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The Role of Homogenate Hepatic Tissue in Myogenesis

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Abstract

Two groups of albino rats were exposed to identical tibialis anterior muscle crush injury. Small pieces of the liver were obtained from both groups. The animals of the first group were left without implantation with the liver tissue. Those of the second group received homogenized autogenous liver mince labeled with Indian ink. From the follow up, it was noticed that the muscle regenerative process was faster in the animals of the second group when compared with those of the first, and that the labeled liver cells participated in the formation of myotubes, which formed mature muscle fibers and possibly new satellite cells in the crushed skeletal muscles. This suggests that the labeled liver tissue homogenate implanted at the injured site has a positive regenerative effect on the skeletal muscles. The results of this experiment may eventually revolutionize therapeutic procedures for some forms of muscle diseases.

Keywords: Stem cells, Homogenized liver tissue, Muscle regeneration**.**

1. Introduction

Muscle injuries constitute one of the most challenging problems of sports traumatology, since although common, their treatment is still controversial and often inefficient (Jarvinen *et al*., 2005). Long periods of leave of absence are usually necessary for athletes, and a full recovery is sometimes difficult (Armfield *et al.*, 2006).

The muscle regenerative process has been demonstrated to reproduce myogenesis by the proliferation of myogenic stem cells, followed by their fusion to form multinucleated cells, and their further differentiations into mature muscle fibers (Carlson, 1973; Al- Hadithi *et al*., 2002; Al-Yawer *et al.*, 2004).

The origin of these stem cells was widely considered to arise within the damaged tissue from the quiescent satellite cells (Kang and Krauss, 2010). These cells are activated in response to injury, and are claimed to be the source of myoblasts (Alameddine *et al*., 1989). These myoblasts proliferated, fused and formed multinucleate myotubes that matured into myofibres which replaced the damaged and dead muscle fibers (Kuang and Rundnicki, 2008; Kang and Krauss, 2010).

Al-Azzawi, (1972) suggested that some muscle precursors are possibly of local origin, but may have moved into the site of muscle injury from elsewhere. Recently, few authors reported that some of these stem cells have been proposed to be phagocytic cells of blood origin (Weissman, 2002; Al-Yawer *et al.*, 2004); or from the bone marrow (Seal and Rudnicki, 2000). These undifferentiated cells show high phagocytic activity engulfing various types of foreign substances as trypan blue, various acridine dyes, and horse radish peroxidase (Al-Azzawi, 1972).

The muscle tissue healing process usually starts promptly as soon as the injury occurs; however, it can evolve slowly and irregularly, hindered by an extensive formation of connective scar tissue (Buckwalter and Cruess, 1993). This scar tissue inhibits the complete regeneration of the preexisting muscles, since it leads to the destruction, exhaustion and depletion of the local myogenic cells, which may necessitate the recruitment of additional myogenic cells from another source other than the injured muscle itself.

The limitations described earlier gave rise to surveys investigating the biological measures capable of stimulating the muscle regeneration process and of preventing fibrosis formation (Huard *et al*., 2002). Among these new techniques, two lines of implantation have been studied extensively. The first was through **t**he local application of the following promoting agent: i. White blood cells (Al-Yawer *et al*., 2004; Denapoli *et al.*, 2016); ii. Platelet-rich plasma (Utomo *et al*., 2018); iii. Bone-marrow centrifugate (Ferrari *et al*., 1988; Matziolis *et al*., 2006). These promoting agents are substances used in the tissue culture of skeletal muscles to enhance myogenesis due to their supposed healing properties, and attributed to the ability to recruit, proliferate and differentiate cells involved in tissue repair. On the other hand, therapies are based on the direct local addition of embryonic stem cell cultures, in the hope that the latter will differentiate in the cells of the target tissue (Al-Hadithi *et al*., 2002; Musaro *et al*., 2004). The aim of this study is to shed light on tissues, apart from blood or bone marrow, such as liver, because of its well-known power of regeneration, and since it contains growth factors especially those affecting division and further the differentiation of myogenic progenitors like transferring and hypoxanthine related compounds (De La Haba *et al.*, 1975; It *et al*., 1982 and 1985; Mac Sween and Whaley, 1992).

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2. Materials and Methods

2.1. Animals Used and the Obtaining of Tissue Specimen

A total number of eighty mature albino male rats that are eight weeks old and weighing 150–200 g were used in this study. The animals were lightly anesthetized by intraperitoneal injection of 0.1 mL (equal to 6 mg) pentobarbitone sodium. Right paramedian incision, 2 cm long, was done in the right hypochondrium of the anesthetized animals. A small piece of the liver (0.25 × 0.5 cm) was obtained using fine scissors. Then the injured site was covered with a layer of gel foam. Finally, closure of the abdominal wall was achieved by using 2/0 silk.

2.2. Preparing the Hepatic Tissues

Manual mincing of the hepatic tissue into very minute pieces using a scalpel's blade after placing the tissue in 3–4 ml of normal saline (Al-Hadithi *et al.*, 2002). The homogenate was centrifuged for fifteen minutes at 3000 rpm (Al-Yawer *et al*., 2004).

2.3. Incubation Procedures of the Homogenate

The homogenized tissues were incubated in an incubation media (1/3 Minimum Essential Medium, EAGLE-modified) with 2/3 normal saline. Fifteen drops of 25 % diluted Indian ink were added to the above mixture. The mixture was then incubated for ninety minutes in a water bath at 37°C (Al-Yawer *et al*., 2004). The incubating medium was then centrifuged for five minutes at a rate of 1500 rpm. The supernatant was discarded, and the precipitate was resuspended with 1 ml of 0.9 % normal saline and was centrifuged again at a rate of 1500 rpm for five minutes. The supernatant was again removed, and the precipitate was washed thrice with 1 mL of normal saline, until the supernatant was clear enough (Alwan, 2004). The remaining precipitate was suspended in 1 ml of normal saline. One drop of this suspension was put on a microscopic slide (two microscopic slides were prepared), and was dried and fixed in 95 % methanol. The first slide was stained with Harris's hematoxylin and eosin stain, and the other slide was stained with Mayer's carmalum solution. These slides were prepared to study the general morphology, and the type of cells that phagocytosed the label (Al-Yawer *et al*., 2004).

2.4. Induction of Injury and Implantation of Labeled Cells

While the animals were under anesthesia, the upper, middle and lower third of the tibialis anterior muscle were crushed and the already labeled liver cells suspension (each its own) was implanted in the tibialis anterior muscle by inserting the needle of a 1ml syringe along the longitudinal axis of the muscle. 0.33 mL of labeled cell suspension was injected to each third of the muscle. Animals were sacrificed in groups of seven animals, each, after 1, 2, 3, 5, 7, 14, 21 and 28 days from induction of injury. In the remaining animals (control animals) the same procedure was repeated (small piece of liver was obtained) but without implantation of liver tissue obtained to the injured tibialis anterior muscle. Three control animals were used with each experimental group. After sacrifice, serial frozen section 8 microns thickness was prepared to study muscle regeneration by a light microscope.

3. Results

The examination of the hepatic tissue homogenate revealed that the main cells involved in the uptake of the label were monocytes and macrophages, but the uptake of the label was not equal in these cells (Figure l and 2).

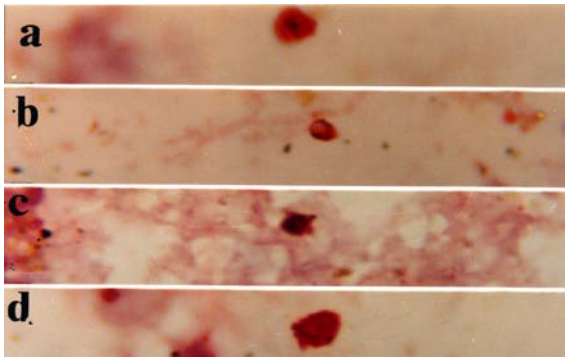

Figure 1. Hepatic tissue homogenate smear after labeling with Indian ink, showed different labeled cells: Macrophages engulfing fine-coarse carbon particles (a and d). Polymorphs showed poor uptake as only fine granules were present in their cytoplasm (b). Other cells, which were small with relatively large nucleus occupying almost all the cytoplasm, showed good uptake of carbon particles. These cells could be stem cells or mature lymphocytes (c). Mayer's carmalum stain (X1000).

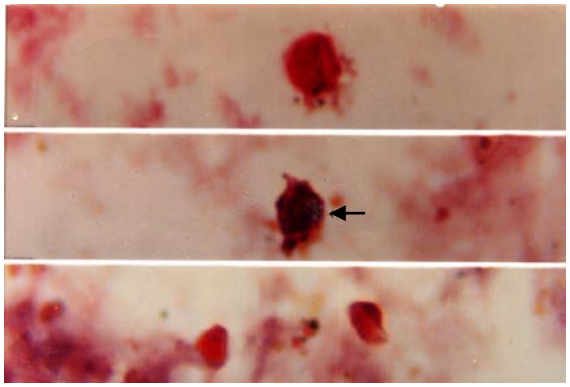

Figure 2. Photograph of hepatic tissue homogenate smear after labeling with Indian ink, showing large polyhedral cells (arrow), which could be heapatocyte or their precursors, engulfing coarse carbon particles; were as the cytoplasm of the others cells contains fine carbon particles. Mayer's carmalum stain (X 1000).

Large polyhedral cells, with centrally located nuclei, were also shown to engulf the Indian ink. Fine carbon particles were found distributed in their cytoplasm. These cells could be hepatocytes or their precursors.

Some cells, small in size with relatively large nuclei occupying almost all the cytoplasm revealed a good uptake of the label. These cells could be mature lymphocytes or undifferentiated stem cells. The polymorphs did not show any good uptake where only scarce granules were present.

On the first postoperative date, the injured area of the muscle tissue of the experimental group showed (Figure 3 and 4) necrotic fibers and inflammatory cell infiltration, mainly polymorphs, together with labeled cells of the implanted hepatic tissue. Some of the labeled cells were fusiform, others were rounded. Some of them showed fine carbon particles in their cytoplasm, while others showed coarse carbon particles indicative of the phagocytic activity of these cells.

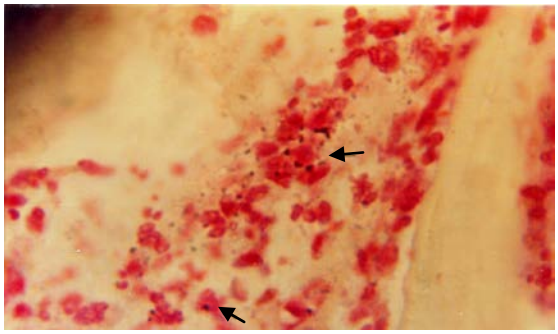

Figure 3. Injured tibialis anterior muscle implanted with hepatic tissue homogenate – Day one after injury. Labeled cells (arrows) were found among the inflammatory cells invading the injured area. Mayer's carmalum stain (X 1000).

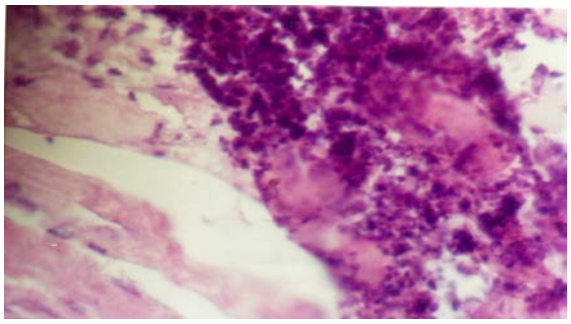

Figure 4. Injured tibialis anterior muscle implanted with hepatic tissue homogenate – Day one after injury. Huge amount of artificially administrated cells invaded the injured site of the muscle. H & E stain (X 400).

On the second postoperative date (Figure 5), numerous macrophages were profusely distributed throughout the injured area. Some of them were artificially introduced into the lesion since they contained carbon particles in their cytoplasm. Some of the labeled cells can be recognized as myoblasts, which are fusiform mononucleated cells with ovoid nuclei. Their cytoplasm was filled with relatively fine carbon particles. Other labeled cells appeared as elongated fusiform cells with elongated nuclei and sparse cytoplasm. These cells were in the process of transformation to either satellite cells or fibroblast.

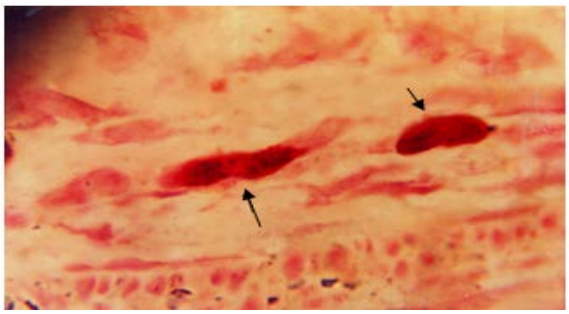

Figure 5. Injure tibialis anterior muscle implanted with hepatic tissue homogenate – Day two after injury. Labeled myoblast (arrows) participated in the formation of doublet. Mayer's carmalum stain (X 1000).

On day three after the injury (Figure 6), myoblasts populated the injured area. Some of them were artificially introduced into the lesion since they were labeled. All of them were just about to fuse to form multinucleated myotubes. Liver specific cells such as hepatocytes can also be recognized.

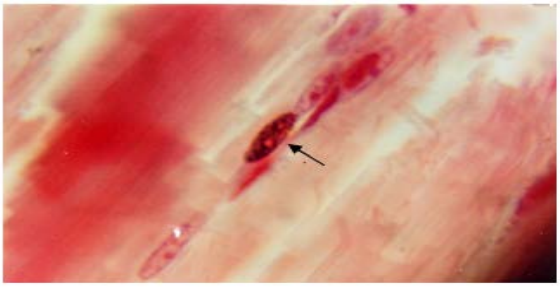

Figure 6. Injure tibialis anterior muscle implanted with hepatic tissue homogenate – Day three after injury. Myoblasts were just about to fuse or had begun to fuse into multinucleated myotubes. Many of them are labeled (arrow). Mayer's carmalum stain (X 1000).

On the fifth (Figure 7), seventh (Figure 8) and fourteenth (Figure 9) postoperative days, the necrotic area is diminished in size as a result of the progressive phagocytic activity of the macrophages. The infiltrating cells were decreased in number, and are mainly macrophages and fibroblasts. The labeled myoblasts in fortuitous area were seen attached to each other longitudinally forming strands termed the myotubes. These were parallel to the general alignment of muscle fibers. These myotubes containing the labeled cells have grown larger and larger at the end of day seven, and formed regenerating muscle fibers at day fourteen, since they appeared striated. However, they appeared smaller in diameter than mature fibers and their nuclei were mainly central.

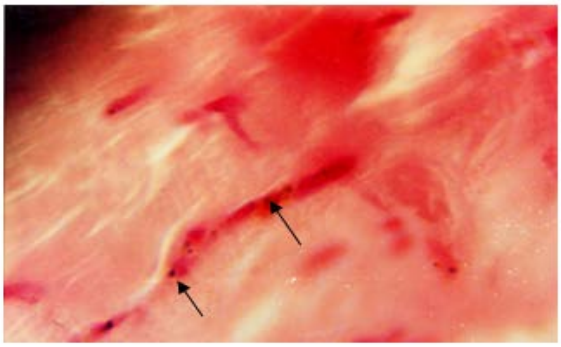

Figure 7. Injured tibialis anterior muscle implanted with hepatic tissue homogenate – Day five after injury. Early formation of myotubes can be recognized. Labeled myoblasts were found in some of these myotubes (arrows). Mayer's carmalum stain (X 1000).

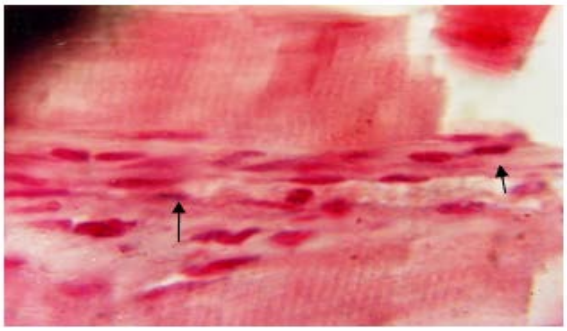

Figure 8. Injured tibialis anterior muscle implanted with hepatic tissue homogenate – Day seven after injury. Long myotubes were recognized parallel to the general alignment of the muscle fibers. Some of them contained labeled myoblasts (arrows). Mayer's carmalum stain (X 1000).

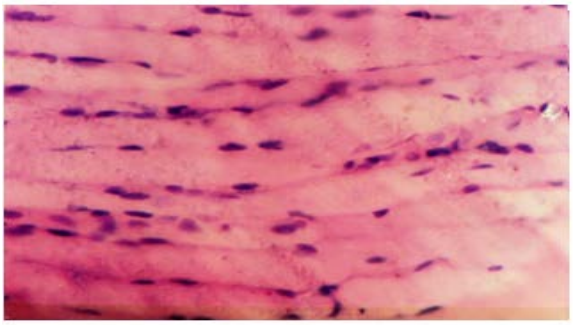

Figure 9. Injured tibialis anterior muscle implanted with hepatic tissue homogenate – Day fourteen after injury. Numerous regenerating muscle fibers were seen. They appeared smaller in diameter than mature fibers and their nuclei were central. H & E stain (X 400).

On day twenty one after injury (Figure 10), the fibers appeared more mature; their nuclei were either central or peripheral. Many of these regenerating fibers were labeled with carbon particles, indicating the engagement of labeled cells in the muscle regeneration. Some labeled cells were identified as satellite cells from their morphology.

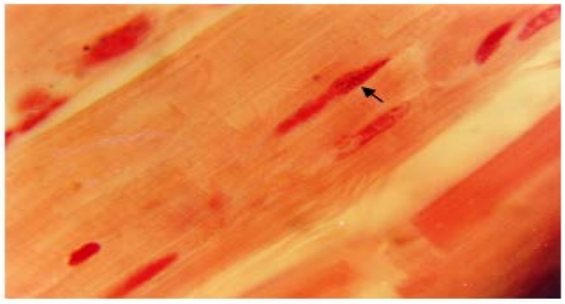

Figure 10. Injured tibialis anterior muscle implanted with hepatic tissue homogenate – Day twenty-one after injury. Regenerating muscle fibers with central or peripheral nuclei can be recognized. Some of them were labeled (arrow). Mayer's carmalum stain (X 1000).

On day twenty eight after injury (Figure 11 and 12), the labeled regenerating muscle fibers showed an increase in their diameter. The fiber diameter nearly equaled a normal one, and striations were clear. Their nuclei were peripherally located. However, some myonuclei were still centrally located.

The regenerative process in the muscles of the control animals, where no liver tissue has been implanted, shows a less formation of myoblasts and myotubes with delayed signs of maturity and excessive fibrosis.

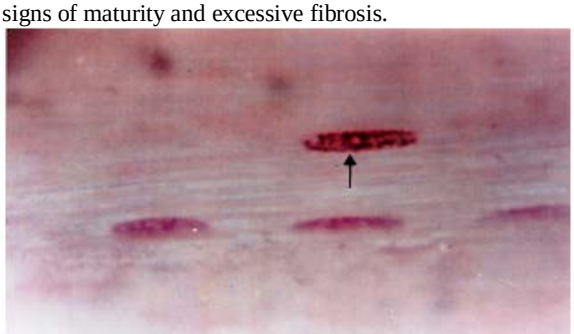

Figure 11. Injured tibialis anterior muscle implanted with hepatic tissue homogenate – Day twenty-eight after injury. Labeled regenerating fibers nearly equaled the normal one, with clear striations and peripheral location of their nuclei (arrow). Mayer's carmalum stain (X 1000).

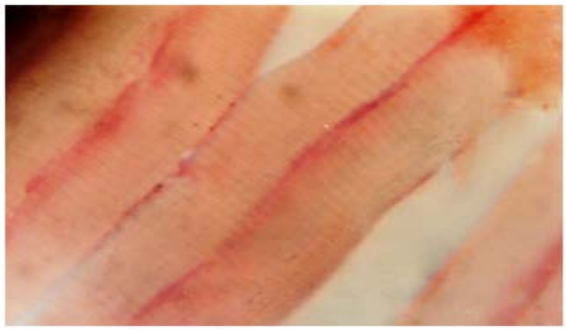

Figure 12. Injured tibialis anterior muscle implanted with hepatic tissue homogenate – Day twenty-eight after injury. Labeled regenerating fibers reached full maturity, showing clear striation with peripherally located nuclei. Mayer's carmalum stain (X 1000).

4. Discussion

Animals used in this study were mature young adult rats due to the presence of more satellite cells in the muscles at a younger age than at an older one (Allbrook, 1981); in addition, the viability of the tissue and its capacity to regenerate can be more in the young adult rats than in the older ones (Allbrook, 1981).

Indian ink was used for labeling the cells of the hepatic homogenate in this study, since cells labeled by Indian ink can be followed and traced for a long time (more than one month) when implanted at the site of the muscle injury. This is because the carbon particles are taken up by the cells (endocytosis) and endocytic vacuoles containing the undigested carbon particles fuse with the primary lysosomes to form secondary lysosomes. Eventually the residual undigested, insoluble contents remain within the cell as storage excretion (Al-Yawer *et al*., 2004). In addition to that, Indian ink was engulfed by the cells of the liver tissue homogenate.

The current results show that the monocytes and macrophages are the main types of cells involved in the phagocytosis of carbon particles in the hepatic homogenates. Monocytes are macrophages in the process of passing from the bone marrow, where they are formed, to peripheral tissues via the blood stream. These cells pass into extravascular sites through the walls of capillaries and venules (Bannister, 1995b). The author stated that macrophages were originally given different names according to their location. In the blood, they are represented by monocytes, whereas in the liver they are called littoral cells of the sinusoid (Von Kupffer cells).

Polymorphs showed poor uptake of the carbon particles, which is represented by the presence of scarce fine granules in their cytoplasm. This finding agreed with that obtained by Al-Yawer *et al*., 2004; Junqueira and Carnneiro, 2005. Polymorphs are active phagocytes of small particle like trypan blue (Al-Yawer *et al*., 2004). Carbon particles are relatively large, therefore, the uptake of this label by the polymorphs was relatively less than the uptake of trypan blue (Al-Yawer *et al*., 2004).

Some cells of a small size and with a relatively large nucleus occupying almost all the cytoplasm showed good uptake of the label in the hepatic homogenate. These cells could be mature lymphocytes or multipotent stem cells, since both of them have nearly the same size (Bannister,

1995b; Junqueri and Carnneiro, 2005). This finding was also reported by Vogelstein *et al*., 2001, who stated that the uptake of carbon particles by these cells can be attributed to their content of lysosomes, and in the case of stem cells, these cells may be differentiated into myoblast.

The tibialis anterior muscle was used for studying muscle regeneration in this project because this muscle is characterized by having a reasonable size with distal and superficial position which makes it easily accessible, and identifiable. Also it lacks interchangeability with other neighboring muscles, and there are no important structures related to it. Moreover, this muscle contains the three major fiber types, white, red and intermediate (Al-Azzawi, 1972). Crushing the muscle with a clamp is commonly used as a mean of studying the reaction of muscle to injury (Al-Hadithi *et al*., 2002; Al-Yawer *et al*., 2004).

After implantation of the hepatic tissue homogenate at the site of muscle injury in the experimental animals, the injured muscle retained its intrinsic capacity to undergo regeneration in response to injury. Therefore, liver enhances muscle regeneration. This may be due to the high concentration of growth factors that hepatic tissue contains. This finding agrees with the results obtained by De La Haba, *et al*., 1975; Bannister, 1995b who concluded that cells of the liver were themselves the manufacturer of growth factors.

In addition, this study shows that the labeled cells of the homogenate of the hepatic tissue participate in muscle regeneration, since the implanted labeled cells succeeded to form labeled myoblasts, labeled myotubes, and finally labeled regenerating muscle fibers. This finding was also reported by Al-Yawer *et al*., 2004, who concluded that the labeled cells of the buffy coat implanted at the site of muscle injury participate in the regeneration of muscle by forming labeled myoblasts, labeled myotubes, and finally labeled regenerating muscle fibers.

The hepatic tissue contains hepatocytes, Kupffer cells, multipotent stem cells, endothelial lining of sinusoids and arterioles, in addition to different types of white blood cells (Janqueira and Carnneiro, 2005). Most of these cells have the ability to ingest a foreign body (Al-hadithi *et al*., 2002). With the exception of multipotent stem cells, liver cells have no ability to be differentiated into other cell type. The role of hepatic homogenate in the regeneration of muscle tissue could be explained as:

(a) The liver is the main source of multipotent stem cells in addition to the bone marrow and the circulating mesenchymal cells. In the present study, labeled stem cells had participated in the process of muscle regeneration by the formation of labeled myoblasts. This finding is supported by other researchers (Vogelstein *et al*., 2001; Alwan, 2004) who pointed out that the multipotent stem cells have the ability to differentiate into multiple cell lineages, one of which is the myogenic pathway. In addition, Al-Azzawi, 1972; Bannister, 1995b; Malouf *et al*., 2001; Shafrtiz and Dabeva, 2002, reported that hepatic stem cells constitute a type of adult multipotent stem cells, which have the ability to be pluripotent stem cells, and differentiate into new cells, other than the cells of the organ from which it was obtained. On other hand, Al-Azzawi, 1972; Al-Taie *et al*., 1994; Caterson *et al*., 2001, concluded that mesenchymal stem cells are a rare population of undifferentiated cells isolated from adult tissue sources, and have the capacity to differentiate into mesodermal lineages, including muscle, bone, cartilage, tendon, fat and marrow stroma. This cell population may be expanded in culture, and subsequently permitted to differentiate into desired lineages.

(b) Other type of cells circulating in the blood and transferring to other tissue is the monocytes or macrophages (Jee and Nolan, 1963; Yarom *et al*., 1976; Turgon, 1988). i. Macrophages act as a scavenger engulfing dead cells and necrotic tissue and keep the field clear to regenerate (Leeson and Leeson, 1985); ii. Macrophages secreted a number of factors, which are chemo attractants for muscle precursor cells (Robertson *et al*., 1993); iii. Macrophages might stimulate the proliferation of muscle precursor cells (Junqueri and Carnneiro, 2005; Robertson *et al*., 1993).

(c) Some of the labeled cells appeared as elongated fusiform cells with elongated nuclei. These cells could be fibroblasts. This is in agreement with the finding of other researchers who found that undifferentiated mesenchymal cells are present in the circulating blood (Leeson and Leeson, 1985; Weissman, 2002; Caterson *et al*., 2001) or bone marrow (Ferrari *et al*., 1988; Al-Taie *et al*., 1994; Seale and Rudnicki, 2000). These cells give rise to fibroblasts when stimulated by injury (Leeson and Leeson, 1985; Caterson *et al*., 2001).

(d) The growth factor which is secreted by hepatic cells enhanced the regenerating process.

(e) Therefore, the only cells that could be differentiated into myotube are the multipotent hepatic stem cells, which can be detected easily by the presence of carbon particles in their cytoplasm.

Based on these observations, it can be said that when liver mince is added at the injured site can promote muscle regeneration. Similar results were reported after implantation of: i. Isolated white blood cells (Al-Yawer *et al*., 2004; Denapoli *et al*., 2016); ii. Platelet-rich plasma (Utomo *et al*., 2018); iii. Bone-marrow centrifugate (Ferrari *et al*., 1988; Matziolis *et al*., 2006).

The delayed appearance of maturity signs and the amount of fibrous cicatrix formed in the control animals indicate that the removal of liver tissue per se does not have any effects on the regeneration of crushed muscle unless the liver mince is added at the injured site.

5. Conclusion

It can be concluded from the present study that liver mince appears to promote the regeneration of crushed muscles by increasing the amount of regenerating tissue, which is recognized in the early days as increased myoblasts and myotubes, and by increasing the speed of regeneration, which is recognized in the late stages as the presence of more advanced signs of maturity. This effect of liver mince on the regeneration of injured skeletal muscles is explained by the effects of growth-promoting factors which are present in high levels in the liver tissue.

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