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Cytogenesis in the Regeneration of Striated Muscles

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CYTOGENESIS IN THE REGENERATION
OF STRIATED MUSCLES

by

Carlos S. Anguizola

A Thesis Submitted to the Faculty of the Graduate School of Loyola University in Partial Fulfillment of The Requirements for the Degree of Master of Science

June

1965
TO MY CHILDREN:

Carlos Santiago,
Juan Antonio,
Maria Guadalupe,
Luis Eduardo
Carlos S. Anguizola was born in Panama, Republic of Panama on September 26, 1926. His primary education was received in David, Province of Chiriqui. His Bachelor Degree was obtained at the Instituto Nacional de Panama in 1946.

From 1948 to 1953 attended the Dental School at the Universidad de Guadalajara, Mexico, where he obtained his D.D.S. Degree.

Upon graduation, ten years were spent in private practice and during the last five years was also working at the Santo Tomas Charity Hospital in Oral Surgery.

In July, 1963, he began a two year graduate program at Loyola University leading to a Master of Science in Oral Biology Degree, and a postgraduate Certificate in Oral Surgery.
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# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>CHAPTER</th>
<th>INTRODUCTION</th>
<th>REVIEW OF THE LITERATURE</th>
<th>MATERIALS AND METHODS</th>
<th>EXPERIMENTAL RESULTS</th>
<th>DISCUSSION</th>
<th>SUMMARY</th>
<th>CONCLUSIONS</th>
<th>BIBLIOGRAPHY</th>
<th>APPENDIX</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>A. Tables</td>
</tr>
<tr>
<td>II</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>B. Figures</td>
</tr>
<tr>
<td>III</td>
<td></td>
<td></td>
<td>A. Experimental design</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>B. Histologic preparation</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>C. Autoradiographic technique</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>D. Method of counting labeled cells</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IV</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>V</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>VI</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>VII</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Page

1
2
24
24
25
27
28
30
41
52
57
59
63
65
CHAPTER I

INTRODUCTION

The biologic concept that the mammalian skeletal muscle following an injury regenerates under favorable conditions, has been well established. Among the various investigators there is an overall agreement on the overlapping stage during muscle regeneration; nevertheless, one problem remains controversial, namely, the origin and identification of the accumulation of nuclei at the growing end of the injured striated muscle producing new muscle tissue, and their mode of reproduction.

The present investigation has been undertaken with the aim of identify those cells capable of such regeneration and to determine their origin and means of duplication. The study was conducted using young albino rats in which striated muscle was injured and then examined microscopically utilizing autoradiographic preparations, and further supplemented by both histochemical and routine histologic techniques.

As result of this investigation, there is a strong evidence to support the concept that the regenerative cell is of connective tissue origin and its mode of division is mitotic.
CHAPTER II

REVIEW OF THE LITERATURE

Striated muscle can regenerate under favorable conditions (Miller 34, Speidel 38, Altschul 42, Le Gros Clark 46, Bets 51, Gay and Hunt 54, Godman 57-58, Bintliff and Walker 60, Zhinkin and Andreva 63, Price, Howes and Blumberg 64). Furthermore, the type of experimental injury used, i.e. incision, crushing, vitamin E deficiency, interruption of blood or nerve supply and various chemical insults, made no significant difference in the capacity for repair.

A constant finding throughout the various investigations has been the accumulation of nuclei centrally located in injured striated muscle. In fact, the literature repeatedly alludes to Waldeyer (1865), who was the first to describe such an accumulation of nuclei in injured muscle. In addition to their increased number, Waldeyer described their pattern in rows; the so-called "Waldeyer cell tubes".

In the years that have followed Waldeyer, many investigators have theorized about the increased number of subsarcolemmal nuclei and in particular about their mode of reproduction.
Tower (1935), believed the nuclear increase to be due to a nuclear stimulant set free by the degenerating nerve. Speidel (1938), believed a loss of "chemical equilibrium" to be the existing factor behind nuclear accumulation. Soon afterward, Altschul (1942), attributed the numerical increase of subsarcolemmal nuclei to a decrease in intracytoplasmic tension due to the loss of cell particulate material following injury. Again, Altschul (1947), suggested the cause of nuclear proliferation resulted from a loss of equilibrium in pressure between the nuclei and sarcoplasm brought about by waste products of the degenerating sarcoplasm and the myofibrils. He emphasized that this increase in the number of nuclei was not only a relative one due to a decrease in the volume of the parenchyma, but also a real and absolute increase.

Lash (1957), tentatively proposed that the accumulation of the centrally located nuclei was the result of mobilization, not the extensive proliferation within the regenerating myotube. However, because of the difficulty in analyzing the movements of muscle nuclei, he offered a second theory which he favored: that mononucleated cells proliferated in the wound region and secondarily coalesced to form myotubes.

Although cell proliferation, accumulation, and regeneration
undoubtedly occurs in striated muscle, there is an overall disagreement among investigators as to the cell source for new myotubes and the mode of accumulation of these cells.

Forbes (1926), injected powerful irritants such as alcohol, phenol or boiling water into muscle or interrupted the blood supply to a voluntary muscle in rats for a three hour period to produce degenerative changes. His main purpose in these experiments was to study the origin of the cells that contributed to the ensuing regeneration. He determined that the sarcolemmal tube of damaged muscle fibers contained both phagocytic cells as well as groups of other cells which were morphologically different and he believed these cells to be of muscle origin. Moreover, these cells did not ingest previously injected vital dyes, but if the administration of dyes was continued during the experimental period such cells stored dyes in large quantities.

Forbes concluded that phagocytic cells were of extramuscular origin but the regenerating muscle fibers arose from nuclei and sarcoplasm of the preserved muscle fibers.

Miller (1934), studied regeneration in the rectus femoris of the rabbit which he either crushed or incised. He felt that the accumulation of histiocytes was the most striking feature and remarked that few mononuclear cells were seen from three to five
days after injury. Miller believed the muscle nuclei to be in active proliferation since they were present in chains of three to eight or more, in closed contact or joined.

In his interpretation of mononuclear cells which he called muscle giant cells, Miller cited Dawson (1908), who earlier pointed out that these cells were formed by muscle substance. Miller found that the majority of these muscle segments died and were reabsorbed. However, some muscle segments survived and demonstrated multiplication of nuclei and increased in their content of sarcoplasm.

Miller believed the nuclei divided amitotically and felt this concept to be in harmony with Dawson's findings as well as those of Schmincke (1908). Miller found no trace or sign of mitotic proliferation.

Tower (1935), who studied the regeneration of injured muscle in cats over a period of two weeks to one year, held the opinion that after denervation and atrophy, one activity reasumed by the nuclei was division. Mononuclear round cells also were present; small cells not more than 10 microns in diameter, with the nucleus filling more than half a cell were identified as monocytes. Larger cells were identified as plasma cells on the basis of their chromatin arrangement. A few such mononuclear cell
types were always found in the connective tissue surrounding normal muscle and felt it possible that they arose from this source. However, the concentration of these monocytes and plasma cells within and just outside of blood vessels, and frequently in the process of passing through the vessel wall, spoke more strongly of their blood origin.

Tower (1939), returned to the problem of muscle nuclei proliferation and concluded that they changed in form, increased in number, and in spite of the fact that cell division was not actually observed, the increase in nuclei was attributed to amitotic division.

Altschul (1942), produced atrophic changes in selected leg muscles of cats and rabbits by severing the sciatic nerve. He concluded that the numerical increase of subsarcolemmal muscle nuclei in atrophy was due to the decrease in tension inside the fiber resulting from the loss of cell material. He reported that there occurred a metaplasia of muscle nuclei into connective tissue cells similar to fibroblast in morphology. During the course of this study which extended to 113 days, an increase in muscle nuclei was evident and was attributed to amitotic cell division.

Le Gros Clark (1946), studied the microscopic changes following crushing injuries to muscle in rats over a period of
three months. At three days, the first sign of regeneration was seen as a plasmodial outgrowth of granular sarcoplasm that protruded from the stump of the injured muscle fibers. Accompanying this regeneration was a local increase in histiocytes and fibroblasts as well as an infiltration of acute inflammatory cells from the blood.

An important feature during the first few weeks was the establishment of a new endomysial scaffolding and the phagocytosis of degenerated muscle fibers, which filled gaps between the sarcoplasmal outgrowths.

The muscle outgrowth from either edge of the wound characteristically presented chains of centrally positioned nuclei which maintained this relationship until the opposing extensions met and fused. Le Gros Clark proposed that the increase in muscle nuclei arose by amitotic division and that the principal source of new fibers was by direct and continuous outgrowth from surviving fibers. Some mitosis was seen within regenerating fibers, but this was attributed to the histiocytes and not the muscle nuclei.

During the latter stages of healing the connective tissue elements, cells and fibers, decreased until at three months healing was considered to be complete.
Le Gros Clark contrasted his results with those of Lavander (1941-45), who observed the differentiation of connective tissue cells by inductions into myoblasts. Lavander's concept was supported by Chevremont (1940), who had reported the direct transformation of histiocytes into myoblast in vitro.

In a review of muscle regeneration, Altschul (1947), described a swelling of the terminal portion of injured muscle fibers, into a "club-like" enlargement. The terminal swelling was felt to be the result of amitotic division of the nuclei at this site followed by the elaboration of new cytoplasmic material.

Amitotic division leading to budding and reformation of muscle fibers was described by Constance (1955), following experimental injuries by incision, excision and crushing of the leg muscle in seven guinea pigs. The animals were sacrificed at intervals from three to forty two days.

Constance identified most of the nuclei in the myotubes as histiocytes concerned with the removal of necrotic muscle substance. The muscle nuclei within the new formed fiber showed considerable degree of proliferation and their division was essentially amitotic. Whenever a mitotic figure was seen in the muscle fiber, it was attributed to histiocytes and fibroblasts.
Degenerative changes in skeletal muscle of the rabbit during avitaminosis "E" and their subsequent regeneration was studied by Gatz (1952). He found that some atrophic muscle fibers presented central rows of spherical nuclei while other such fibers showed mitotic figures as well, in this new central position. Gatz believed that normal muscle nuclei migrated from a peripheral position as a result of injury, and subsequently divided mitotically.

Following the obstruction of the nutrient vessels to the tibialis anterior of fifty adult rabbits, Godman (1957), studied the induced areas of infarction and resulting coagulation necrosis. In many fibers there was an increase in muscle nuclei surrounded by new sarcoplasm. The fate and development of the surviving muscle nuclei and sarcoplasm from segments whose myofibrils had degenerated depended upon local conditions. Muscle nuclei centrally located within degenerating fibers, migrated toward the cut end of the muscle, forming a terminal cluster.

Isolated macrophages and fusiform sarcoblasts and myoblasts were seen formed by the transformation of the nuclei in the sarcoplasm of muscle.

Individual cells, short chains of cells, slender plasmoidal slips, broad plasmoidal ribbons, and the multinucleated muscle
buds (muscle giant cells) all developed from the surviving muscle protoplasm.

Mitotic figures were commonly seen in the isolated round cells (macrophages).

He concluded that the general absence of mitosis in muscle nuclei, the juxtaposition of muscle nuclei in longitudinal doublets, triplets, etc. and the partial separation of nuclei by membranes and contractions, were evidence of amitotic division.

Two electron microscopic studies, Allbrook (1962), Price, Howes and Blumberg (1964), agree on the characteristic features of degeneration and regeneration of striated muscle, but are distinctly different in their interpretation of the cell type responsible for new fiber formation. Aside from this lack of agreement, both studies greatly extend the information compiled by experiments restricted to routine histologic studies at the optical level.

Allbrook produced crushing and ischemic injuries to the skeletal muscle of mice and rabbits and studied the electronmicrograms of the regeneration over a three week period.

By six days, lymphocytes and granular leucocytes were seen both inside and outside of muscle fibers. Macrophages were present, as were young fibroblasts identified by their associa-
tion with collagen fibrils. Myoblasts also were seen, some of which were spindle shaped and binucleated.

Under the light microscope both the young fibroblast and myoblast were typified by a basophilic cytoplasm. The mononuclear cells seen in the early stages of regeneration showed different amounts of cytoplasmic basophilia and nuclear density. These characteristics as seen through the light microscope were not considered reliable enough to differentiate between myoblasts, myocytes, fibroblasts or endomysial cells. With the electron microscope, however, it was possible to classify individual mononuclear cells by their relation to other elements such as myofibrils or collagen fibrils.

In regard to the sarcolemmal tube it was established that following severe injury the entire sarcoplasm, including the sarcoplasmic membrane was phagocytized, but the basement membrane was resistant and remained as an intact sarcolemmal tube which limited and guided the growth of regenerating fiber. Within the regenerating sarcolemmal tubes there were clusters of phagocytes, some of which fused by breaking down their cytoplasmic membrane, thus forming a syncytium. It seemed that within a sarcolemmic tube the myotube and its nuclei were derived from a fusion of the original muscle nuclei, which may or may not have passed through a phagocytic phase. Isolated myocytes were incorporated into the
growing syncytium of the myotubes.

Allbrook concluded that striated mammalian skeletal muscle regenerates from the pre-existing muscle nuclei. Many cells in the adjacent connective tissue had one or two nuclei, basophilic cytoplasm, small myofibrils and two discs in an intensely granular background identified as myocytes; such myocytes were observed in mitosis in the connective tissue. Neither individual myocytes nor an individual myotube alone was responsible for the production of mature muscle fibers. This was suggested by the lateral break down of the intervening sarcolemma between adjacent myotubes. Hence, myotubes were formed by the fusion or coalescence of myocytes.

Price, Howes and Blumberg (1964), conducted a series of experiments using cold to produce controlled lesions of the tibialis anterior muscle of rats. They divided the study in two parts: first, the degenerative changes occurring in skeletal muscle that had been injured by cold; second, the participation of cells of the sarcolemmal tube in regeneration and myofibril formation.

The sarcolemma was described as having a double membrane: the plasma membrane and a basement membrane. The basement membrane of the muscle fibers was the most resistant to injury by
cold. The plasma membrane at twenty four hours after injury had disappeared, while the basement membrane survived as the principal component of the sarcolemmal tube. However, there was no basement membrane around the myoblasts during regeneration when cell fusion and mobility were essential. Price, Howes and Blumberg explained fusion as an aggregation of myoblasts by loss of their contacting plasma membranes followed by reformation of the basement membrane.

The second part of his work was directed toward muscle regeneration which emphasized the differentiation of the isolated cells of the sarcolemmal tube, which favored the multicellular theory of muscle fiber origin and supported the "discontinuous" mode of skeletal muscle regeneration as against the "continuous or budding" regeneration theory.

At four days, using the light microscope, three different types of free single cells were identified: macrophages, myoblasts and polymorphonuclear leucocytes and lymphocytes. The most prominent was the macrophage.

It was reported that in electromicrograms the macrophages had an irregular contour not limited by a basement membrane structure.

A fusiform cell type located against the inner wall of the
sarcocremmal tube contained an elongated cytoplasm that appeared to touch similar adjacent cells where their ends met or overlapped. These uni- and bi-nucleated spindle cells were identified as muscle fibers precursors and classified as myoblasts. At eight or nine days these elongated cells were seen to contain an alignment of nuclei. The fusiform cell type which he called early myoblasts, eventually became multinucleated along the periphery of the sarcocremmal tube. No mitosis or amitosis was observed. However, few mitotic figures were identified in this investigation and those seen were attributed to histiocytes.

Differentiation between mononuclear cells destined to become myoblasts and typical fibroblasts was made. The early mononuclear myoblast did not form any absolute part of a plasmoidal extension arising from muscle at the margin of the lesion. They were isolated cell entities that eventually fused to form a multinucleated muscle cell.

Three reasons favoring the cell fusion theory were observed by these investigators: first, the spacing of nuclei within the multinuclear cell corresponded to the spacing of mononuclear cells before fusion; second, the absence of mitotic or amitotic figures; and third, the break down of the plasma membrane of adjacent mononuclear cells.
The remainder of this review is devoted to four investigations in which tritiated thymidine (TH₃) was used to study either the embryonic development or regeneration of striated muscle.

The use of thymidine H₃, for autoradiographic studies has become a valuable aid in histologic and histopathologic studies.

Thymidine is a specific desoxiribonucleic acid (DNA) precursor and is incorporated into the nucleus in the cromosomal replication, phase G-1, prior to mitosis, (Taylor, et al. 1957; Amano, et al. 1959; Lajtha, et al. 1959). In this way the different cell population and migration can be traced autoradiographically, (Hughes, et al. 1958; Leblond, et al. 1959; Messier and Leblond, 1960).

Bintliff and Walker (1960), used thymidine H₃ to label cell nuclei in a series of experiments in which the tibialis anterior in mice was injured.

The experimental animals were divided into three groups. A control group was not injected with thymidine H₃; a second group A was injected at varying time intervals prior to injury; and a third group B was injected at varying time intervals following injury. In both group A and group B, the animals were then sacrificed at various selected intervals throughout the experiment.
After two days, the border of the experimentally injured area was defined by the formation of the muscle stumps. Surviving endomysium extended from the borders of the muscle stump into the wound area. The cellular infiltrate consisted primarily of granular leucocytes, but endomysial cells and macrophages were present.

One and one half days after injury the first morphological changes of the muscle nuclei appeared: enlargement and vesiculation and hypertrophy of nucleoli. The altered nuclei were found in muscle stumps and fibers near the wound area. A large unclassified basophilic cell, found in the coagulum, was observed to undergo mitotic division. Also they observed that mitosis never occurred in the nuclei found in the muscle stump.

Row alignment of the muscle nuclei within the sarcolemmal tube containing basophilic cytoplasm occurred at four days after injury. Between four and twenty six days healing and regeneration gradually occurred.

In group A thymidine H^3 was administered far in advance of injury to the muscle. This allowed polymorphonuclear leucocytes labeled in the bone marrow to be in the circulating blood at the time of injury. Thymidine H^3, however, was no longer available to muscle cells in DNA synthesis by the time of injury. In this
way, it was shown that most cells in the wound site were of vascular origin, and while they contributed to the healing, they did not contribute to the formation of new muscle fibers.

In group B, a large radioactive cell population appeared as a consequence of injury since non-injured muscle fibers showed almost no radioactive cells. They observed two labeled cell populations aside from the blood cells. One such cell population was composed of fusiform cells with moderately elongated nuclei and light chromatin density. Some such cells formed part of capillary walls, while others were endomysial, and a few of them belonged to no defined structure. Capillaries and endomysium underwent repair as indicated by the number of labeled cells present in these tissues.

The second population of radioactive cells, which was distributed similarly to the first, had a slight variation in nuclear morphology and a radical variation in cytoplasmic morphology. The investigators combined these two morphologically different cell populations into a single group because as one cell type decreased the other showed a proportionate increase. This shift in cell morphology was interpreted as a sequence of differentiation and redifferentiation.

Since the fate of all other radioactive mononuclear cells
in the wound area had been accounted for, these two related cell populations were believed to have been derived from injured muscle. Once these cells entered the wound area they became myoblasts, proliferated and subsequently produced a larger but more weakly labeled population of cells. Eventually, the number of myoblasts diminished and myotubes with radioactive nuclei appeared. The latter must have been derived from the former either by fusion or by karyokinesis without cytokinesis. The authors believed enough evidence was available to demonstrate that myotube formed by cell fusion.

Walker (1961), studied the regeneration of tibialis anterior muscle in seven mice with hereditary muscular dystrophy. Tritiated thymidine was given two days after injury and the animals were killed at different times ranging from twelve hours to twenty-eight days after injection.

The radioautographs of regenerating fibers were distinguished from the surrounding distrophic fibers by the presence of many labeled nuclei. Initially, radioactive myoblasts were seen in the injury; and at four days radioactive labeled nuclei were seen in basophilic myotubes. At seven days radioactive nuclei were present in narrow eosinophilic fibers containing myofibrils. Between fourteen and thirty days after injury labeled nuclei were
centrally positioned in normal muscle fibers.

Gerber et al. (1982), used tritiated thymidine in an autoradiographic study of striated muscle following degeneration due to vitamin E deficiency in two series of Golden Hamsters.

Thirteen Hamsters were deprived of vitamin E, while seven Hamsters were maintained on the same vitamin E deficient diet but supplemented with tocopherol. The animals were killed at different days after thymidine H^3 injection. Specimens were taken from the rectus abdominus, pectoralis major and masseter muscles.

Labeled nuclei were seen only in the areas of degenerative lesions in vitamin E deficient muscle. The labeled cells were mostly macrophages, leucocytes and fibroblasts. Labeled cells decreased markedly from three to seven days after thymidine H^3 injection.

In vitamin E supplemented and previously vitamin E deficient animals, labeling was also observed in fibroblasts and macrophages. However, because of the absence of any lesion, a considerably smaller number of cells per field of view was labeled.

Aligned nuclei were not labeled to any great extent. Rowed nuclei represented displaced sarcolemic nuclei that had undergone dedifferentiation to a myoblastic stage, but did not
undergo cell division.

Zhinkin and Andreva (1964), using tritiated thymidine autoradiograms studied the interrelation between mitosis and DNA synthesis in muscle of albino rats and mice.

Albino rat embryos fourteen to twenty one days old were injected with thymidine H\(^3\) and killed at intervals between four to twenty four hours after injection. Over a twenty four hour period the number of labeled nuclei double but the intensity of the labeling decreased by one half. Therefore, in a twenty four hour period most of labeled nuclei had divided at least once in the developing muscle fibers of the seventeen days old embryo. Later, a decrease in mitotic activity as well as a parallel decrease in the number of labeled cells was attributed to normal embryonic growth. This was accompanied by an increasing number of nuclei which underwent differentiation and subsequently engaged in the synthesis of specialized contractile proteins without undergoing further reproduction.

Muscle development was accompanied by what could only be diagnosed as mitotic division. In their opinion, the amitotic division described by other investigators was ascribable to nuclear movement within the developing muscle fiber and a temporary pairing of nuclei. This pairing was believed to be a "random colli-
sion" within the fiber and in no way related to mitotic or amitotic duplication.

In the second experimental series, the rectus femoris muscle was cut in mice, while in rats the sartorius muscle was incised.

The rats were killed four hours after injection of thymidine H³ at two, four and six days after operation. One rat was injected with thymidine H³ four days after injury and killed two days later.

The albino mice were treated with thymidine H³ at two, four and six days after operation; and four, twenty four and forty eight hours prior to killing.

In mice, the first signs of activation of muscle nuclei were an increased nuclear size and a rise in the DNA content of the cytoplasm. At four days, myotubes were forming at the basophilic stained ends of the cut fibers. Large nuclei frequently were arranged in rows within the traumatized fibers. This nuclear alignment also was found in fibers not directly traumatized but near the area of inflammation or adjoining traumatized fibers.

At six days, the muscle buds had grown in length, and when in contact with the granulation tissue they became broadened.

Similar features were observed in the experimental rats but
the regenerative process here proceeded at a slower rate.

In rats and mice, small labeled nuclei with an elongated shape were also described adjoining the sarcolemma tubes from the outside. The largest number of labeled nuclei seen synthesizing DNA were found at the ends of cut muscle fibers where they acquired a particularly large size. Their number decreased proportionally to the distance from the wound. There was an appreciable difference in the number of labeled muscle nuclei depending upon the elapsed time between injury and time of thymidine $H^3$ injection, and time of thymidine $H^3$ injection and sacrifice.

In mice, at four days when muscle buds had already formed, and after thymidine $H^3$ was injected four hours prior to sacrifice, there was a sharp reduction in labeled nuclei.

In rats, labeled nuclei in the myotubes were observed at two and four days after the operation. At six days after operation, when thymidine $H^3$ was given forty eight hours prior to death, aggregations of large numbers of labeled muscle nuclei in muscle buds were seen.

These findings in rats agree with those of Bintliff and Walker (1960), namely, the synthesis of DNA, chromosomal replication and formation of the bulk of the nuclei, have already
occurred prior to the formation of muscle buds. Zhinkin and Andreva concluded that both during embryonic development and during muscle regeneration, the nuclei must first undergo a period of DNA synthesis and reproduction in the same manner as in any other tissue.

In the seventeen day rat embryo, the mitotic cycle in muscle took about twenty hours and the duration of mitosis itself was two and one half hours. Comparison of the number of nuclei participating in the mitotic cycle with the mitotic index suggested that as differentiation proceeded some nuclei synthesize specialized proteins while others had undergone reproduction.

At two days after operation in regenerating muscle, most labeled nuclei were found at the ends of injured muscle fibers, and correspondingly the mitotic index was high. The number of labeled nuclei and mitotic figures decreased with the distance from the wound.
Experimental Design

Twenty young adult Sprague Dawley rats, weighing one hundred and fifty to two hundred grams were used. They were maintained on a Purina Lablox and water diet.

The animals were divided into two series and placed in wire bottom cages marked Series 1 and Series 2; furthermore, in Series 1 the rat's ears were notched according to Billingham and Silver technique (1961), to identify properly the individual animals.

All animals underwent surgery on the same day following ethyl ether inhalation anesthesia. The abdominal hair was shaved and a skin incision and dissection performed in order to expose the right external oblique muscle. Once exposed, each right external oblique muscle was experimentally injured at selected sites by producing either an incision, crush or punch wounds. Lifting the muscle by its superficial surface between the beaks of a mosquito forceps caused the crush injury; then by cutting into the muscle with the tips of a pointed scissors, after it had been lifted to form a ridge-like fold, an oblique incision was made approximately 8.0 mm in length across the long axis of
the muscle. Finally, a punch wound about 2.0 mm in diameter was produced in a second fold of muscle which was squeezed between the pinion and recess of a dental rubber-dam punch. No approximation of any tissue was made except the skin which was repositioned and sutured with number 4-0 silk thread.

The animals in the Series 1 were injected intraperitoneally with tritiated thymidine (specific activity 1.9c/mM)\(^1\) six hours after surgery, at a rate of 1 c for each gram of body weight. The animals in Series 2 were given identical injections of thymidine H\(^3\), but were not injected until one hour prior to sacrifice.

The two series of animals were anesthetized by inhalation of ethyl ether and killed by decapitation at: 10, 13, 16, 24, 36, 44 hours and at 3, 4, 6, 8 days after injury.

The skin incision was reopened, the overlying fascia dissected, and the injured muscle exposed and excised in toto. All specimens were identified and placed in Carnoy's fixative.

**Histologic Preparation**

The specimens were kept in Carnoy's fixative from one to two hours. They were washed three times for one half hour in one hundred per cent ethyl alcohol. The specimens were then washed

\(^1\)Schwarz Bioressarch Inc., Orangeburg, N. Y.
in three physiologic saline changes each ten minutes in duration. The specimens finally were embedded in three one half hour paraffin changes, (paraffin melting temperature 56.5°C,) and poured into blocks. The specimens then were sectioned at three to five microns using a rotary microtome.

All sections were stained by the following methods:

Silver impregnation
Periodic acid Schiff (P.A.S.)
Alcian Blue - P.A.S. (PH-2.6)
Methyl Green Pyronine
Toluidine Blue
Hematoxilin and Eosin
DNA

Gamori silver impregnation of reticular fiber
McManus
McManus
Methyl green pyronin ribonuclease method for RNA (Brachet)
Gurr's Methachromatic stain
Harris's alum hematoxilin
Tomassi's method

The sections were then mounted on standard 7 1/2cm x 2 3/4cm glass slides and cover-slip affixed.

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2Tissuemat from Fisher Scientific Co., Chicago, Ill.
Autoradiographic Technique

To prepare the autoradiographs NTB₃ Emulsion was used.

The equipment and supplies needed for the autoradiography included: NTB₃ emulsion, slides, water bath, metal warming plate over with hole in the center for a coplin jar, Lithium chloride for storing with film, plastic tape for sealing the boxes and paper towels to drain excess of emulsion from slide.

As NTB₃ emulsion is stored below 30°C before use in a gel state, it is allowed to stand to come to room temperature. Then the emulsion is placed in a water bath for fifteen minutes at 43°C, slowly and gently rotating the emulsion to mix its contents without making any bubbles; it is then poured into a coplin jar (approximately 40 ml.) to which is added one to two drops of Tween 20, stirred gently and allowed to stand until the NTB₃ has reached a temperature of 43°C.

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4Laboratory Procedures for Tritium Autoradiograph compiled by Emil R. Adamik, Division of Experimental Pathology, Medical Research Center, Brookhaven National Laboratory, Upton, N. Y.

5NTB₃ Emulsion from William A. Sykes, Research Division, Special Products, Rochester, N. Y.

6Lipshaw water bath by Lipshaw Manufacturing Co., Detroit, Michigan

7Fisher Scientific Co.
The slides were placed on a metal plate heater warm to 43°C and then transferred to a coplin jar containing the NTB₃ emulsion for fifteen seconds. The slides removed from the emulsion were then set in a drying rack with the frosted end up in order to permit the NTB₃ emulsion to drain down on a wet paper towel for fifteen seconds.

The emulsion was permitted to gel on the slide for a period of five to ten minutes and placed to dry before air circulated by a fan. When dried, the slides were stored in boxes with Lithium chloride, and the box lids were sealed with black tape. Such boxes of slides were refrigerated for two weeks for purposes of exposure before development of the autoradiograph.

Under safe light illumination, Wratten number 2, the slides were transferred from the storage boxes to D 19 Kodak developer for two minutes; rinsed in water five to ten seconds; placed in Kodak fixative for two minutes; and, finally, were washed in water for ten minutes; dried and placed in slide storage boxes.

Methods of Counting the Labeled Nuclei

The area of injury was arbitrarily divided into three slightly overlapping zones: The exudate Zone A; the injured and surviving muscle fibers Zone B; and the immediately adjacent
uninjured muscle Zone C.

The number of labeled nuclei in each of fifteen adjacent oil immersion fields was counted within each of the above zones. The mean of the labeled nuclei was calculated. A table and chart of the frequency distribution of labeled nuclei in each zone was prepared for each sacrifice interval in Series 1 and Series 2.

The criteria for labeled nuclei was based upon the number of type grains found above each cells. A minimum of five grains above background was selected as the basis for counting.
CHAPTER IV

EXPERIMENTAL RESULTS

The morphologic changes in injured muscle leading to regeneration as observed in Series 1 and Series 2 were essentially identical. Furthermore, such changes as seen in cutting, crushing and punching also were identical. Therefore, the description to follow can apply equally both to Series 1 and Series 2 and to the different modes of injury.

The changes observed in the injured muscles included the epimysium, perimysium, endomysium and the muscle fibers. The supporting tissue of muscle contains small arteries, veins, lymphatics, nerve fiber bundles, loosely bound by loose connective tissue. Capillaries and perivascular undifferentiated mesenchymal cells formed the principal supporting tissue in the endomysium.

The endomysial capillaries are intimately related to the sarcolemma of the muscle fibers by acid mucopolysaccharides cementing substance as seen in sections stained metachromatically with Alcian blue-P.A.S. and by neutral mucopolysaccharides as seen in P.A.S. stained sections.

Argyrophilic fibers in a regular arrangement surround each
capillary and each muscle fiber as seen in silver impregnated sections. (Figure 3)

The muscle fibers were multicellular with subsarcolemmal location of nuclei. The myofibrils and sarcoplasm appeared homogeneously pyronineophilic as seen in Methyl green-pyronine stained sections.

Mononuclear cells resembling endothelial cells or fibroblast-like cells could be found in the mucopolysaccharides between the capillaries and the muscle fibers. Such cells had nuclei of round to slightly ovoid shape. The chromatin was reticulated and pale. The nuclear sap appeared vacuoloted. There was at least one and frequently two nucleoli. The cytoplasm contained pyronineophilic granules. The cell outline was undefined. There was evidence of mucopolysaccharides in some such cells as seen in the Toluidine blue stained sections. (Figure 4)

The capillaries of the endomysium and the accompanying mononuclear cells coursed together, sometimes parallel to the long axis of the muscle, sometimes obliquely or at right angles to the long axis of the muscle fibers. In contrast, the muscle nuclei always coursed along the long axis of the muscle fibers.
The morphologic difference between muscle fiber on one hand, and perivascular mononuclear cells and endothelial cells on the other hand was the presence of pyronineophilic granules in the cytoplasm of the latter, but diffuse pyronineophilia in the former.

The changes in the injured muscle over the observed period of eight days can be divided into three overlapping phases: necrosis and inflammation; proliferation; and differentiation.

The earliest changes in injured muscle were characterized by a break in the continuity of the striated muscle fiber bundles, (Figure 5) created by the experimental injury. The least severe break in the continuity of the muscle fibers was seen in the crush type injury.

The sarcoplasm appeared to "spill" out of the muscle fibers into the interstitial tissue spaces and into the gap created by the discontinuity of injured muscle. The muscle fibers significantly lost some or all pyronineophilia but the interstices correspondingly gained in pyronineophilia as a diffuse distribution could be seen with Methyl green pyronine stain. (Figure 6)

The injured muscle fibers, Zone B, and the adjacent intact muscle fibers, Zone C, which appeared in direct continuity
separated along their long axis from neighboring muscle fibers giving a splayed-out appearance. This observation was enhanced in many areas as the vascular components of the endomysium clearly stood separate from the sarcolemma.

The argyrophilic fibers were lost or greatly disorganized both around the injured and apparently intact muscle fibers, as well as from around the capillaries of the endomysium in the area of injury. (Figure 7) Neutral mucopolysaccharides were present in the interstitial tissue as seen with the P.A.S. reaction, but a loss of acid mucopolysaccharides as indicated by loss of gamma metachromasia (red-violet color) as seen in sections stained with Alcian Blue-P.A.S. (Figures 8 and 8A)

The capillaries were dilated to three or four times their normal diameter in the endomysium, perimysium and epimysium. The endothelial cells were rounded instead of their normal fusiform shapes. The areas between the "splayed" muscle fibers, the gap in the muscle created by the injury, and the epimysium were filled with a fibrin clot and extravasated erythrocytes caused by the direct injury to the capillaries.

Polymorphonuclear neutrophilic leucocytes were found diffusely infiltrating the epimysium, perimysium and endomysium.
They could be found within the injured muscle fibers, and in addition, were seen within the sarcoplasm of the adjacent intact muscle. Serving as macrophages, cells of the blood were apparently phagocytizing the myofibrils, contributing these to sarcolytic changes already caused by the injury. Accompanying the polymorphonuclear leucocytes within the muscle fibers were mononuclear macrophages.

The mononuclear cells were few in number during the first twenty four hours as compared to preponderance of polymorphonuclear neutrophilic leucocytes. It appeared that the macrophages arose from the perivascular indifferent mesenchymal cell, i.e., reserve cell, and penetrated the sarcolemma of the adjacent reacting intact muscle and were accompanied by polymorphonuclear neutrophilic leucocytes.

Sarcolysis and phagocytosis (Figures 9 to 12) continued through forty-eight hours. There was only a slight increase, if any, in macrophages contributing to the phagocytosis of the muscle fibers during this time.

The epimysium and the zone of loss of muscle continuity showed the greatest volume of inflammatory exudate. It was infiltrated by polymorphonuclear leucocytes, monocytes of the blood
and lymphocytes. The exudative Zone A could be distinguished, if not precisely defined, in all sections examined. However, such exudate extended slightly into the perimysium and endomysium of the injured muscle fibers.

The injured muscle fibers with loss of sarcoplasm, Zone B, and their separation from adjacent parallel injured muscle fibers also could be recognized in all the sections examined. This injured zone continued almost imperceptibly into the adjacent reactive intact muscle fibers of Zone C.

In the latter zone, intact muscle fibers also became separated and splayed out and were attacked both by polymorphonuclear leucocytes and macrophages.

The distribution of tritiated thymidine labeled cells of the exudate Zone A, and the labeled cells of the endomysium of injured muscle fibers Zone B, and intact adjacent muscle fibers Zone C, (Figure 1) as seen in autoradiograms of Series 1 and Series 2 are found in Tables 1 and 2.

In Series 2, only few lightly labeled (1-3 grains) polymorphonuclear leucocytes ever were seen in the dense exudate, while in Series 1, labeled polymorphonuclear leucocytes rarely were seen during the first sixteen hours, but rose rapidly
betwen sixteen and twenty four hours. In Series 1 and Series 2, the mononuclear perivascular cells, while rarely seen, were heavily labeled. The grains were so closely packed that counting them was impossible.

The distribution of labeled PMN's in Series 1 which rose after sixteen hours, held steady until thirty six hours when they began gradually to fall. (Table 1, Figure 1)

The distribution of labeled mononuclear cells showed a rise after sixteen hours both in Series 1 and Series 2. (Figure 2) Endothelial cells were found labeled in both series. Without exception, preexisting muscle nuclei were never found to be labeled in either series.

The labeled mononuclear cells in Series 1 showed a sharp rise in number after a lag period of approximately sixteen hours. This sharp rise levels off to a plateau after three days and extended through the duration of the experiment. (Figure 2)

The grains over the labeled nuclei in the endomysium in Series 1 are fewer in number after four days as compared to those seen during the first sixteen hours.

The grains over the labeled nuclei of the endomysium are always dense and numerous in Series 2. However, in contrast to
Series 1, the number of such labeled nuclei is low and furthermore, begin to fall after six days. (Figure 2)

The rise in labeled mononuclear cells as seen in Series 1 while initially low in number, clearly shows that the synthesis of dextrosepyrimidine acid began after a sixteen hour lag phase. (Figure 2)

The fibrin clot is invaded by indifferent mononuclear cells and capillaries at twenty eight hours. The clot shows organization throughout the epimysium, perimysium and endomysium. The number of capillaries increased through four days; and remained widely dilated through eight days. Some endothelial cells and the accompanying mononuclear cells organizing the blood clot were labeled.

The mononuclear indifferent perivascular connective tissue cells rapidly increased in number between two and four days. (Figure 2). Labeled figures were frequently seen in the interstices around capillaries. (Figure 14) Such perivascular cells were the source of the mononuclear cells invading the fibrin clot with the capillaries.

The intimacy of such indifferent cells with their accompanying capillary had suggested that some capillaries may have
arisen from such cells early in clot organization. However, as endothelial cells are labeled in Series 2, it is clear that they also proliferate.

The proliferative activity of interstitial undifferentiated connective tissue cells resulted in local aggregations of such cells between the capillaries and the surviving muscle fibers at three days. (Figure 15) Such aggregates of cells were found parallel to the muscle fibers and over the cut ends of injured muscle fibers.

The injured muscle fibers stained pale with Methyl green and pyronine. (Figures 6 and 16) However, the mononuclear cells showed intensely pyronineophilic granules in their cytoplasm with this stain. In addition, the mononuclear cells stained basophilic with either Alcian blue-P.A.S. or Hematoxylin and Eosin. (Figure 17)

The injured muscle fibers and the adjacent intact muscle fibers showed both polymorphonuclear leucocytes and macrophages within the sarcoplasm which contributed to continued sarcolysis. The polymorphonuclear cells showed lysis in all areas of distribution of the exudate.

The presence of polymorphonuclear leucocytes and macro-
phages remain in the injured muscle through eight days duration of the experiment. However, there is notable reduction of PMN's.

The aggregates of indifferent cells appear intimately attached to the sarcolemmal surface and the cut ends of the surviving injured muscle fibers. They appear as solid cords of cells with common cytoplasm. Some of the nuclei of such cell aggregates are labeled but only in Series 1. (Figures 18 and 19) This observation of labeled cells increased to a peak at four days. Thereafter, mononuclear cells in the interstices and most nuclei of the aggregates were labeled in Series 1. In contrast in Series 2, only the mononuclear interstitial cells were labeled. (Figure 20)

While evidence of inflammatory exudate persisted in the presence of proliferating perivascular undifferentiated cells and endothelial cells, the aggregated cells showed differentiation. The aggregates of mononuclear cells were intimately apposed to the sarcolemmal surface of the surviving muscle. They showed a change from granular pyronineophilia to homogeneous pyronineophilia as is usually seen in striated muscle in Methyl green-pyronine stained sections.
Many other mononuclear interstitial cells showed an eccentric nucleus and a tear-drop shaped cytoplasm with a pale halo at the centrosome. Such a cell is a myoblast.

After six to eight days, there was a reduction in labeled cells in Series 2. Thus, the synthesis of deoxyribonucleic acid was significantly diminished in endothelial cells and perivascular undifferentiated cells.

The epimysium and perimysium showed fibroblasts and associated delicate collagenous fibers. In Series 1 such fibroblasts, like endothelial cells, were labeled. There was evidence of acid mucopolysaccharides in the epimysium; the wound gap now filled in with fibroblasts, delicate collagenous fibers and capillaries as well as the perimysium, and the endomysium of the intact muscle adjacent to the injured muscle fibers.

The injured muscle fibers do not show any increase in acid mucopolysaccharides except where dense aggregates of cells were well developed as seen in Alcian blue-P.A.S. reaction. Accompanying the evidence of increased acid mucopolysaccharides was the regular arrangement of argyrophilic fibers around the regenerating muscle fibers. Similarly, capillaries found in the endomysium and epimysium which showed normal diameters are supported by acid mucopolysaccharides and regular argyrophilic fibers.
CHAPTER V

DISCUSSION

The injury caused by crushing, cutting or punching of striated muscle in the rat leads initially to local defense reactions characterized by a blood clot, and polymorphonuclear leucocytic infiltration. The blood clot seals the wound by filling in the interstices between splayed muscle fibers, the area of loss in continuity of injured muscle fibers, the epimysium and the subcutaneous surfaces, and extends into the skin incision. The fibrin also seems to cement all such tissues anchoring them together.

The polymorphonuclear leucocytes are immediately available to the injured tissue. They diffusely infiltrate all of the tissues bound by the blood clot and also enter the sarcoplasm of injured and the immediately adjacent normal muscle fibers. The polymorphonuclear leucocytes appear to gain access to the muscle fibers through the injured cut surface and directly through the sarcolemma. They contribute to sarcolysis of the muscle fibers.

The loss of sarcoplasm by direct injury and by the phagocytosis and subsequent lysis of the polymorphonuclear leucocytes
contributes to the increased pyronineophilia of the interstices in the injured muscle. As the pyronineophilia is ribose nucleic acid of striated muscle, the injured tissue site becomes rich with this substance.

The hemorrhage in the wound site accompanied by the increased capillary vasodilatation and hyperpermeability also contributes to additional blood contents in the wound site.

The wound site is in a virtual shock state during the first sixteen hours following injury as only regressive or cytolytic changes are evident. However, even during this period of cytolysis, DNA synthesis is evident as a few heavily labeled perivascular mononuclear cells are seen in the endomysium of injured muscle.

The loss of argyrophilic fibers both around the capillaries and the injured and intact muscle is directly related to the loss of acid mucopolysaccharides in such tissue. This loss of support contributes to the separation or splaying out of muscle fibers. Moreover, as neutral polysaccharides, stainable by the P.A.S. reaction, still exist in injured tissue, it is possible that they are not essential to the binding of the argyrophilic fibers.

This loss of support by acid mucopolysaccharides and
Argyrophilic fibers lead not only to dilatation and hyperpermeability of capillaries but to loss of sarcoplasm from muscle fibers. It is felt that the polymorphonuclear leukocytes and the observed macrophages migrate through the unsupported sarcolemma of the injured muscle. Price, Howes and Blumberg (1964) recognize the loss of the plasma membrane but stated that the basement membrane was resistant to cold. Our findings show that the "basement membrane" is significantly altered both in injured and adjacent muscle by cutting, crushing and punching.

The endothelial cells and perivascular undifferentiated connective tissue cells begin to synthesize deoxyribose nucleic acid at an accelerated rate after the shock phase, that is, sixteen hours following injury. While there is no notable mononuclear cell increase, the number of such cells labeled with tritiated thymidine does increase. This rise of labeled cells in the endomysium indicates that mitotic division soon will begin within a few hours.

The labeled cells in the endomysium in Series 1 showed a rapid rise after sixteen hours, climbed to a peak and remained at a plateau. The quantitative differences in labeled cells of the endomysium in Series 1 and Series 2 is attributed to mitotic
division. In Series 1, the labeled cells showed decreased grains over the nuclei but increased numbers of cells. Mitotic figures were numerous in the endomysium particularly at three and four days. It can be conservatively estimated that the DNA synthesis in the endomysium increases by one hundred per cent between sixteen hours and twenty four hours as seen in Series 2. Furthermore, as the number of labeled cells in the endomysium rises from less than one to more than fifteen by four days in Series 1, it indicates the response by mitotic division. This is further supported by the observed dilution of grains over the labeled nuclei in Series 1.

The rapid rise of labeled cells in the endomysium as seen after sixteen hours resulted in a large accumulation of mononuclear cells. It is essential that the raw materials for new cell production be available at the site of muscle injury in order that mitotic division can occur in rapid sequence involving many cells. In Table II, it is seen that in Series 1, Zone B, the number of labeled cells rise from 7.0 at twenty-four hours to 15.8 at four days. This doubling of labeled cells is an absolute cell increase. It does not, however, quite agree with a doubling of cells in a single twenty four hour period as
reported by Zhinkin and Andreva (in developing muscle). (1963)

All the reports of muscle regeneration which included the early periods between one and six days, clearly were in agreement with the increased cell population. It is suggested that the early period of cytolysis, hemorrhage, vascular hyperpermeability prepares a rich substrate for the production requirements of the large numbers of cells found at the site of muscle injury.

The polymorphonuclear leucocytes probably contribute to the substrate by sarcolysis and subsequently their own cytolysis. It can be seen that while such polymorphonuclear leucocytes rise rapidly following injury, few such cells are labeled during the first sixteen hours. The circulating cells are not labeled at the time of injury and thus infiltrate the injury site unlabeled. However, after twenty four hours the number of labeled leucocytes rises sharply. This shows that cells forming in the marrow at the time of tritiated thymidine injection are released into the blood and find their way to the injured site within twenty hours. The polymorphonuclear leucocytes begin to fall in number after three days, which can be attributed to the cytolysis of such cells found throughout the tissues. Moreover, as such
cells have labeled nuclei, it is quite probable that this furnishes additional labeled precursor thymidine in the form of soluble strands of ribonucleic acid hydrolysate to the substrate. Therefore, it is probable that the endothelial and perivascular undifferentiated cells may become labeled by utilization of such radioactive material. The rise in labeled endomysial cells is inversely proportionate to the fall in labeled exudate cells which principally are polymorphonuclear leucocytes.

The presence of labeled polymorphonuclear leucocytes increases in the exudate between twenty four hours and forty hours as seen in Series 2. Since these animals were killed only one hour after tritiated thymidine injection, it indicates that polymorphonuclear leucocytes find their way to the injured site within one hour in a muscle injured for a period of twenty three hours. Moreover, labeled leucocytes are found to arrive at the site of injury from the bone marrow hourly through the entire eight day experimental period. However, the number of such labeled leucocytes is significantly reduced by eight days.

The labeled mononuclear cells around capillaries increase at three days. Such cells migrate into the blood clot. The close proximity of such cells to the capillaries suggest that
they may serve as cells for capillary budding. This suggestion is based upon the observation that endothelial cells are not labeled during the first sixteen hours, while few perivascular undifferentiated connective tissue cells were found labeled. However, when endothelial cells were found penetrating the fibrin interstices they were labeled after three days, they must be considered as part of the proliferative cells at the site of muscle injury. As the clot is phagocytized by the macrophages and polymorphonuclear leucocytes it is replaced by many new capillaries and fibroblasts. The delicate argyrophilic fibers found on the epimysial subcutaneous surfaces indicates the repair of such tissues is by fibrosis.

At approximately the same time, three days, the mononuclear cells found between the capillaries and the injured muscle assume the characteristics of myoblasts. They show increase granular pyroninophilic material, the nucleus becomes large, vesicular and contains several prominent nucleoli. Allbrook, (1962) and Price, Howes and Blumberg, (1964) describe the individual myoblast by the presence of myofibrils as seen in electronmicrographs. However, Allbrook (1962) pointed out the unreliability of differentiating between the fibroblast and the
myoblasts with the light microscope. Nevertheless, Zhinkin and Andreva (1963) describe labeled mononuclear cells as adjoining the sarcolemma from without the muscle fibers. Lash, (1957) also describes a mononuclear cell in injured muscle which proliferates and coalesces to form myotubes. Lavander (1945) earlier reported the change of connective tissue cells into myoblasts by induction. The only criteria of myoblasts is the presence of myofibrils in the cytoplasm. This can be seen with the light microscope only after considerable synthesis of such myofibrils. In this regard, the electron micrographs can make the differentiation when only small numbers of myofibrils are present in the myoblast.

The aggregates of such myoblasts on the surface or cut ends of the injured muscle increases over eight days. Some nuclei of such aggregates of cells are labeled. This finding shows that the aggregates indeed result from fusion by individual mononuclear cells. This observation generally agrees with those of Lash, (1957) Walker, (1962) Bintliff and Walker, (1960) Allbrook, (1962) Price, Howes and Blumberg, (1964) Zhinkin and Andreva, (1963).

As labeled nuclei were observed in aggregates of myoblasts
in Series 1, but not in Series 2, the source of the labeling could be only from the labeled perivascular undifferentiated connective tissue cells. It is clear that as the perivascular undifferentiated cell undergoes mitotic division, it gives rise to myoblasts in addition to fibroblasts and macrophages. The myoblasts are immediately adjacent to the injured muscle. It is conceivable that the differentiation of the myoblasts could result from utilization of muscle ribonucleic acid liberated by sarcolysis. The reaction of such undifferentiated cells with genetic equipment for differentiation to myoblasts is induced by injured muscle as in a regional specific induction system. This explanation is compatible with a similar system described by Holtfreter (1933).

The failure to find labeled nuclei in the myoblast aggregates in Series 2 clearly shows that such cells do not synthesize DNA. Therefore, they could divide neither mitotically nor amitotically. This was suggested by Lash (1957) in his cyto-photometric study of DNA synthesis. The lack of labeling of such myotubes was described also by Bintliff and Walker, (1960) Gerber, (1962) and Zhinkin and Andreva (1963).

It is clear that neither mature injured and uninjured muscle nuclei nor aggregating myoblasts synthesize deoxyribo-
nucleic acid or undergo mitotic or amitotic division. Thus, all the early investigations explaining muscle regeneration by migration of the nuclei of surviving muscle must be understood as incorrect. In addition, more recent investigations by Allbrook, (1962) Price, Howes and Blumberg, (1964) Zhinkin and Andreva, (1963) Bintliff and Walker, (1960) who explained the origin of myoblasts from surviving muscle nuclei also must be considered incorrect. However, the concept of fusion of the individual myoblasts to form the myotubes is generally accepted by such investigators.

The change of myoblasts from granular pyronineophilia to homogeneous pyronineophilia occurs after fusion of such cells. This can be described as a second phase of differentiation of the myoblast. The individual granular, pyronineophilic myoblast might undergo a primary induction by the high muscle ribonucleic acid in the substrate; after aggregating it loses the granular form of this substance as myofibrils increase in quantity and synthesizes the soluble form. Electronmicrograph studies of Allbrook (1962) and Price, et al. (1964) clearly show a dissolution of the plasma membrane of the myoblasts when aggregation occurs. Moreover, the ergastoplasm seen in electronmicrograms disappears after the myoblasts fuse.
There is a reconstitution of both the argyrophilic fibers and acid mucopolysaccharides around myotubes and new capillaries. Such support is not seen in myoblasts either with the light microscope or in electronmicrographs as shown by Price, Howes and Blumberg (1964). It is evident that injury to muscle brings about a loss of such "basement membrane" in muscle. Furthermore, when regeneration of muscle occurs, there is a reconstitution of the basement membrane only around myotubes.
CHAPTER VI

SUMMARY

The right external oblique muscle of twenty Sprague Dawley young rats weighing 150 to 200 grams were injured by incision, crush or punch at the same time. The animals were then divided into two series: Series 1 and Series 2 and intraperitoneally injected with TRH at a rate of 1 c per gram of body weight in the following manner: Series 1, six hours after surgery and Series 2, one hour prior to sacrifice. In this way the cell population, migration and proliferation was traced.

The animals were sacrificed at 10 - 13 - 16 - 24 - 36 - 44 hours, and 3 - 4 - 6 and 8 days.

The histological specimens were fixed and embedded in paraffin. Sections from three to five microns were made and stained with Hematoxilin and Eosin, Toluidine blue, Methyl green pyronine, Periodic Acid Schiff, Silver, DNA and Alcian blue-P.A.S. For the autoradiograms the sections were first deparaffinized and treated with NTB3 emulsion and placed in refrigeration for two weeks, after which they were developed, fixed, washed and finally stained with Hematoxilin and Eosin.
The morphologic changes observed in Series 1 and Series 2 regardless of type of injury, cut, punch or crush were essentially identical.

The morphologic difference between muscle fibers and perivascular mononuclear cells and endothelial cells was the presence of pyronineophilia granules in the cytoplasm of the latter and diffuse pyronineophilia in the former indicative of a loss of pyronineophilia in the injured muscle fiber. There was a change of granular pyronineophilia to homogeneous pyronineophilia in the myoblasts that occurs after fusion of myoblasts to form myotubes. The individual granular pyronineophilic myoblast might undergo a primary induction by the high content of muscle RNA in the cytoplasm.

Acid and neutral mucopolysaccharides as seen with Alcian blue-P.A.S. reaction and P.A.S. reactions were seen in the supporting tissue in injured muscle fiber. A loss of this acid mucopolysaccharides as indicated by loss of gamma metachromasia (red violet color) in sections stains with Alcian blue-P.A.S. was observed.

In normal muscle argyrophilic fibers in regular arrangement are seen surrounding each capillary and muscle fiber with
silver impregnation. This arrangement in injured muscle is lost or greatly disorganized.

The loss of acid mucopolysaccharides and argyrophilic fibers contributes to the hyperpermeability of the capillaries, separation of the muscle fibers, and loss of sarcoplasm providing a perfect media for the mobilization of cells.

A rise of acid mucopolysaccharides was observed in the latest hours of the experiment where there was an aggregation of cells, a reconstruction of argyrophilic fibers around the myotubes and capillaries was also observed.

The injured area in Series 1 and Series 2 was arbitrarily divided into three zones: Zone A, the exudate; Zone B, the injured and surviving muscle fibers and Zone C, the adjacent uninjured muscle. The number of labeled nuclei was counted in each of fifteen oil immersion fields, within each of the zones mentioned and from each sacrifice interval. After a repressive period of sixteen hours in both Series 1 and Series 2, a marked rise in labeled nuclei per series and zones was observed.

In Series 1, Zone A, the distribution of PMN's rose rapidly after a sixteen hour period and held steadily until thirty six hours when they gradually fell.
The labeled mononuclear cells show a gradual rise in Series 1 after the shock period of sixteen hours, and then levels off to a plateau and remains through the experiment.

The grains over the nucleus in the endomysium in Series 1 were fewer in number after four days as compared to those seen during the first sixteen hours. While in Series 2, the grains over the endomysial nuclei were dense and numerous; however, in contrast to Series 1, the number of labeled endomysial nuclei is lower in Series 2 and began to fall after six days.

The quantitative difference between labeled cells of endomysium in Series 1 and Series 2 is attributed to mitotic division, which were numerous between three and four days.

DNA synthesis that during the lag period started slowly showed a great increase after sixteen hours. It is essential that the raw materials for cell production be available at the site of injury in order that mitotic division can occur. It is suggested that the early period of cytolysis, hemorrhage, vascular dilatation and hyperpermeability prepare such a substance for the production of a large population of cells at the site of injury.

Mononuclear undifferentiated perivascular connective tissue
cells rapidly increased in number between two and four days, and as some of them showed diffuse labeling and only in Series 1, is obvious that they proliferate. Aggregation of such cells at three days were found between the capillaries and the surviving muscle fiber at the muscle cut ends, and over the muscle fiber where they appeared as solid cells cords with a common cytoplasm.

The failure of finding labeled nuclei in aggregated myoblast in Series 2, clearly showed that such cells once aggregated do not synthesize DNA; therefore, do not divide neither mitotically nor amitotically.

The undifferentiated perivascular connective tissue cells also gave rise to macrophages and fibroblasts.
CHAPTER VII

CONCLUSIONS

1. The changes observed in the injured striated muscle in an eight day period included the epimysium, perimysium and endomysium. They could be divided into three overlapping phases: necrosis and inflammation, proliferation and differentiation.

2. The undifferentiated perivascular connective tissue cells divide mitotically and migrate to the muscle cut ends, and differentiate into myoblasts which aggregate and fuse forming myotubes after which they do not divide either mitotically or amitotically.

3. The differentiation to myoblasts occurs in two phases: a primary phase, induction by the high content of granular RNA in the cytoplasm, and a secondary phase, the change of granular pyronineophilia to homogeneous pyronineophilia after the fusion of the myoblast.

4. There is a lost and desorganization of argyrophilic fibers around the injured and adjacent apparently intact muscle fiber, as well as from around the capillaries from the endomysium.
5. This lost of argyrophilic fibers is directly related to the lost of acid mucopolysaccharides. This lost of connective tissue substance contributes to the separation and splaying out of the muscle fiber also contributes to facilitate the cell movement.

6. There is a reconstitution of both, argyrophilic fibers and acid mucopolysaccharides around myotubes and capillaries.
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### TABLE I

**AVERAGE NUMBER OF LABELED NUCLEI IN ZONE A**

<table>
<thead>
<tr>
<th>Time</th>
<th>Series 1</th>
<th>Series 2</th>
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<td>15.4</td>
</tr>
<tr>
<td>6 days</td>
<td>10.4</td>
<td>16.5</td>
</tr>
<tr>
<td>8 days</td>
<td>9.8</td>
<td>9.8</td>
</tr>
</tbody>
</table>

*Average of fifteen oil immersion fields.*
### Table II

**Average Number of Labeled Nuclei in Zones B and C**

<table>
<thead>
<tr>
<th>Time</th>
<th>Zone B</th>
<th>Zone C</th>
<th>Zone B</th>
<th>Zone C</th>
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<td>.1</td>
<td>.1</td>
<td>.1</td>
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<tr>
<td>13 hours</td>
<td>.1</td>
<td>.1</td>
<td>.1</td>
<td>.1</td>
</tr>
<tr>
<td>16 hours</td>
<td>.1</td>
<td>.1</td>
<td>.1</td>
<td>.1</td>
</tr>
<tr>
<td>24 hours</td>
<td>7.0</td>
<td>2.8</td>
<td>1.4</td>
<td>.1</td>
</tr>
<tr>
<td>36 hours</td>
<td>4.4</td>
<td>2.5</td>
<td>2.2</td>
<td>.7</td>
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<tr>
<td>44 hours</td>
<td>9.2</td>
<td>4.0</td>
<td>1.6</td>
<td>.8</td>
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<td>3 days</td>
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<td>6.0</td>
<td>3.2</td>
<td>.2</td>
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<tr>
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<td>5.7</td>
<td>1.7</td>
<td>.5</td>
</tr>
<tr>
<td>6 days</td>
<td>10.4</td>
<td>6.1</td>
<td>3.2</td>
<td>.8</td>
</tr>
<tr>
<td>8 days</td>
<td>15.0</td>
<td>6.8</td>
<td>.5</td>
<td>.0</td>
</tr>
</tbody>
</table>

*Average of fifteen oil immersion fields.*
FREQUENCY OF LABELED NUCLEI IN REGENERATING STRIATED MUSCLE
ZONE A

SERIES 1  ---  TH3 INJECTED 6 HRS. AFTER INJURY
SERIES 2  ---  TH3 INJECTED 1 HR. PRIOR TO SACRIFICE
FREQUENCY OF LABELED NUCLEI IN REGENERATING STRIATED MUSCLE ZONE B AND C

SERIES 1 — TH³ INJECTED 6 HRS. AFTER INJURY
SERIES 2 — TH³ INJECTED 1 HR. PRIOR TO SACRIFICE
Argyrophilic fibers in a regular arrangement around capillaries and muscle fibers. (Silver impregnation X400)
Beta-metachromasia in cytoplasm of fused myoblasts.  
Note undefined cells outline in non fused myoblast.  
(Toluidine blue X530)
FIGURE 5

Break in continuity of muscle fiber bundle
(H and E X100)
Loss of pyronineophilia of the muscle bundle and gain of pyronineophilia in the injured area.
(Methyl green pyronine X450)
Loss and disorganization of the argyrophilic fiber.
(Silver impregnation X400)
Loss of acid mucopolysaccharides indicated by loss of gamma metachromacia. (Red-violet color) Alcian blue-P.A.S. X450
FIGURE 8A

Loss of acid mucopolysaccharides.
(Alcian blue-P.A.S. X400)
FIGURE 9

Sarcolesis and phagocytosis
(H and E X530)
Sarcolysis and phagocytosis.
(H and E X530)
Sarcolysis and phagocytosis.
Note phagocytosis in muscle bundle.
Note endomysium cells.
(H and E X530)
Almost complete sarcolysis and phagocytosis. Note proliferation of endomysium cells. (H and E X450)
FIGURE 13

Zones A, B and C
X60
Perivascular labeled mononuclear cells.
ToIoudine blue X1000
Proliferative activity of interstitial undifferentiated connective tissue cells between the capillaries and the surviving muscle fiber.
(H and E X530)
Pale aspect of injured muscle fiber stained with methyl green pyronine (X530)
FIGURE 17

Basophilic mononuclear cells.
Note mitotic figures.
(H and E X530)
Fused myoblasts forming myotubes. Note labeled nuclei, Series I. (Autoradiograms X400)
Fused myoblasts. 
Note labeled nuclei, Series 1 
(Autoradiograms X400)
Myotubes formation, Series 2
Note labeling only in interstitial mononuclear cells.
(Autoradiograms X530)
APPROVAL SHEET

The thesis submitted by Carlos S. Anguizola has been read and approved by three members of the faculty of the Graduate School.

The final copies have been examined by the director of the thesis and the signature which appears below verifies the fact that any necessary changes have been incorporated, and that the thesis is now given final approval with reference to content, form and mechanical accuracy.

The thesis is therefore accepted in partial fulfillment of the requirements for the Degree of Master of Science.

Date May 17, 1965

Patrick D. Toto
Signature of Advisor

Patrick D. Toto, M.S., D.D.S.