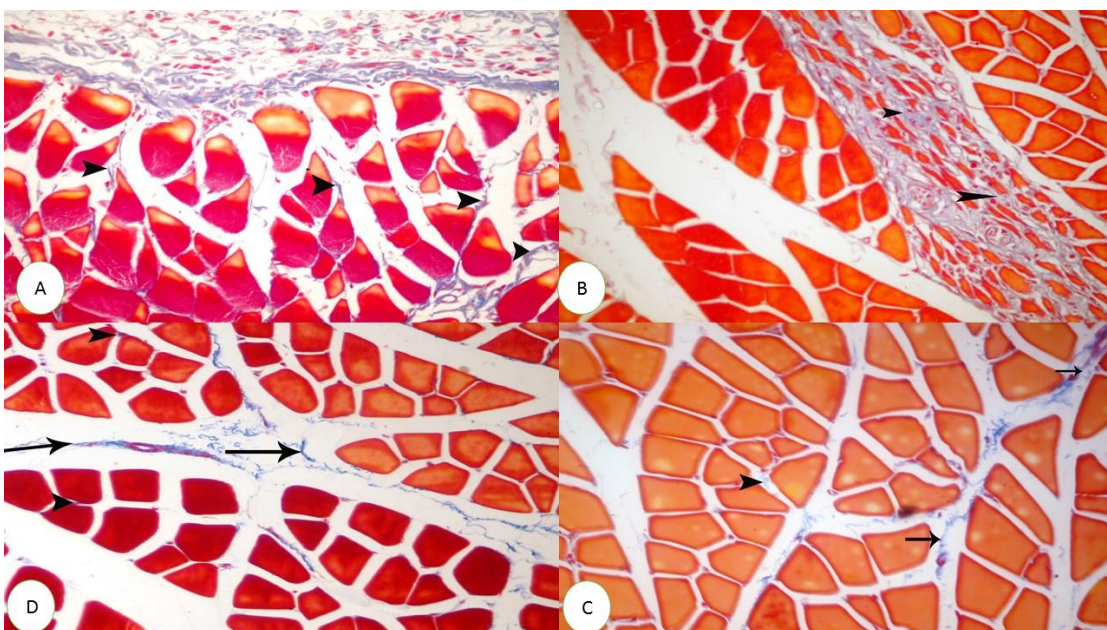
**Figure.2** Sections in the gastrocnemius muscles of subgroup IC  
A: after one day showing blue collagen fibers scattered between inflammatory cells (arrow heads). B: after 7 days, denotes blue fine collagen fibers in endomysium (arrows) and perimysium (arrow heads).  
(Mallory's trichrome, A and B x200)



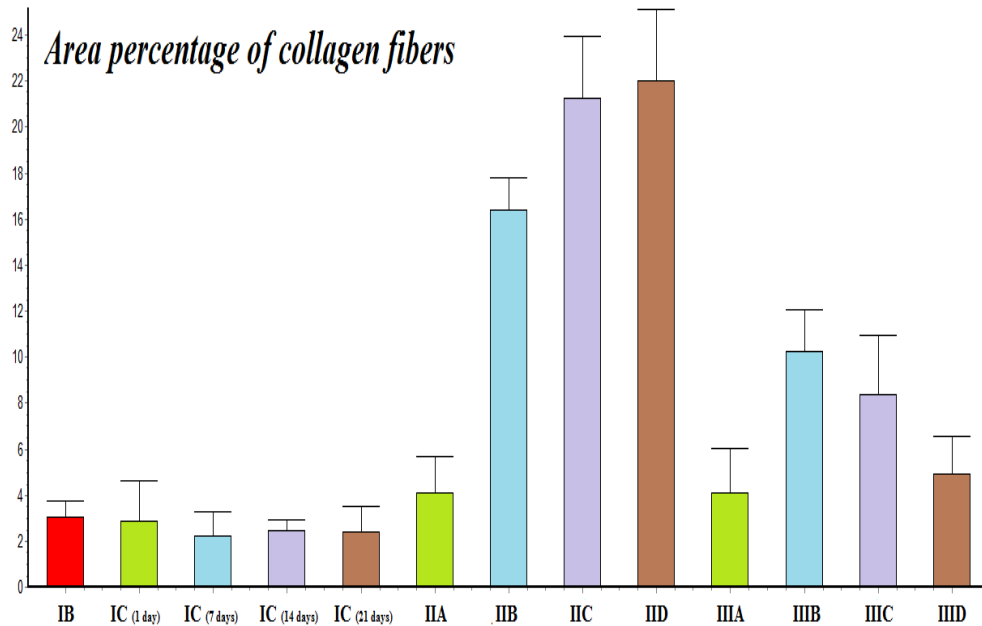
**Figure.3** Sections in the gastrocnemius muscles of group II A: after 1 day showing blue collagen fibers inbetween and around muscle fibers (arrow heads). B: after 7 days reveals more collagen fibers (arrow heads). C and D: after 14 and 21 days respectively demonstrate prominent increase in collagen fibers (arrow heads). (Mallory trichrome, A, B, C and D x400)



**Figure.4** Sections in the gastrocnemius muscles of group III. A: after 1 day shows blue collagen fibers in between muscle fibers (arrow heads). B: after 7 days depicts more collagen fibers (arrow heads) inbetween newly formed muscle fibers. C and D: after 14 and 21 days respectively demonstrate less collagen fibers in endomysium (arrow heads) and in perimysium (arrows) to be more or less similar to control. (Mallory trichrome, A, B, C and D x400)



**Histogram.1** Comparison between the studied groups as regard mean of area percentage of collagen fibers



Muscle injuries are common and may be associated with impaired functional capacity, especially among athletes. Pain and restricted range of motion due to these injuries can lead to decreased performance and limited ability to play. With the exception of muscle complete ruptures/ avulsions, complications like myositis ossificans and the persistence of uncomfortable symptoms in chronic injuries, almost all the acute muscle damages are usually treated non-surgically. Conventional therapy including rest, ice, compression, elevation, is often considered the treatment of choice. Experimental and clinical studies demonstrated that myogenesis is not restricted only to the prenatal period but may also occurs during the healing period after muscle tissue damage (Järvinen *et al.*, 2005; Mosca and Rodeo, 2015; and Benazzo *et al.*, 2017).

Concentrated growth factors (GFs) within platelet rich plasma, act synergistically during the different phases of the healing processes

when compared with the use of isolated GFs. Platelet rich plasma is simply obtained and easily prepared with a little risk of developing an immune response (Borrione *et al.*, 2010).

Therefore, this study was carried out to evaluate the role of platelet rich plasma in the healing of experimentally induced skeletal muscle injury in adult male albino rat model.

Remarkably, the muscle specimens of group II (non-treated) that were obtained after 7, 14 and 21days depicted increased amount of collagen fibers in endomysium and perimysium which were measured and statistically analyzed. This was evidenced at day 21 when compared to days 7 and 14 within the same group and when compared to control and treated group with evident fibrosis. The same findings were observed by Fisher and Rathgaber, (2006) who found that, at 6 days post trauma, muscles appeared to regenerate with focal interstitial fibrosis and

multiple subsarcolemmal nuclei or central located nuclei with prominent nucleoli. With persistence of residual focal areas of fibrosis after 14 days.

These changes were explained by Järvinen *et al.*, (2005); and Järvinen *et al.*, (2007) who reported that satellite cells can proliferate and mature into myoblasts, which can form multinucleated myotubes and ultimately myofibers. The ends of the ruptured myofibers are typically prevented from reuniting completely by the scar tissue that forms during healing. Järvinen *et al.*, (2005) also mentioned that the process of scar formation begins almost immediately following injury. Inflammatory cells degrade the blood clot while fibrin cross-links form an initial extracellular matrix that functions as an initial scaffold to support a reparative response.

In this study, morphometric and statistical results also showed significant decrease of the mean of area percentage of collagen fibers in PRP treated group at day 21 when compared with other subgroups within the same group and when compared to the same period in group II (Muscle injury induced group). But this difference wasn't significant when compared with the control group. This was explained by Quarteiro *et al.*, (2015) who mentioned that, during repair and remodeling phases, deposition of collagen in an organized and gradual manner is the most important characteristic for assuring balance between lysis of the old cell matrix and synthesis of the new matrix. This is an essential condition for successful regeneration of the injured muscle tissue. In addition, the initially produced collagen is thinner than the collagen from the healthy tissue; this initial collagen is then reabsorbed and thicker collagen is produced along the tension lines, and this is positively correlated with increase in tensile strength.

From the previous discussion, it was observed that PRP had a significant effect on enhancement of muscle regeneration after injury without fibrosis as compared to non-treated group. The same finding was documented by Sanchez *et al.*, 2009 who stated that full recovery of functional capabilities was restored in half the expected time, and images showed full regenerated muscle tissue after PRP treatment. According to Hamilton and Best, 2011 Platelets are rich in growth factors that can stimulate myogenesis and mitigate inflammation.

In conclusion, treatment with PRP resulted in enhanced regeneration of skeletal muscle injury without fibrosis.

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