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Change in the Number of Fibers and Nuclei in the Rat Soleus Muscle During Normal Development and Increased Work Load Exercise

John K. Pearson

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CHANGE IN THE NUMBER OF FIBERS AND NUCLEI IN THE RAT SOLEUS MUSCLE DURING NORMAL DEVELOPMENT AND INCREASED WORK LOAD EXERCISE

by

John K. Pearson

A Thesis in Partial Fulfillment of the Requirements for the Degree Master of Science in the Field of Orthodontics

May 1971

1 7 0 9 3 2
Each person whose signature appears below certifies that he has read this thesis and that in his opinion it is adequate, in scope and quality, as a thesis for the degree of Master of Science.

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ACKNOWLEDGEMENTS

I wish to express my sincere appreciation to the members of my guidance committee and all those having any part in the preparation of this thesis.

Special recognition must go to the following:

Dr. John P. DeVincenzo and Dr. Paul J. McMillan. Thanks.
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NOTICE

The following manuscript was prepared as a partial fulfillment of the requirements for a graduate degree from Loma Linda University Graduate School under the discipline of the School of Dentistry.

While the format in general is governed by the criteria of a conventional Graduate School Thesis, it is in actuality a manuscript which readily is amenable for publication in a scientific journal.
CHAPTER I

INTRODUCTION

All orthodontists have had the patient with a gummy smile resulting from a relatively short upper lip and many have wished that they could lengthen that upper lip. The classic concept of the relationship between birth and change in exercised muscle mass is well appreciated. It is reasoned that even if lip exercise could increase lip length, on cessation of exercise the lip would probably return to its original length because the number of muscle fibers were established at birth.\(^2\)

In recent years several studies have appeared which indicate that muscle deoxyribonucleic acid (DNA) continues to increase long after birth and that an increase in muscle cell nuclei is in part responsible.\(^1,7\) Could this finding suggest that the number of muscle fibers are not established at birth but are subject to a post-parturition increase? Would it be possible to increase the number of muscle fibers, muscle nuclei or both by additional exercises? If it would be possible to demonstrate in animals that skeletal muscle cells and corresponding DNA increased with increased exercise, clinical studies designed to increase fiber length of the muscles comprising the upper lip would be in order.

It is the purpose of this study to investigate the relationship between additional work load exercise on selected muscles of the albino rat and the increase in muscle nuclei.
In addition the findings of a companion report,\textsuperscript{3} demonstrating significant increases in muscle tissue DNA during normal development and development with increased work load exercise, will be analyzed and discussed.
CHAPTER II

METHODS AND MATERIALS

Forty-two male Sprague Dawley albino rats (Holtzman Company, Madison, Wisconsin) were used in this study. Twenty-one rats were prepuberal (24 days) weighing approximately 60 grams. The other 21 rats were young adults (80 days) weighing approximately 300 grams.

To increase the work load of the soleus and plantaris muscles, rats under ether anesthesia underwent a surgical procedure in which the synergistic gastrocnemius tendon was cut, and sutured to the belly of the muscle near its origin. Control animals received a sham operation in which the muscle bellies were separated but the tendons were left intact. A suture was placed in the belly of the gastrocnemius muscle to simulate the suturing of the reflected tendon of the gastrocnemius in the experimental groups. Identical procedures were performed on both hind legs of each animal used, in order to prevent the possibility of greater use of one leg over the other. All incisions were closed with ooo silk suture.

Two experimental groups were included in the study. In the first group ten prepuberal (24 days) and ten young adult (80 days) rats received the tenotomy operation. Five prepuberal and five young adult rats served as sham controls. In the second group, six prepuberal and six young adult rats served as animals in the study of normal development of muscle tissue. The animals of this group were sacrificed at 24 and 80 days and samples were taken as described later.
The animals were housed in four cages at a constant temperature and fed Purina Chow and water ad libitum. The cages were 26 x 20 x 7 inches. A 26 x 9 inch portion was added to one end of each cage. This 16 inch high addition contained a 30 inch ramp which ran to the top of the cage where the food was contained. Water was placed at the sides of cages near the top of the added portion.

The two groups of ten experimental animals were each housed in an individual cage. The five prepuberal and five young adult sham control rats were housed in separate cages with five more rats of the same age in each cage.

After six weeks of development the 30 experimental and sham control animals were deprived of food and water four hours prior to sacrificing with chloroform. Both soleus muscles of each animal were excised with care to remove all excess connective tissue including scar tissue. The muscle was immediately weighed on an analytical balance to ± one mg. Six muscles form both of the experimental groups (10 animals in a group), two muscles from both of the sham control groups (5 animals in a group), and four muscles from both of the normal development groups (6 animals in a group) were taken for this histological study. In this study only one leg was used per animal, alternately taking right or left leg muscles. All the remaining muscles were used in a companion study.\(^3\)

The muscles for this study were removed and weighed as described above. The muscles were pinned to wooden sticks to retain their original length, and fixed in Zenkers solution for four and a half hours. The specimens were then placed in 70% alcohol overnight. Twelve
cross sections of 10 and 20 μ each were taken at mid and quarter length of the muscle. Longitudinal sections of 10 μ were also prepared. The sections were stained with the periodic acid Schiff technique and counterstained with hematoxylin. This technique gives definition to the nuclei and the membrane which envelops individual muscle fibers.

The length of a muscle fiber nuclei was determined on the 10 μ longitudinal sections by averaging 20 nuclear measurements per muscle. Ten μ cross sections at the midlength of the muscle bellies were used to count the number of muscle nuclei, connective tissue nuclei and muscle fibers in a field defined by an ocular grid. Ten fields per section were arranged in a checker board fashion thus providing a systematic sample of the muscle. The 1st, 6th, and 12th section of each muscle was counted in this manner. The 24 and 80 day old muscle bellies were counted at 100x and 40x respectively, in order to obtain approximately 15 fibers per field. In the 24 day old muscles one field was equal to 5.6 x 10\(^3\) μ\(^2\) and in the 80 day old muscles it was equal to 3.3 x 10\(^4\) μ\(^2\). The reason the other experimental and operated control groups were not counted is discussed later.

The muscle fiber nuclei counts were corrected by the formula of Eranko.\(^8\)

\[
N = \frac{an}{a + 2r - 2k}
\]

where:
- \(N\) = number of particles in each mm\(^3\) tissue
- \(a\) = section thickness
- \(r\) = length of particles (radius)
- \(k\) = vertical height of smallest fragment observable
- \(n\) = number of particles counted per mm\(^3\)

The values were used to determine the percentage of increase in muscle fiber nuclei. Then the total number of muscle fiber and connective
tissue nuclei per muscle was determined with the aid of the following formula.\(^6\)

\[
\text{Number of nuclei (millions) = \frac{\text{Total muscle DNA (mg)} \times 10^3}{6.2}}
\]

Values for ugm of DNA used in this determination were obtained from a concurrent study.\(^3\) With use of values for specific gravity of muscle, muscle mass and corrected numbers of muscle nuclei per \(\text{mm}^3\), a percentage change in connective tissue nuclei number of young and old rats was determined.

Photomicrographs of the total cross sections of the muscles at 24 and 80 days were taken at mid section. The total number of fibers per muscle belly were counted from these photomicrographs. Fiber counts were given the t test for significance.
CHAPTER III

RESULTS

In the group used to study normal development the average length of a muscle fiber nuclei was found to be 15.2 u, with no significant difference between prepuberal and young adult rats. With this value and values for number of nuclei per units of volume of tissues (Table 1) calculations were carried out as previously discussed. A 67\% increase in muscle cell nuclei and a 74\% increase in connective tissue cell nuclei was found.

Further study of development revealed a significant decrease of 26\% in the number of fibers of the rat soleus muscle from 24 to 80 days (Table 2 and Figure 1).

Counts and calculations to determine the percentage increase in connective tissue and muscle nuclei in the experimental groups were not done due to the presence of a densely nucleated lesion found on histological examination (Figure 2). Although the lesion occurred more frequently and to a greater extent in the experimental groups there were also lesions in the operated controls.

The lesion had the appearance of densely nucleated connective tissue with no identifiable cell form to make a more definitive description. The lesion appeared to have fragments of muscle fibers and multinucleated giant cells within its mass (Figure 8). There was also evidence of mitotic activity (Figure 3).

Associated with the lesion are fibers similar in appearance to
normal fibers with size and shape characteristic of this stage or
development (Figures 2 and 4). The fibers appear to be normal with
their nuclei peripherally located against the cell membrane. Fibers
of much smaller diameters with some nuclei more centrally located are
also abundant (Figure 4). Smaller fibers were also found in areas not
associated with the lesion (Figures 5 and 6). Either these smaller
fibers had normal muscle fiber morphology and are approximately of equal
size (Figure 6), or they have abnormally positioned nuclei and a wide
variation of fiber size (Figures 4 and 5).

Trichrome and reticulum stain were used, but the trichrome stain
revealed no further findings (Figure 8). The reticulum staining
revealed reticulin fibers in the lesion and a "halo" appearance within
the individual muscle fibers (Figure 9).

The presence of lesions in with the experimental and sham
controls called for a careful examination of the animals used to study
normal development. This examination revealed a few peripherally
located densely nucleated fibers with atypical grouping of nuclei and
fibers with more centrally located nuclei were observed. This grouping
of nuclei and fibers was small and is seen in its entirety in Figure 11.
<table>
<thead>
<tr>
<th></th>
<th>Muscle* Nuclei Number</th>
<th>Connective Tissue* Nuclei Number</th>
<th>Muscle* Fiber Number</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean±S.D.</td>
<td>176.17±18.43</td>
<td>234.92±41.67</td>
<td>138.33±27.66</td>
</tr>
<tr>
<td>24 Days</td>
<td>Standard Error of Mean</td>
<td>5.32</td>
<td>12.03</td>
</tr>
<tr>
<td>Mean±S.D.</td>
<td>257.42±38.00</td>
<td>648.25±125.39</td>
<td>151.17±23.38</td>
</tr>
<tr>
<td>80 Days</td>
<td>Standard Error of Mean</td>
<td>10.99</td>
<td>36.20</td>
</tr>
</tbody>
</table>

The histological determination of muscle nuclei, connective tissue nuclei, and muscle fiber number in the rat soleus muscle, during development from animals taken at 24 and 80 days.

*These counts represent the mean values of observed nuclei on 12 muscle sections. On each section 10 fields defined by an ocular grid were counted systematically.
<table>
<thead>
<tr>
<th></th>
<th>Mean± S.D.</th>
<th>Standard Error of Mean</th>
<th>Significance p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>24 Days</td>
<td>3172.25±359.19</td>
<td>179.59</td>
<td>.0088</td>
</tr>
<tr>
<td>80 Days</td>
<td>2360.25±228.27</td>
<td>114.13</td>
<td></td>
</tr>
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Total number of fibers per muscle belly of the rat soleus muscle during normal development at 24 and 80 days. Counts were made from four different muscle belly cross sections at each age.
Figure 1. The mean value of total number of fibers in the rat soleus muscle at 24 and 80 days of development. Standard error of the mean is represented by the solid line at the top of each bar on the graph.
Figure 2. One of the large densely nucleated lesions (L). H. & E. x40.

Figure 3. Note mitotic activity within the lesion (L). H. & E. x158.
Figure 4. There are apparently normal fibers (F) and small atypical fibers (S) associated with the lesion (L). H. & E. x158.

Figure 5. Variable fiber size and atypical located nuclei within the fiber (N) are noted. These fibers are similar to fibers found in some human pathologies in which nuclei are more centrally located. H. & E. x158.
Figure 6. A bundle of small fibers (B) found in an area of normal appearing fibers. Note one normal appearing fiber (under arrow) which appears to have a sub fiber splitting from it (arrow). H. & E. x250.

Figure 7. Multinucleated necrotic fibers (N) are seen associated with normal fibers (F). H. & E. x158.
Figure 8. A large area of lesions with what appears to be multinucleated giant cell (G) and what possibly might be fragments of fibers (F). Note the difference in staining and nuclei in what are assumed to be giant cells and fiber fragments. Trichrome. x158.

Figure 9. The areas of the lesion with lighter stain reticulin fibers (R) and a "halo" appearance within the muscle fibers (arrow). Reticulum. x158.
Figure 10. A few densely nucleated necrotic (N) fibers can be seen among normal fibers. H. & E. x158.

Figure 11. Areas of apparent lesion (L) and necrotic fibers (N) in an unoperated animal. H. & E. x158.
The finding of a 26% decrease in total number of muscle fibers in the soleus of 80 day rats compared to 24 day old animals was surprising. That this was a significant decrease was supported by t test analysis. Other investigators showed no significant change in fiber number.\(^2, 5, 9\)

The histologic sections were not taken at a precisely determined location on the muscle although the general mid length area was used. Since the sample size was small, four muscles from older rats and four from younger animals, variations in the location of the sections to be examined could have given these results. Unfortunately, although the correctness of this explanation would have been easy to verify by making fiber counts on sections taken along the entire length of one muscle, this was not done.

If animals not subjected to increased exercise showed no increase in fiber number with development, any increase in fiber number in the experimental animals could be attributed to increased exercise. This part of the investigation was not carried to completion due to the occurrence of densely nucleated lesions which appeared to be replacing fragments of degenerating muscle fibers.

It was possible that these fragments of fibers were actually newly developing fibers, but the observation that replacement of fibers was occurring is supported by histological evidence. There are areas
of some of the lesion where fragments of degenerating fibers appear to be associated with multinucleated giant cells. These cells are believed to be phagocytizing the breakdown products of fibers (Figure 8). There are lesions in which fiber fragments or giant cells were not found (Figure 3), but the overall appearance of the lesion (Figure 2) however is one of extreme loss of fiber number.

Histological examination eliminated the possibility of malignancy. It was also possible that the lesion was an abscess caused by the introduction of microorganisms at the time of surgery. What was seen on the slides therefore was a fibrous scar replacing an abscess. The absence of chronic inflammation and the possibility of finding an abscess thus centrally located, when no incision was made into the muscle are reasons for eliminating the possibility of infection.

The lesion may have been fibrous tissue replacement of an infarct caused by ischemia at the time of surgery. Evidence supporting this explanation includes, lighter staining reticulin fibers (Figure 9), and mitotic figures which are both characteristically seen in young tissue. Possible evidence of phagocytic multinucleated giant cells (Figure 8) do support the possibility of infarction but all these findings could be seen in an abscess.

More convincing evidence is related to the fact that there was increased occurrence of the lesion in animals receiving the more extensive surgical procedure. This involved reflection of the gastrocnemius with possibly greater ischemia from increased manipulation of and interruption of blood supply. Added ischemia might have been imposed on the soleus of both experimental and control groups due to the increased
exercise required in obtaining food and water. The soleus of the experimental animal might well have more functional demand on it resulting from the tenotomy of the synergistic gastrocnemius. Ischemia may also be the cause of what appeared to atrophied fibers seen associated with the lesion (Figure 4).

Fibers normal in histologic appearance except for their small size, were present in occasional "bundles" (Figure 6). These fibers are similar in appearance to ones found in a study relating fiber splitting and increased exercise. Yet the possibility that they were an expression of atrophy due to denervation cannot be ignored. That this was actually fiber splitting is the more reasonable explanation because of the phenomena of "sub fiber", perhaps an example of which is seen in Figure 6. This information on the "sub fiber" phenomena was not available until after the histologic sections were made and continuous histological cross sections of the muscle were not done. This type of sectioning possibly would have demonstrated actual separation of a sub fiber from normal fibers. If this were demonstrated in these experimental animals it would be interesting to investigate the possibility of complete separation of the "sub fiber" and eventual development to normal fiber size.

Although small and necrotic fibers were associated with the lesion its frequency of occurrence and numerous nuclei are of particular interest. The more frequent and extensive occurrence of the lesion in the experimental groups would probably account for the findings of the companion study. Namely, that there was a significant increase in DNA in the increased exercised soleus muscle of the experimental when
compared to the controls. Without histologic evidence of a lesion this increase in DNA would suggest that exercise did increase normal cellular components of muscle.

Further information on DNA and normal development was related to increases in cellular components of soleus muscle. A significant increase in DNA in muscle taken from 80 day old rats compared to 24 day old animals was related to a 74% increase in connective tissue nuclei, and a 67% increase in muscle fiber nuclei. These findings are in partial agreement with findings of Enesco, who found a 387% increase in connective tissue nuclei and a 194% increase in muscle fiber nuclei in rat soleus muscle from 19 to 95 days. There is a 4.5 times greater increase in connective tissue nuclei when compared to muscle fiber nuclei of the soleus during development.

It is possible that muscle fiber nuclei increase is directly related to increase in muscle fiber cytoplasm. The theory being that each nuclei controls a determined volume of fiber cytoplasm. The possibility that fibers may actually be able to increase was also proposed. If the orthodontist were able to produce both or either of these increases in muscle mass by use of exercises, a permanent improvement of a short upper lip might be expected.

Although this study was unable to give answers to basic questions about change induced in muscle by exercise the results would not discourage one from attempting a pilot clinical study on the use of exercise to lengthen a short upper lip.
CHAPTER V

SUMMARY

In a study using albino rats quantitative changes in skeletal muscle fiber and nuclei were investigated. The results were compared to changes in DNA content of the muscles from the same animals.

It was concluded that increased DNA found in the exercised group may not necessarily indicate an increase in skeletal muscle nuclei but more logically may represent an increase in DNA resulting from an unexpected pathological lesion.

Previous studies and subsequent studies using DNA to interpret cellular change in muscle should be viewed with care because of possible densely nucleated lesions.
REFERENCES
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CHANGE IN THE NUMBER OF FIBERS AND NUCLEI IN THE RAT SOLEUS MUSCLE DURING NORMAL DEVELOPMENT AND INCREASED WORK LOAD EXERCISE

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An Abstract of a Thesis in Partial Fulfillment of the Requirements for the Degree of Master of Science in the Field of Orthodontics

May 1971
ABSTRACT

Many orthodontists have desired to produce a permanent lengthening of a short upper lip. To produce a permanent increase in skeletal muscle mass, changes associated with hyperplasia would probably have to occur. If it would be possible to demonstrate in animals that skeletal muscle cells and corresponding deoxyribonucleic acid (DNA) increased with exercise, clinical studies on the upper lip would be in order.

Male Sprague Dawley albino rats were used to study the effects of exercise and normal development. Increased work load exercise of the soleus muscle was induced by cutting the tendon of the synergistic gastrocnemius. Histological sections of the soleus were used in the studies.

Changes of a 67% increase in muscle cell nuclei, a 74% increase in connective tissue cell nuclei with a 26% decrease in fiber number of the soleus found in normal development of rats taken at 24 and 80 days.

In the rats with increased work load exercise a significant increase in DNA was found by a companion study using the same animals.

It was concluded that increased DNA found in the exercised group may not necessarily indicate an increase in skeletal muscle nuclei, but more logically may represent an increase in DNA resulting from an unexpected pathological lesion.