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CASE WESTERN RESERVE UNIVERSITY

GRADUATE STUDIES

We hereby approve the thesis of

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Date JUNE 26, 1985

THE EFFECT OF STRETCHING ON HAMSTER CHEEK POUCH MUSCLE

by

KORNTIP AKSHARANUGRAHA

Submitted in partial fulfillment of the requirements

for the Degree of Master of Science in Dentistry

Department of Orthodontics

School of Dentistry

Case Western Reserve University

June 1985

THE EFFECT OF STRETCHING ON HAMSTER CHEEK POUCH MUSCLE

Abstract

by

KORNTIP AKSHARANUGRAHA

The objective of this research project was to investigate the effect of mechanical stretching on the longitudinal muscle of the Golden hamster cheek pouch. A sample of twenty-eight male animals was divided into two main groups, one for muscle histochemistry and the other for radioisotope labeling. Each main group was further divided into four sub-groups, with four animals in the groups for muscle histochemistry and three animals in the groups for autoradiography. One additional animal of each group was sham-operated

In all experimental animals the left pouch was stretched by inserting a latex balloon, filled with rubber base impression material In the sham-operated animal, the pouch orifice was sutured closed without placement of the stretching device. The pouch on the right side of all animals was left intact and served as control sites. The experimental periods were one week for group I, two weeks for group II, three weeks for group III and four weeks for group IV. In group IV the stretching device was

removed after 21 days which allowed for one week of recovery. For the autoradiographic study, tritiated thymidine was injected intraperitoneally at a dose of 1 Ci/g. of body weight every seventh day and the animals were killed 24 hours after the last injection

Interpretation of the data was based on intergroup comparison of diameter and number of muscle fibers and myosin ATP-ase activity to show muscle fiber type. Quantitative data from the autoradiography were recorded as average percentage of labeled nuclei found in anterior, middle and posterior parts of the cheek pouch muscle.

The findings from this experiment were:

1. Mechanical stretching from the device did not have an effect on transformation of muscle fiber types.

2. Mean diameter and number of the muscle fibers from the experimental sides were less than the contralateral control sides but data showed statistical non-significance.

3. When the stretching device was removed, there was a rapid recovery of the muscle.

4 Higher frequency of labeled nuclei of both muscle and connective tissue cells were found in the anterior part of the experimental pouch than in the middle or posterior parts and the contralateral control sides.

DEDICATED

TO

My parents, Rear Admiral Som and Mrs. Benjapong Aksharanugraha, for their unselfish love and guidance which profoundly influenced my educational decisions and goals.

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INTRODUCTION

Many studies have been done in order to explain the interrelationship between muscular function and bone growth. However, the exact nature of this relationship is still under investigation and there are many unanswered questions regarding musculoskeletal interaction Does change in muscle function have direct effects on skeletal form? Or conversely, can skeletal change result in altered muscle function? McNamara (1972) has raised the question whether change in muscle and bone can occur simultaneously in response to a stimulus but without a direct cause-and-effect relationship between the two tissues?

As orthodontic mechanotherapy is almost exclusively concerned with morphological problems, the biomechanics of growth is of primary relevance in clinical practice. Fränkel (1980) has suggested that local biomechanical conditions can be altered by craniofacial orthopedics. He evaluated statistically a large number of cases treated with the functional regulator. These patients showed remarkable stability in the postretention phase which lends strong support to the concept that space conditions as determined by the orofacial soft tissue capsule are decisive factors in the morphogenesis of the dentoalveolar structures (Fränkel, 1980). Thus, possible direct mechanical effects on the related muscular structures must be considered.

Some investigations have shown that structural adaptation of muscles may occur with treatment (McNamara et al., 1978, Oudet and Petrovic, 1978) Clinical studies indicate that relief of buccal musculature from contact with the teeth may result in widening of the dental arches (Fränkel, 1966, 1969). Treatment with vestibular shields may not only be a temporary mechanical expansion of the soft tissue capsule It may also have direct structural effects on the oromusculature Appliances that exert tension on orofacial muscles can have effects on this tissue similar to those which result from muscle exercise in general orthopedics.

The method of functional cranial analysis, which was formulated by van der Klaauw (1948, 1951), appears to be of particular interest His concept was that size and shape of the various osseous parts of the cranium are determined on the basis of their functions and thus should be regarded as "functional components". Moss (1962, 1968, 1970) extended van der Klaauw's ideas and postulated the "functional matrix concept" which stated that there is no direct genetic control of form and position of skeletal tissues. Rather, the functional matrices play the primary morphogenetic role. He placed primary importance upon the adaptibility of osseous structures to functional patterns and environmental conditions. Bone tissue is passive since it has only a secondary response to contiguous structures.

Solow and Kreiborg (1977) have also suggested that growth and the ultimate size and shape of facial osseous struc-

tures are closely associated with the facial soft tissue draping. They suggested that relief of tension of soft tissues might potentially allow for alterations in skeletal craniofacial development

A number of studies on the effect of muscle stretching and immobilization have been done using the leg musculature of laboratory animals as the experimental site. On the other hand very few studies have involved the oral musculature.

Muscle fibers may adapt to alterations in functional length by addition or deletion of sarcomeres, by rearrangement of their orientation, or both (Crawford, 1954, 1961; Williams and Goldspink, 1978). Muscular tissue may also respond in several other ways to functional alterations: Changes in fiber size, shifts in the distribution of fiber types, changes in sarcolemmal nuclei; degeneration and regeneration, and changes in the cytochemical architecture

Clinicians, especially orthodontists, routinely deal with forces exerted by the perioral-musculature. They may attempt to manipulate these muscles in order to improve treatment results and long-term stability. Thus, with the intent to answer some questions related to muscle adaptation in response to induced tension, the present study was undertaken to analyze the effects of stretching on facial skeletal muscle.

STATEMENT OF THESIS

The objective of this research project is to investigate the response of mechanical tension on the cheek pouch muscle in the hamster regarding fiber type, fiber diameter, fiber number and mitotic activity of satellite cells.

The left cheek pouch will be exposed to mechanical tension by the insertion of a stretching device, the contralateral side will serve as control. The animal sample will be divided into two main groups, i.e., one for muscle histochemical study and one for autoradiography.

Data will be obtained regarding (1) muscle fiber types, fiber diameter and fiber number from the histochemical analysis, and (2) mitotic activity by double blind count of autoradiographically labeled satellite cells and of non-labeled cells at randomly selected areas of the cheek pouch muscle.

LITERATURE REVIEW

One significant aspect of muscle behavior is its inherent adaptability. Boos (1940) stated that any muscle or muscle group which has been elongated within physiological limits will seek to reestablish functional homeostasis. Such adaptive mechanisms may involve elongation of the muscle fibers themselves, establishment of altered neuromuscular feedback mechanisms, migration of muscle attachments along bony surfaces, an occurrence of changed muscular dimensions due to displacement and rotations of the bony elements (McNamara, 1975; McNamara et al., 1978).

A study of clinical relevance was done by Ghafari (1984), who studied palatal sutural response to buccal muscular displacement in the rat By using buccal shields in 30-day-old rats to displace the buccal musculature from the maxillary molar teeth he found that the intermolar distances increased significantly 15 days following insertion of the appliances With further treatment a decrease was observed, and Ghafari concluded that the response may be limited by the nature of the mechanical stress exerted upon, and/or the growth potential of, the suture.

Many investigators have performed experiments regarding muscle response to environmental changes. Schiaffino et al. (1979) studied the rat soleus muscle following tenotomy of the synergistic gastrocnemius. They also studied the extensor digi-

torum longus muscle following extirpation of the synergistic tibialis anterior They observed that there was an increase in the number of fibers rather than pure fiber hypertrophy. The stretch effect was presumably also implicated in the activation of interstitial cells The authors described a sequence of events beginning with satellite cell proliferation and leading to the formation of branched and new fibers. They suggested that there was a possibility for the muscle fibers to undergo some form of covert damage as a result of sustained stretching that might stimulate a kind of regenerative response. Tabary et al. (1972) have shown that if a muscle is fixed in a lengthened position, sarcomeres are added, and if it is fixed at a shorter length, sarcomeres are lost. These results were confirmed in another study by Williams and Goldspink (1978) who studied the changes in sarcomere length and physiological properties in immobilized adult mouse soleus muscle. After a three week period of immobilization in lengthened position sarcomere number was increased and sarcomere length was shorter than controls. On the other hand, immobilization in shortened position resulted in reduced sarcomere number and increased sarcomere length. The results from this study were slightly different from an earlier investigation (Williams and Goldspink, 1971) in which young mice, including newborns, were used. The investigators immobilized the soleus muscle in the same way for periods of two to six weeks and found that the

effect of immobilization during the growth period was a reduction in the rate and extent of sarcomere addition. The number of sarcomeres in fibers of muscles which had been immobilized in the shortened position was approximately half that in fibers from the normal, contralateral muscles. In fibers from muscles which had been immobilized in the extended position, the number of sarcomeres was reduced to almost the same extent as in those which had been immobilized in the shortened position. Following removal of plaster casts in both types of immobilization an increase in sarcomere number to normal levels was observed within a period of four weeks. In this study Williams and Goldspink also investigated fiber growth by injection of [^{3}H] adenosine. They found that most of the label was at the ends of the muscle fibers. This result indicates that the newly formed action and ribosomes, hence new sarcomeres, are found mainly in the end regions of growing muscle fibers. The finding is consistent with those of other researchers who have studied postnatal increase in muscle fiber length (Holtzer et al., 1957; Ishikawa, 1965; Mackay, Harrop and Muir, 1969).

Williams and Goldspink (1973) stated that even muscles which have undergone extremely long periods of immobilization have the same ability to recover as younger muscles. There seems to be no point in development beyond which the muscle cannot recover from the effects of immobilization by producing

more sarcomeres in series. Immobilization also had an effect on the number of muscle fiber muclei. After a two week period of immobilization muscle fibers contained significantly fewer nuclei than fibers from contralateral control muscles. However, when the plaster casts were removed the number of nuclei increased to that of normal fibers

Holly et al (1980) studied stretch induced growth in chicken wing muscles and Laurent et al. (1980) studied protein turnover during skeletal muscle hypertrophy. These authors found that stretching of skeletal muscle produces significant hypertrophy.

In 1981, Maxwell and coworkers studied histochemical properties, muscle fiber cross-sectional area, muscle fiber length, and the oxidative capacity of masticatory muscles of female rhesus monkeys These parameters were assessed following alteration in the functional length of the muscles which was accomplished by an intraoral appliance with or without simultaneous detachment of the muscles. The experimental period was 4 to 48 weeks. The intraoral appliance displaced the mandible infero-posteriorly and caused a 15% lengthening of masseter and temporalis muscle. The authors found that stretching these muscles within physiological limits did not significantly alter the proportion of fiber types. However, it did appear to cause a reduction in the oxidative capacity of type I and type IIA fibers. They also found that

surgical detachment of the masseter muscle with surgical reattachment resulted in a significant decrease in the mean cross-sectional area of type I fibers in the anterior masseter muscle. The length of individual fibers within the temporalis muscle increased following muscle lengthening in conjunction with masseter myotomy. The number of sarcomeres in muscle fibers of the deep and superficial masseter muscles was not significantly altered from control values by the experimental procedures, whether the functional length of the masseter was increased by the appliance, the masseter was detached, or detached and reattached. Significantly more sarcomeres in temporalis muscle fibers were observed in those animals which had surgical manipulation of masseter muscle in addition to the bite-opening appliance compared to controls. The authors concluded that neither surgical manipulation of the masseter alone nor the appliance alone were sufficient to induce significant response. However, the combination of the two stimuli resulted in marked effects.

The use of [^{3}H]-Thymidine has played an important role in investigations concerned with the renewal of cell populations. Such studies have shown that satellite cells, described by Mauro et al. (1961) and Kanji and Allbrook (1965), are stem cells to myoblasts. They may divide and are eventually incorporated to form new fiber after injury or damage to the muscle (McConnachie et al., 1964; Church, 1970; Moss and Leblond, 1971; Bischoff,

1974; Konigsberg et al., 1975; Snow, 1977 a and b; and Shafiq et al., 1980). Mazanet et al. (1982), who studied the frog sartorius muscle, concluded that satellite cells have the ability to fuse with muscle fibers until adulthood and they can become motile and replicate following muscle fiber injury. Satellite cells are also thought to be responsible for muscle tissue regeneration which has been shown both in vivo (Snow, 1977 a and b) and in vitro (Bischoff, 1975, Konigsberg et al , 1975).

Presently the nature of orofacial soft tissue response to so-called functional orthodontic treatment is still essentially unclear and further studies focusing on adaptation of these tissues are needed

MATERIALS AND METHODS

Sample

Twenty-eight male Syrian hamsters (Mesocricetus auratus), (Charles River Breeding Lab., Wilmington, MA) were used. They were 30 days old and weighed 45-83 gm. at the beginning of the experiment. Only males were used in order to eliminate the effect of the estrous cycle on mitotic activity (Bullough, 1943).

The Syrian hamster is an ideal model for research in many areas of gross and microscopic anatomy. For instance, the musculature of the hamster cheek pouch offers a unique site for experimental manipulation. It is readily accessible and the superficial thin layers of muscle fibers are easily exposed and biopsied.

All animals were kept in standard laboratory housing under similar conditions, i.e., room temperature 65-75° F, humidity 55-60% and a light cycle of 14 hours per day. The animals were fed commercial laboratory chow (Formulab chow #5008, Ralston Purina Company). Body weights were recorded daily throughout the experiment.

Description of the Hamster Cheek Pouch

The cheek pouches of the hamster are paired, internal muscular sacs, opening into each side of the mouth and extending dorso-caudally over the shoulder (West, 1958). The blind (caudal)

end of the pouch is devoid of muscle fibers. The wall of each pouch is composed of four distinct layers (Fulton et al., 1947):

1. Stratified squamous epithelium
2. Dense fibrous connective tissue
3. Longitudinal striated muscle fibers which originate from the oral region as a thin strap and flaring out as it approaches its insertion. The muscle layer is derived principally from the buccinator muscles.
4. Loose areolar connective tissue joining the wall of the pouch to the surrounding tissues.

Blood vessels and nerves are found in the connective tissue and muscle layers.

There are two other striated muscles of the pouch which, however, were not involved in this study, i.e., the retractor muscle and the circular muscle The retractor muscle is a long strap-shaped muscle originating from the lumbodorsal fascia and spinous processes of the last three thoracic vertebrae and inserts proximally at the blind end of the pouch. The circular muscle consists of fibers which form part of the pouch opening into the oral cavity. The longitudinal and circular muscles are controlled by branches from the facial nerve. In addition to these muscles, the superficial muscles of the face and neck indirectly aid in the deposition and removal of material from the pouch. In a mature hamster the distended pouch ranges in length from 2.5 to 5.5 cm. The width is approximately 1 cm. The pouch has "rugae" and these longitudinal folds expand when the pouch is being filled.

Grouping and Experimental Period

The sample was divided into two main groups, one for muscle histochemistry and the other for autoradiography. Each of these two groups had four sub-groups (Tables I and II).

Each of the four groups for the muscle histochemical study consisted of four Syrian hamsters. Three hamsters had the stretching device in the left cheek pouch while the right one was left untouched and served as control One hamster in each group was sham-operated by suturing the opening of the left pouch. The contralateral side was left untouched.

In order to study the mitotic activity of cells, the radio-isotope labelling study was performed. There were three animals in each of the four groups. Two of them had the stretching device in the left cheek pouch. The third animal in each group was sham-operated by suturing the left cheek pouch The right cheek pouch served as control in all animals.

Anesthesia

For surgical procedures, each animal was anesthetized by an intraperitoneal injection of Diabutal^{TM} (Sodium Pentobarbital) (Diabutal^{TM} 60 mg per ml., Diamond Laboratories, Inc., Des Moines, Iowa) at a dose of 6-9 mg/100 gm body weight. With this dose, the anesthetic period lasted 30-45 minutes.

TABLE I

GROUPS AND EXPERIMENTAL PERIODS FOR HISTOCHEMISTRY STUDY

Group	Experimental Period
I	7 days of stretching
II	14 days of stretching
III	21 days of stretching
IV	28 days (stretching device removed after 21 days, allowing for a one week recovery period)

TABLE II

GROUPS, EXPERIMENTAL PERIODS AND TIME OF INJECTION FOR RADIOISOTOPE LABELLING STUDY

Group	Experimental Period	[^{3}H] Thymidine[1] Injection Dose: 1μCi/gm body weight
I	8 days of stretching	1 injection on day 7 (24 hours prior to sacrifice)
II	15 days of stretching	2 injections on days 7 and 14 (the second injection was 24 hours prior to sacrifice)
III	22 days of stretching	3 injections on days 7, 14 and 21 (the third injection was 24 hours prior to sacrifice)
IV	22 days of stretching 7 days recovery	4 injections on days 7, 14, 21 and 28 (the fourth injection was 24 hours prior to sacrifice)

[1]Thymidine (Methyl-3H) 0.025 MC in 0.5 ML, Schwarz/Mann, Orangeburg, N.Y.

Design of Device for Stretching the Cheek Pouch

The device was made of a finger from a latex glove and was about 1.5 inches in length. The opening was sealed with alpha cyanoacrylate, into which a piece of latex tubing had been inserted to function as a valve. After the animal was anesthetized, the pouch was cleaned and the device was inserted into the left cheek pouch so that the blind end of the device was pushed into the caudal part of the pouch. The latex tube was left protruding from the entrance of the cheek pouch.

In order to stretch the pouch, about 3.5-4 0 cc of rubber base impression material (Citracon, Kerr Sybron, Romulus, Michigan) was mixed and injected through the tubing into the stretching device with a 10 cc syringe. After the material was set, the orifice of the pouch was sutured and the tubing was cut off. Suturing was made with 4-0 black silk and about 3-4 mm of tissue was picked up with each stitch to prevent laceration.

In the sham-operated animals, the left pouch was cleaned and the opening closed by suturing under anesthesia.

Procedures for Radioactive Isotope Labelling Technique

Tritiated thymidine (Methyl-3H 0.025 MC in 0.5 ML, Schwarz/Mann, Orangeburg, N.Y.) was injected intraperitoneally in a dose of 1.0 micro Ci/gm body weight. All injections were administered between 5:00 and 7:00 P.M. in order to minimize the effect of diurnal variation of cell proliferation (Bullough, 1948).

Figure 1 Gross anatomy of the cheek pouch showing three different muscles
1 Circular muscle
2 Longitudinal muscle
3 Long retractor muscle

Figure 2. Procedure for placing stretching device in the cheek pouch

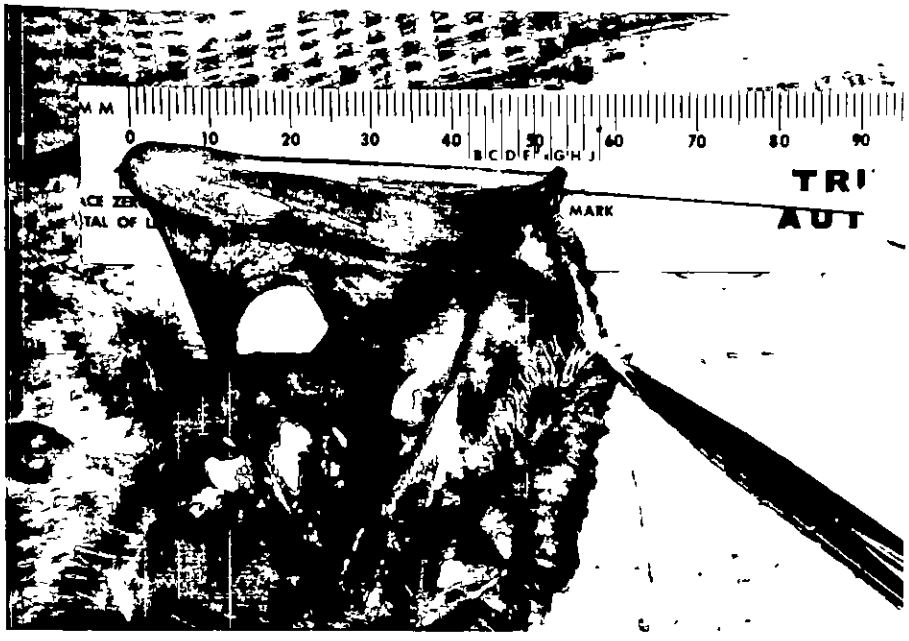
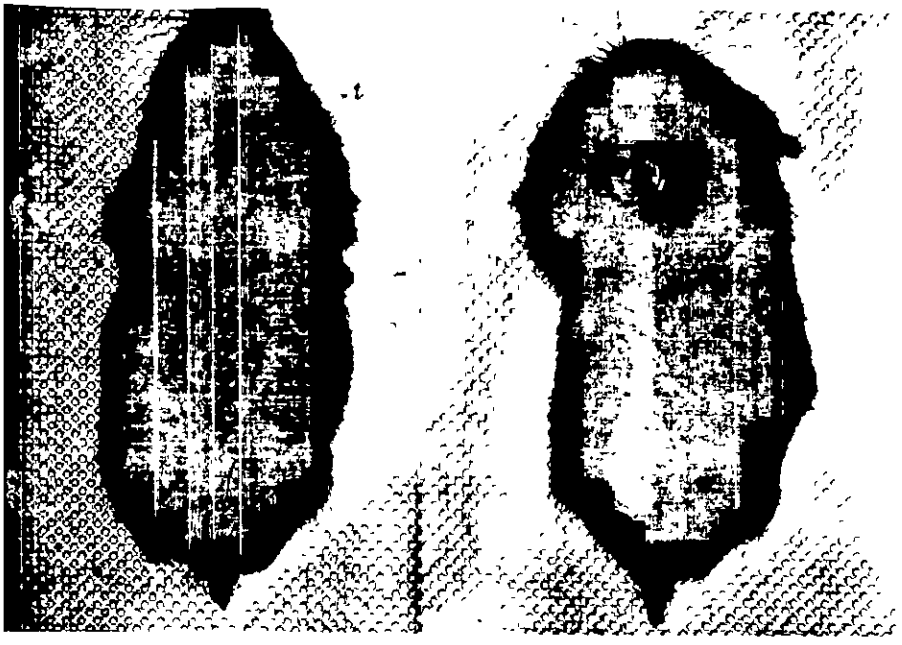
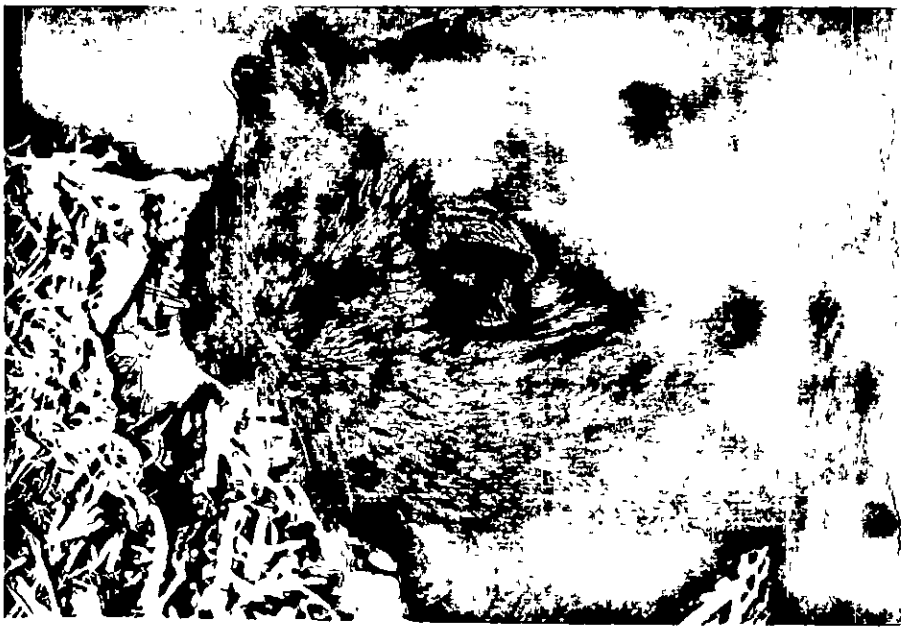

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Figure 3 Comparison between experimental and sham-operated animals.

Figure 4. Experimental animal with a stretched pouch





Sacrifice and Specimen Preparation for Autoradiography

The animals were anesthetized by an intraperitoneal injection of Diabutal. The cheek pouch was surgically exposed and removed by a circular incision at the orifice of the pouch. A longitudinal incision was made along the full length of the pouch to unfold and flatten it. The tissue was pinned out flat on Sylgard in 10% buffered formalin and fixed for at least twenty-four hours.

Small blocks of tissue about 3 mm by 2 mm, were excised from the anterior, middle and posterior parts of each pouch. Blocks were dehydrated in a graded series of ethanol (50%, 70%, 80%, 90%, 95% to 100%), fifteen minutes in each solution.

The dehydrated specimens were infiltrated in methacrylate plastic (JB-4 Polysciences, Inc., Paul Vally Industrial Park, Warrington, PA) overnight and then embedded. There were three specimens (from the anterior, middle and posterior parts of each cheek pouch) in the same tissue embedding block The embedded tissue blocks were allowed to polymerize at least twelve hours. The cured blocks were sectioned in a longitudinal plane with a glass knife on a JB-4 microtome at 3.5 microns thickness and mounted on acid cleaned slides.

Autoradiography for Light Microscopy Coating Technic

(Kopriwa and Leblond, 1962)

The slides with the labeled specimens were dipped in

Eastman Kodak NTB2 emulsion in a light proof dark room with safe light condition. The following procedure was employed for emulsion coating

1. Heat water bath to 40° C, then transfer melted emulsion to a pre-warmed, acid cleaned slide container which has been placed in the water bath and held constant at 40° C. Pour emulsion into container to a level where the tissue on the slide will be covered

2 Immerse the lower 2/3 of the slides (the part with tissue sections) into the molten emulsion vertically without tilting and leave for 1-2 seconds. Slowly withdraw slides Remove excess of emulsion by letting the lower edge of the slide lightly touch a wet filter paper.

3 Keep slides vertically and let the emulsion air-dry for two hours. (Too rapid drying produces uneven films causing clumping of silver grains and may also cause a heavy background level of grains due to build up of stresses in the emulsion during drying.)

Exposure

After drying, the coated slides were stored in light proof boxes containing silica gel (Dendrite) at 4° C. The desiccant minimizes the tendency for latent image fading during the exposure period Latent image fading is more likely to be troublesome when having low levels of radioactivity and when long exposure times are necessary. In this study, 25 days was estimated to be an optimum duration of exposure.

Processing

After 25 days of exposure, the slides were transferred to glass slide trays and processed in the dark room.

The following sequence of procedures was employed for processing the coated slides:

1. Develop in freshly prepared Kodak Dektol developer at 17° C for 2 minutes and 30 seconds. Agitate gently every 30 seconds.
2. Rinse in stop bath at 17° C for 15 seconds.
3. Fix in Kodak rapid fixer at 17° C for three minutes.
4. Wash thoroughly in running filtered tap water for 20 minutes.
5. Dry in well-ventilated area, whereafter the preparations are ready for staining.

Staining Procedure (Hematoxylin-Eosin Staining)

By suitable staining procedures the underlying section may be stained for visualization of important features, and thus the distribution of grains can be related to specific tissue structures

Slides were transferred to a metal slide tray and stained by hematoxylin-eosin staining procedure as follows:

1. Place slides in Gill's hematoxylin #3 for 45 minutes.
2. Rinse in distilled water by dipping 10 times.
3. Place in acid water (10 drops of 1 M HCl in half gallon of distilled water) and agitate gently for two minutes.
4. Place in Scott's tap water substitute for one minute and agitate gently.
5. Place in 0.5% Eosin for three minutes.

Initial examination of labeled cells was at X680 magnification with oil immersion to verify proper labeling (about 10 grains or more) Cell counts were made at a magnification of X400.

Procedures for Muscle Histochemistry

The purpose of this part of the study was to examine any changes in fiber type, and in size and number of fibers of the cheek pouch muscle under mechanical stretch compared to the normal pouch.

Muscle Biopsies

The middle part of left and right cheek pouches from all experimental and sham-operated animals were prepared for histochemical examination. The middle part of the pouch was presumed to receive the most mechanical stretching. The soleus muscle was also biopsied and used as reference material since its normal histochemical profile is known (Goldspink and Ward, 1979).

The following steps were employed for the preparation of muscle tissues prior to the histochemical procedures (Dubowitz and Brooke, 1973):

1. The animal was anesthetized by intraperitoneal injection of Diabutal.
2. The middle portion of the cheek pouch was removed A biopsy from the soleus muscle was also taken.
3. The muscle specimen was placed in Cryomold (Tissue-Tek II, Lab-Tek Division, Miles Laboratories, Inc.,

Naperville, IL), which was half filled with Cryoform (Tissue-Tek II, Lab-Tek Division, Miles Laboratories, Inc., Naperville, IL). After orientation of the specimen, Cryoform was added to completely cover the tissue.

4. The Cryomold with specimen was immediately frozen by plunging it into isopentane cooled to -160° C with liquid nitrogen The tissue block was stored at -90° C in airtight container.

5. The specimen was cut on a Cryostat (International Cryostat, IEC, Model CTO, Needham Heights, MA), at a thickness of 8 microns. The orientation of the specimens was such that serial cross-sections of muscle fibers were obtained.

6. The sections were positioned on warm, clean, and chrome-alum treated glass slides, air-dried at room temperature for an hour prior to histochemical processing.

Methods for Calcium-Activated ATP-ase

The mechanism of the ATP-ase reaction is as follows (Sheehan and Hrapchak, 1980): Sections are incubated in a solution containing adenosine triphosphate (ATP) substrate and calcium. ATP-ase present in the skeletal muscle tissue hydrolyzes the substrate, and the products formed are adenosine diphosphate (ADP) and orthophosphate. The released phosphate combines with the calcium present to form colorless calcium phosphate. When the tissue is exposed to the cobalt chloride solution, the cobalt is exchanged for the calcium and a colorless precipitate of cobaltous phosphate is now present at the sites of enzymatic activity. Upon treatment with ammonium sulfide, a

black insoluble precipitate of cobaltous sulfide is formed and permits visualization of the products of enzymatic activity.

The procedure (modified from Chayen et al., 1969) involved the following steps

1 Preincubation at pH 4 3, 4 6 and 10.3 for five minutes Reaction medium consists of:

0 2 M sodium acetate (27.2 g/1L), 45 ml

0 2 M acetic acid (11.55 ml gs/1L), 55 ml

The pH is adjusted with 0.1 M HCl and 0.2 N NaOH.

2 Blotting of the preincubation solution remaining on the slides with clean tissue paper.

3. Incubation at pH 9.4. All preincubated sections are incubated in a freshly prepared reaction medium at 37° C for 30 minutes. Reaction medium consists of:

0.1 M sodium barbiturate solution (2.06 g/100 ml) 10 ml

ATP, 76 mg

2.4 dinitrophenol, 30 mg

2% calcium-chloride solution (10.5 g, $CaCl_2 \cdot 2 H_2O$/100 ml), 5 ml

Distilled water, 35 ml.

4. Wash in three changes of 1% calcium chloride for two minutes in each bath.

5. Rinse in running distilled water for two minutes.

6. Transfer to 1% cobalt chloride solution for two minutes Wash in two changes of the cobalt chloride solution for two minutes.

7. Wash thoroughly in running distilled water for four minutes to remove unadsorbed cobalt.

8. Place in dilute ammonium sulfide (0.5% V/V) for one minute.

9. Wash in distilled water for two minutes.

10. Mount the sections with A-Q aqueous mounting medium (Hopkins and Williams, Chadwellhealth, Essex, England).

ATP-ase activity is shown by a brown-black precipitation of cobalt sulphide. All slides were examined to identify muscle fiber types A morphometric procedure was performed in order to compare the size and number of muscle fibers in experimental and control cheek pouches. From all four groups areas on the histochemical slides were selected under low magnification (X 25) and photomicrographs were taken at the magnification of X 103 using Kodachrome-25 film (KM 135-36, Eastman Kodak Company, Rochester, N.Y.).

All the slide pictures were projected onto the wall under standardized magnification. The outlines of the muscle fibers were traced on white paper. On each tracing, three different areas of the cheek pouch muscle were randomly selected and fibers falling within a 10 cm by 10 cm area were counted and measured When a fiber touched the borderline of this area, its entire outline was completed and counted, even though part of the fiber may have extended beyond the outline square. Number of the muscle fibers from these three different areas were counted. The diameters of 100 fibers from each of these separate areas were measured. It has been shown by some researchers

that it is statistically valid to use this size of sample and method of fiber measurements (Meara, 1947, Joubert, 1956). The measurements were taken as the maximum distance across the lesser aspect of the muscle fiber (Brooke, 1973). This procedure was used to avoid artifacts introduced by oblique sectioning of the muscle which will give an artifically large measurement. Measurements were taken by a Boley Gauge with sharpened points.

RESULTS

Weight Gain

The body weight of the experimental animals decreased by 2 to 7% during the first three days after the stretching device was placed (Tables III and IV). However on the fourth day they started to gain weight. The animals adapted readily to the appliance and experienced no significant problems in feeding behavior. Despite this, the stretching device might have had somewhat of an effect on the dietary intake Compared with the sham-operated animals the average weight gain of the experimental animals within groups was less. This might be due to interference with their normal habit of storing food in the cheek pouches.

Muscle Histochemistry

The histochemical profile of the longitudinal cheek pouch muscle was determined by means of myosin ATP-ase activity for all experimental and sham-operated animals (Table V). Normally, this muscle is composed of a homogeneous population of type II A (F.O G.) myofibers. This fiber type is adapted for fast, repetitive contractions and is less subject to fatigue than type II B (F.G.). With the ATP-ase reaction type II A fibers are intensely stained after preincubation at a high pH (10.3) but show no staining with low pH preincubation (4.3 or 4.6).

TABLE III

WEIGHT DATA: MEAN OF INITIAL WEIGHT, FINAL WEIGHT AND WEIGHT DIFFERENCE IN ANIMALS FOR MUSCLE HISTOCHEMICAL STUDY

Group	Mean of Initial Wt. Ex-gr.	SEM for Initial Wt. Ex-gr.	Mean of Initial Wt. Sham-Op.	Mean of Final Wt. Ex-gr.	SEM for Final Wt. Ex-gr.	Mean of Final Wt. Sham-Op.	Mean of Wt. Diff.		Ave. Wt. Gain/Day	
							Ex-gr.	Sham-Op.	Ex-gr.	Sham-Op.
I	48.83	1.153	45.60	64.87	1.889	65.80	16.54	20.20	2.36	2.88
II	59.67	2.839	66.50	83.50	3.400	96.70	23.83	30.60	1.70	2.19
III	80.57	4.684	78.00	103.83	8.095	117.00	23.26	39.00	1.11	1.86
IV	64.93	2.515	68.5	87.00	1.893	109.30	22.07	40.80	0.79	1.46

TABLE IV

WEIGHT DATA: MEAN OF INITIAL WEIGHT, FINAL WEIGHT AND WEIGHT DIFFERENCE IN ANIMALS FOR RADIOISOTOPE LABELLING STUDY

Group	Mean of Initial Wt. Ex-gr.	SEM for Initial Wt. Ex-gr.	Mean of Initial Wt. Sham-Op.	Mean of Final Wt. Ex-gr.	SEM for Final Wt. Ex-gr.	Mean of Final Wt. Sham Op.	Mean of Wt. Diff.		Ave. Wt. Gain/Day	
							Ex-gr.	Sham-Op.	Ex-gr.	Sham-Op.
I	75.20	1.699	69.90	72.15	5.049	82.50	-3.05	12.60	-0.44	1.80
II	78.25	3.150	72.40	82.30	8.700	98.70	4.05	26.30	0.29	1.88
III	76.20	4.199	72.10	101.35	7.050	107.10	25.15	35.00	1.19	1.67
IV	63.95	1.349	65.60	91.05	2.649	112.60	27.10	47.00	0.97	1.68

In all experimental pouches, both in those subjected to stretch only and in those which had a one week recovery period following stretch, the fiber type profile was identical to the non-stretched (control) sides. All fibers exhibited the dark brown cobalt sulphide precipitation at pH 9.4 and 10.3 characteristic of type II A fibers.

Samples of soleus muscle processes simultaneously indicated a distribution of type II A (F O.G.), type II B (F.G.) and type I (SO) fibers

Morphometry of Muscle Fiber

Intragroup comparisons of muscle fiber diameter, between the experimental and control sides, were made to evaluate influence of tension on the longitudinal muscle of the cheek pouch. Mean diameters of 100 muscle fibers from each pouch in three randomly selected areas were statistically analyzed (Table VI). Differences in fiber diameter in the experimental and control cheek pouches within each of the four groups were determined by means of the t-test for paired comparisons. None of these groups showed statistical significance ($p > 0.05$) between experimental and control sides. This is probably, in part, due to small samples and high variance within groups especially with regard to the experimental side. However, the mean values for fiber diameters from the experimental sides in every group were consistently less

TABLE V

SUMMARY OF MUSCLE FIBER TYPES IN THE FOUR SAMPLE GROUPS

Group		ATPase pH				Fiber Type
		4.3	4.6	9.4	10.3	
I	Ex. Side	-	-	+++	+++	Type 2 A
	Control Side	-	-	+++	+++	
	Sham-Op.	-	-	+++	+++	
II	Ex. Side	-	-	+++	+++	Type 2 A
	Control Side	-	-	+++	+++	
	Sham-Op.	-	-	+++	+++	
III	Ex. Side	-	-	+++	+++	Type 2 A
	Control Side	-	-	+++	+++	
	Sham-Op.	-	-	+++	+++	
IV	Ex. Side	-	-	+++	+++	Type 2 A
	Control Side	-	-	+++	+++	
	Sham-Op.	-	-	+++	+++	

TABLE VI

PAIRED-COMPARISON T-TEST FOR DIAMETER OF MUSCLE FIBERS: UNSTRETCHED AND STRETCHED SIDES WITHIN EACH OF THE FOUR GROUPS

Group	Animal #	Diameter of Fiber (mm) Control Side	Diameter of Fiber (mm) Experimental Side	Mean of Diff.	SEM	T Value*
I	1	3.7	3.5	-0.067	0.811	-0.082
	2	3.3	1.9			
	3	3.4	4.8			
II	1	3.1	3.1	-0.167	0.705	-1.512
	2	3.2	2.4			
	3	3.8	1.4			
III	1	3.2	2.6	-1.233	0.633	-1.947
	2	3.8	1.3			
	3	3.4	2.8			
IV	1	4.6	3.7	-0.033	0.491	-0.068
	2	3.6	3.6			
	3	2.0	2.8			

*Critical value of "T" for α = 0.05 and degree of freedom 2 is 4.303

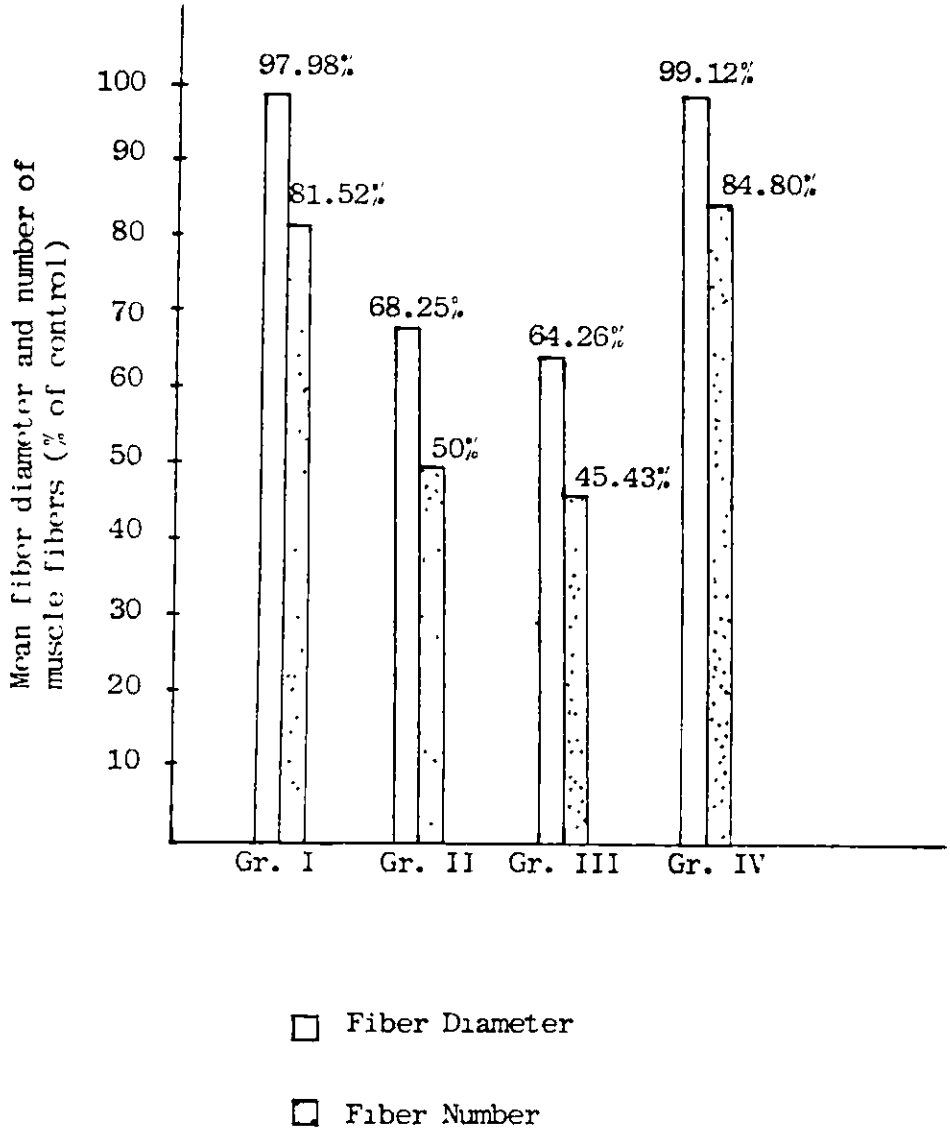


Figure 5. Mean fiber diameters and number of muscle fibers, expressed as percentage of control, after various periods of stretching.

than means from the control sides except for animal #3 in groups I and III. Figure 5 shows mean fiber diameters of experimental pouches, expressed as percentage of control. The mean of fiber diameters in group I was larger than in group II which, in turn, was larger than in group III. Thus, there was a tendency for continuous mechanical stretching to cause progressive decrease in muscle fiber diameter. After the stretching devices were removed from the experimental pouchs (group IV) the mean of fiber diameters increased and equalled the value for the contralateral control side.

Number of Muscle Fibers

The comparison between number of muscle fibers from the experimental and control pouches from the same group was done by means of the t-test for paired comparisons. Only data in group I showed a difference that was statistically significant and difference in the other three groups did not reach levels of statistical significance (Table VII). Again, a reason may be that the sample size was too small. In Table VII it can be seen that in all animals the value of the experimental side is consistently less than the value of the control side of the same animal. Especially in groups II and III, in which tension was exerted for two and three weeks respectively, the decrease is marked.

TABLE VII

PAIRED-COMPARISON T-TEST FOR NUMBER OF MUSCLE FIBERS: UNSTRETCHED AND STRETCHED SIDES WITHIN EACH OF THE FOUR GROUPS

Group	Animal	Control	Experiment	Mean of Diff.	SEM	T Value*
I	1	42.3	29.7	-10.1	2.207	-4.577
	2	42.0	36.3			
	3	79.7	67.7			
II	1	71.0	34.3	-29.67	13.653	-2.173
	2	74.7	25.7			
	3	32.3	29.0			
III	1	44.3	15.3	-22.67	6.839	-3.314
	2	33.0	24.0			
	3	47.3	17.3			
IV	1	47.0	41.0	- 5.33	1.594	-3.346
	2	40.0	32.3			
	3	18.3	16.0			

*Critical Value of "T" for α = 0.05 and degree of freedom 2 is 4.303.

In Figure 5 it can be seen that means of number of muscle fibers from the experimental sides, expressed as percentages of the contralateral control sides, progressively decreased with increase in duration of stretch (81.5% for group I, 50.0% for group II and 45 4% for group III). When the stretch was removed (group IV), number of muscle fibers returned rapidly to normal values (84.80%). These findings show that not only did stretching of the cheek pouch muscle cause signs of smaller fiber diameter but also indications of possible fiber degeneration and reduction in number of muscle fiber.

Quantitative Results from Autoradiography

Tritiated thymidine autoradiography was used to determine if chronic stretch would affect mitotic activity. Three different areas of each pouch, i.e., the anterior, middle and posterior were studied in order to determine if there were regional differences in labeling All data were gathered in a double blind format In five randomly selected areas of each sample, total number of nuclei observed was recorded. The number of muscle cell nuclei (myoblasts and satellite cells) was counted and expressed as a ratio of the connective tissue cell nuclei. Within the same areas the total number of labeled nuclei was counted and recorded as a percentage of the total number of nuclei observed. A ratio between the labeled muscle cell nuclei and labeled connective tissue cells was also recorded (Table VIII).

The anterior part of all the experimental cheek pouches was consistently found to be the area with the highest frequency of labeled nuclei (both muscle cell and connective tissue cell nuclei) when compared to the control and sham-operated cheek pouches within the same group. In group III, the middle part of the experimental pouch in one animal showed almost as many labeled nuclei as the anterior part. Even though the labeling was not entirely consistent, the data indicated that most of the labeled nuclei were located in the anterior end of the pouch on the stretched side This leads to the conclusion that stretching of the pouch muscle may induce fiber growth. This may also stimulate the satellite cells to divide and become incorporated into the fiber. It is reasonable to assume that such incorporation could take place at the end of the fiber as it is known that longitudinal growth occurs by the addition of sarcomeres in this part of fiber.

TABLE VIII

SUMMARY OF CONTROL AND EXPERIMENTAL QUANTITATIVE DATA FOR MUSCLE OF THE CHEEK POUCH

Group	Animal	Part of the Pouch	Total Nuclei Observed	Ratio of M Cells: CNT Cells	Total Labelled Nuclei Observed	% of Labelled Nuclei	Ratio of Labelled M Cells: Labelled CNT Cells
		Experiment.					
I	1	Anterior	571	1:2.04	34	5.95%	1:3.5
		Middle	245	1:2.92	1	0.41%	-
		Posterior	263	1:3.01	2	0.76%	-
		Control					
I	2	Anterior	603	1:0.34	0	0	-
		Middle	577	1:0.61	0	0	-
		Posterior	399	1:4.50	0	0	-
		Sham-Op. Sutured					
I	3	Anterior	209	1:0.50	1	0.48%	-
		Middle	188	1:2.50	1	0.53%	-
		Posterior	206	1:5.00	0	0	-
		Sham-Op. Non-Sutured					
I	3	Anterior	329	1:4.00	0	0	-
		Middle	186	1:0.25	1	0.54%	-
		Posterior	191	1:0.64	2	0.51%	-

TABLE VIII continued

Group	Animal	Part of the Pouch	Total Nuclei Observed	Ratio of M Cells: CNT Cells	Total Labelled Nuclei Observed	% of Labelled Nuclei	Ratio of Labelled M Cells: Labelled CNT Cells
		Experiment.					
II	1	Anterior	625	1:2.30	18	2.88%	1:1.36
		Middle	603	1:1.08	3	0.49%	-
		Posterior	412	1:0.40	3	0.73%	-
		Control					
II	2	Anterior	488	1:1.10	2	0.46%	-
		Middle	460	1:0.70	5	1.09%	-
		Posterior	760	1:0.86	0	0	-
		Sham-Op. Sutured					
II	3	Anterior	196	1:0.30	3	1.50%	-
		Middle	200	1:3.00	1	0.50%	-
		Posterior	289	1:6.00	0	0	-
		Sham-Op. Non-Sutured					
II	3	Anterior	193	1:1.50	0	0	-
		Middle	211	1:5.00	0	0	-
		Posterior	177	1:3.90	1	0.56%	-

TABLE VIII continued

Group	Animal	Part of the Pouch	Total Nuclei Observed	Ratio of M Cells: CNT Cells	Total Labelled Nuclei Observed	% of Labelled Nuclei	Ratio of Labelled M Cells: Labelled CNT Cells
		Experiment.					
III	1	Anterior	601	1:1.92	24	3.99%	1:1.15
		Middle	690	1:3.69	27	3.91%	1:3.67
		Posterior	360	1:2.82	1	0.28%	-
		Control					
III	2	Anterior	624	1:0.14	0	0	-
		Middle	321	1:1.50	0	0	-
		Posterior	298	1:0.48	0	0	-
		Sham-Op. Sutured					
III	3	Anterior	340	1:2.50	4	1.18%	-
		Middle	205	1:2.95	1	0.49%	-
		Posterior	191	1:1.02	1	0.52%	-
		Sham-Op. Non-Sutured					
III	3	Anterior	289	1:3.70	2	0.69%	-
		Middle	169	1:0.63	0	0	-
		Posterior	297	1:5.50	0	0	-

TABLE VIII continued

Group	Animal	Part of the Pouch	Total Nuclei Observed	Ratio of M Cells: CNT Cells	Total Labelled Nuclei Observed	% of Labelled Nuclei	Ratio of Labelled M Cells: Labelled CNT Cells
		Experiment.					
		Anterior	1109	1:2.80	70	6.31%	1:1.64
IV	1	Middle	448	1:5.90	6	1.34%	-
		Posterior	502	1:0.87	7	1.39%	-
		Control					
		Anterior	465	1:2.92	1	0.22%	-
IV	2	Middle	424	1:1.21	1	0.24%	-
		Posterior	534	1:1.54	0	0	-
		Sham-Op. Sutured					
		Anterior	256	1:2.07	4	1.56%	-
IV	3	Middle	396	1:6.58	3	0.76%	-
		Posterior	236	1:5.40	1	0.42%	-
		Sham-Op. Non-Sutured					
		Anterior	262	1:2.60	1	0.38%	-
IV	3	Middle	394	1:3.67	9	2.28%	-
		Posterior	349	1:6.50	1	0.29%	-



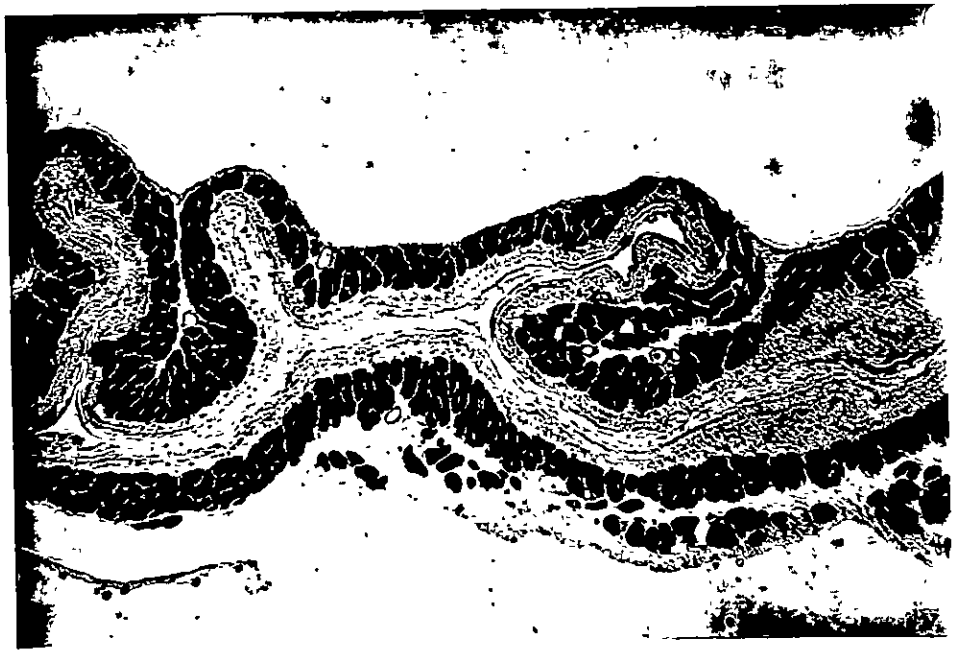


Figure 8 Muscle histochemistry of an experimental pouch in group II animal (ATPase at pH 10 3, X 103)

Figure 9 Muscle histochemistry of a control pouch in group II animal (ATPase at pH 10 3, X 103)





Figure 10 Muscle histochemistry of an experimental pouch in group III animal (ATPase at pH 10 3, X 103)

Figure 11. Muscle histochemistry of a control pouch in group III animal (ATPase at pH 10.3, X 103).





Figure 12. Muscle histochemistry of an experimental pouch in group IV animal (ATPase at pH 10.3, X 103).

Figure 13 Muscle histochemistry of a control pouch in group IV animal (ATPase at pH 10 3, X 103)





Figure 14. Radioisotope labeling study shows labeled nuclei in the experimental pouch (anterior part) of an animal in group I (X 165)

Figure 15 Radioisotope labeling study shows labeled nuclei in the experimental pouch (anterior part) of an animal in group II (X 165).

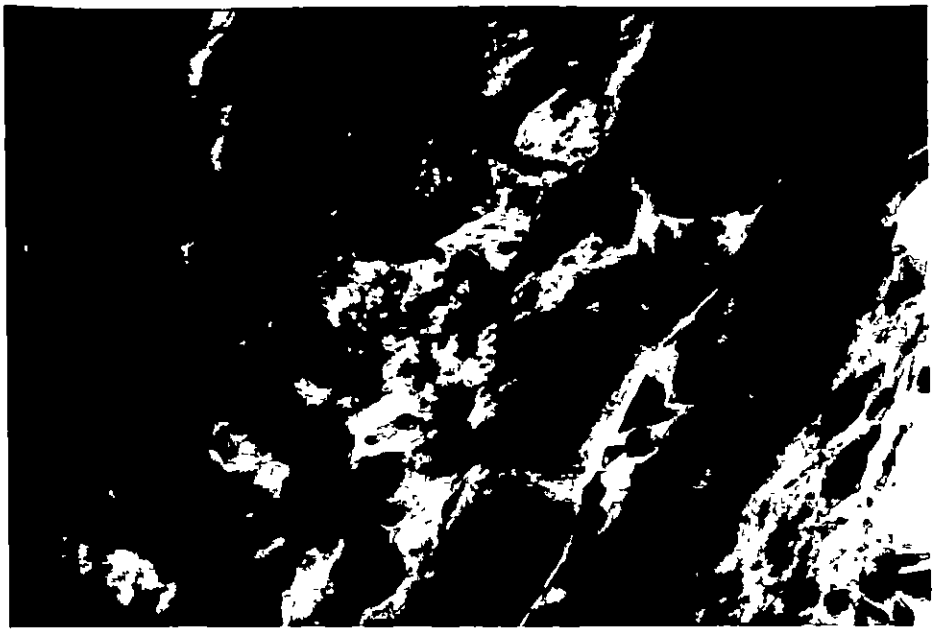




Figure 16 Radioisotope labeling study shows labeled nuclei in the experimental pouch (anterior part) of an animal in group III (X 165).

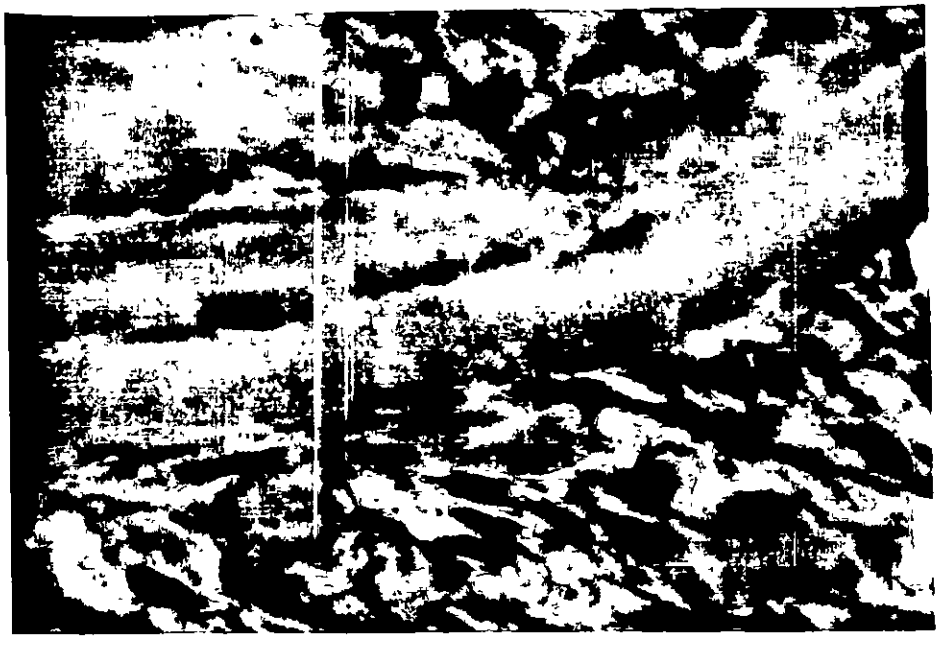
Figure 17. Radioisotope labeling study shows labeled nuclei in the experimental pouch (anterior part) of an animal in group IV (X 165)





Figure 18. Chain alignment of muscle nuclei in the experimental pouch (anterior part) of an animal in group I (X 165)

Figure 19. Muscle of the control pouch (anterior part) of the same animal in Figure 18 (X 165)



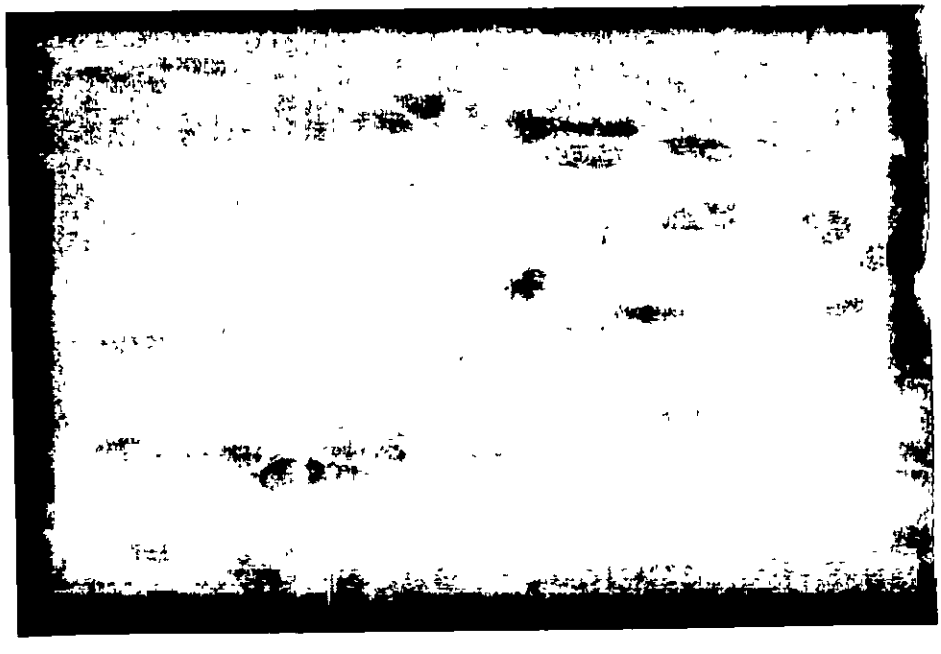
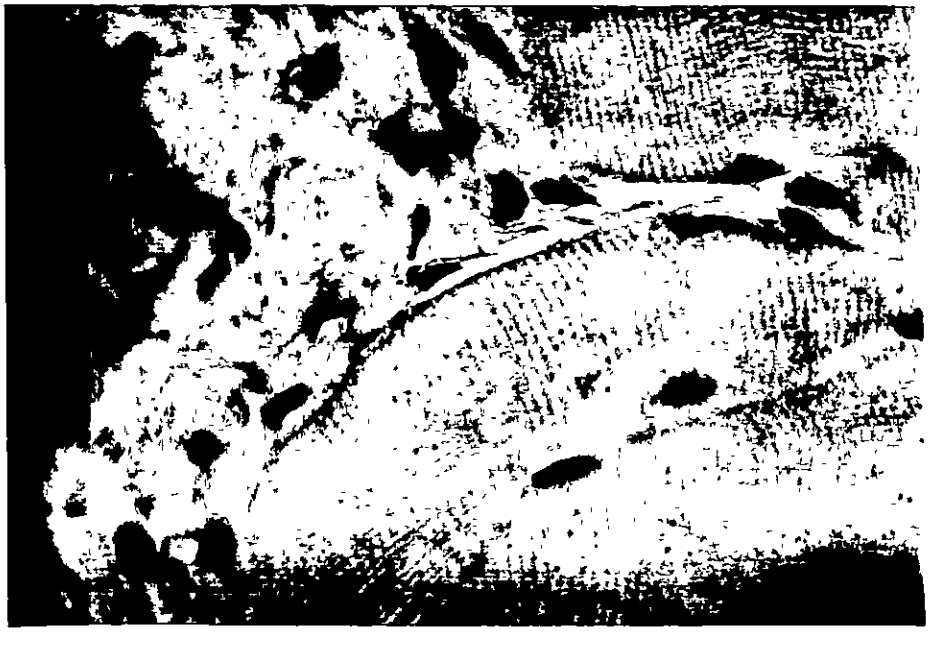


Figure 20 Chain alignment of muscle nuclei in the experimental pouch (anterior part) of an animal in group IV (X 165)

Figure 21. Muscle of the control pouch (anterior part) of the same animal in Figure 20 (X 165).





DISCUSSION

Skeletal muscle is a highly adaptive tissue that must continuously respond to changes in the external environment. Thus, muscle activity leads to many biochemical changes, the type of activity will determine the changes. It has been known for more than 80 years that mechanical stretching of skeletal muscle increases its metabolic rate (Feng, 1932).

The present study describes an in vivo model system concerning the adaptation of the orofacial musculature to mechanical stretch. The results show that facial muscle does, indeed, respond rapidly to tension caused by stretching of the muscle.

As it is part of the buccinator mechanism, the longitudinal muscle of the hamster cheek pouch was selected as the experimental site. The underlying thesis was to delineate the characteristics of the response in which subadult muscle adapts to change in its functional length due to mechanical stretching and to extrapolate from the findings to clinical orthodontics and what the implications may be on therapeutic rationale.

Primarily due to small sample size, the results did not show statistical significance in the comparison of cross-sectional fiber diameter and number of the muscle fibers between experimental and control sides within each group, except for fiber number in Group I ($p < 0.05$). However, the mean values of

both diameter and number of muscle fibers from each group did show the changes which seem to follow a pattern. Figure 5 illustrates the mean fiber diameters and number of muscle fibers expressed as percentages of control fibers after the various periods of stretching. Following seven days of stretching both diameter and number of fibers were slightly less than controls (97.98% and 81.52%, respectively). Stretching for 14 and 21 days resulted in progressively smaller values for both diameter and number of fibers (68 25% and 50% after 14 days; 64.26% and 45.43% after 21 days). When 7 days of recovery was allowed by removal of the latex balloon following 21 days of stretching, the mean values of fiber diameter as well as fiber number increased and approached those of the controls (99.12% and 84.80%, respectively) This finding demonstrates the high adaptive property of the cheek pouch muscle. The reactions for myosin ATP-ase activity indicated the presence of only one fiber type, i.e., type II A fibers. The experimental and control sides in all groups showed the same fiber type. Thus, the mechanical stretching did not induce transformation of muscle fiber types in this study.

The quantitative data from the autoradiographic study showed higher frequencies of both labeled muscle nuclei (myoblasts and satellite cells) and connective tissue nuclei in the anterior part of the pouch compared to the middle and posterior parts. This finding suggests that there was a higher rate of

mitotic activity in the anterior end of the muscle than in other parts. This possibly implies that sarcomeres were added in the anterior end as an adaptation to the lengthening of the muscle fibers due to the mechanical stretching. The sarcomere numbers were not studied because of technical difficulties. It was not possible to tease out single muscle fibers as they intermingled with the connective tissue fibers and the exact origin and insertion of the muscle were difficult to establish.

Petrovic et al. (1975), studied the control processes in the postnatal growth of the condylar cartilage of the mandible in growing rats. They found that the lateral pterygoid muscle plays an important role in the growth of the condylar cartilage. With a passive orthodontic appliance which produced mandibular advancement they found that, as the anatomical length of the lateral pterygoid was decreased, the number of serially arranged sarcomeres was reduced. They also found hypertrophy of the muscle pointing towards an increase in contractile activity. The relationship between muscle and bone was shown by an increase in prechondroblastic multiplication at the mandibular condyle. On the other hand, active advancement of the mandible by extraoral elastic traction did not result in any significant difference in the number of serial sarcomeres compared to controls. Petrovic and coworkers also investigated the effects of mandibular retractive forces. Here they found a significant increase in the number of

serial sarcomeres of the lateral pterygoid muscle, reflecting an increase in the anatomical length of that muscle. The effect of the chin-cup on the condylar cartilage was a reduction in the prechondroblastic activity. These experimentally induced variations in the number of sarcomeres can be explained as an adaptive phenomenon in response to alterations in the length of muscle fibers in situ.

In the present study, the apparatus used to stretch the muscle simulated a vestibular shield of a functional appliance. The appliance created continuous tension on the cheek pouch muscle. The design of the device was such that it allowed the cheek pouch muscle to shorten during activity since neither end of the muscle was fixed or immobilized. Maxwell et al. (1981) studied the adaptation of the masseter and temporalis muscles following alteration in length, with or without surgical detachment in rhesus monkeys. The functional length of muscles was altered by an intraoral bite opening appliance. They found that stretching within physiological limits did not significantly alter the proportion of fiber types, although oxidative capacity of the fibers was reduced and when the appliance was removed, the potential for oxidative phosphorylation recovered to normal limits The cross-sectional area of type I fibers significantly decreased only in the anterior masseter muscles which had undergone surgical detachment and reattachment indicating that recruitment of these fibers is the most sensitive to altered jaw function.

Tomanek and Lund (1974) immobilized the legs of guinea pigs by using plaster casts. The muscles were fixed at their resting lengths and thus less likely to contract. The data showed that after 21 days of immobilization, fiber diameters were markedly reduced in the soleus muscle. Some slight variation in the intensity of staining for ATP-ase activity (a few fibers tended to be slightly darker) was also observed. Similar results have been described by Summers and Hines (1951) and Cooper (1972), who found that atrophy of the soleus occurs rapidly during the first three or four weeks of immobilization. Karpati and Engle (1968) reported atrophy of both type I (SO) and type II (FG and FOG) fibers in the cat gastrocnemius after one month of immobilization. Maier et al (1973), who also investigated the cat gastrocnemius, observed in cross-sections that SO fibers were the most markedly reduced, followed by FOG fibers, while FG fibers were the least atrophic.

In contrast, immobilization of the muscle in a lengthened or stretched position results in hypertrophy. Williams and Goldspink (1978) suggested that in such muscles, maximum tension is increased as compared with controls and this may be due to the hypertrophy induced by the initial stretching of the muscle. Holly et al. (1980) used a spring device to stretch the muscles of six to eight week old chickens. The muscles which were being stretched were neither denervated nor immobilized.

Muscle activity, measured by EMG, was not increased. The results suggested that muscle hypertrophy was induced since muscle weight increased more rapidly in the stretched muscles when compared to the non-stretched contralateral control muscles.

In the present study, the muscle was not fixed or immobilized at either end The stretching device allowed the cheek pouch muscle to contract against increased load for a long period of time The action of the longitudinal muscle is to contract the pouch and thus draw it toward its aperture, serving the probable function of aiding in emptying the pouch (Priddy et al., 1948). Thus, the stretching device altered the function of this muscle. The results showed that means of fiber diameters and number of muscle fibers in experimental sides decreased when compared to their contralateral control sides. Maxwell et al. (1981) showed that changes in fiber cross-sectional area is one of the responses to alterations in functional demand. In my study induced stretching caused a decrease in fiber diameter as well as in number of muscle fibers. This, however, was not caused by disused atrophy but rather by changes in function. The stretching device impaired the contraction of cheek pouch muscle. In a study of denture wearers, Ringqvist (1974) noted a reduced percentage of type II fibers as well as a reduction in the cross-sectional area of fibers in the temporalis muscle of individuals who described their dentures as deficient. She concluded that decrease

in muscle function also leads to reduction in fiber size. Besides decreased function, other possible causes for atrophic tendencies of the experimental pouches from my study are factors such as poor nutrition and impaired elimination of toxic metabolic biproducts which may induce the onset of atrophy. The stretching device could interfere with the pouch blood supply and nerve endings, especially α -motor neurons. Since the muscle of the pouch is very thin, only 182 microns (Priddy et al., 1948), and the thickness of the whole pouch wall is only 0.4-0.5 mm the tissue is very sensitive to any kind of mechanical pressure.

Goldspink (1968) and Williams and Goldspink (1971) have shown that the increase in fiber length which occurs during normal growth is associated with a substantial increase in the number of sarcomeres along the length of the fibers. As the actin and myosin filaments are of a constant length, the adaptation of adult muscles to a different functional length must presumably involve the production or removal of sarcomeres in series in order to maintain the sarcomere length in relation to the total muscle length.

In 1972, Tabary et al. studied physiological and structural changes in the cat soleus muscle due to immobilization at different lengths by plaster casts. They found that muscles which had been immobilized in the lengthened position showed no difference in their length-tension properties compared to normal

muscles. However, they were found to have 20% more sarcomeres in series and decreased sarcomere length. The authors suggested that a change in the number of sarcomere in series seems to be the way in which the sarcomere length of the muscle is adjusted to its new anatomical length. However, they also demonstrated that a muscle which has adjusted to a new anatomical length can rapidly readjust to its original length once the immobilizing cast is removed. Other studies on the effect of immobilization had been done by Williams and Goldspink (1971, 1973, 1978). They demonstrated in the mouse soleus muscle that in the case of immobilization in the lengthened position, different results were obtained according to the age of the animals. Muscle fibers from animals older than two and one half weeks had more sarcomeres in series than fibers from control animals. In very young mice, on the other hand, the fibers contained fewer sarcomeres in series than those of control animals. Regarding recovery they found that even muscles which had undergone extremely long periods of immobilization had the same ability as younger muscles to recover when the casts were removed. With tritiated adenosine these authors located the area on the fiber where the sarcomeres are added during recovery from immobilization in a shortened position. They found that new sarcomeres were added at the ends of the muscle fibers and particularly at the distal end.

There are two theories to explain the origin of the myoblast. Some investigators believe that the mature multinucleated muscle fiber is capable of producing new mononucleated cells after injury by a process involving the formation of a plasmalemma around single peripheral myonuclei with adjacent cytoplasm (e.g., Hay, 1959, 1970; Lee, 1965; Reznik, 1969, 1976; Teräväinen, 1970) Others maintain that the muscle satellite cell is a reserve myogenic stem cell that divides by mitosis after injury and can thus proliferate into a new population of myoblasts (e.g., Church and Norohna, 1966; Church, 1970; Moss and Leblond, 1971).

The present study tends to confirm the findings of Williams and Goldspink (1973) regarding the area of the fiber where longitudinal growth occurs. The findings showed more labeled nuclei on the experimental sides than on control sides with a higher concentration of nuclei in the anterior part of the pouch than in other parts. It seems reasonable to suggest that nuclei are probably incorporated into the fiber in areas where sarcomeres are being added during longitudinal increase. Thus, it is likely that new sarcomeres were formed and added to the muscle fibers at the anterior end, i.e., where most labeled nuclei were observed. Possible explanations for this might be the richer blood supply of the anterior part or that this part of the pouch might have received an optimal magnitude of tension per cross-sectional area.

Because of infrequent injections of thymidine at low doses relatively few labeled nuclei resulted. Kitiyakara et al. (1963), who studied the postembryonic growth of the Gracilis muscle in mice, stated that because of the rapid rate at which [^{3}H] thymidine is incorporated into DNA of muscle nuclei, very few precursor cells will incorporate the radioactive compounds, divide and fuse into muscle fibers. Only precursor cells which are synthesizing DNA at the time of injection become labeled. Thus, relatively few labeled cells may contribute to the formation of the muscle fiber (Cronkite et al., 1959). Moreover, Potter et al. (1958) observed that non-metabolized thymidine is eliminated from the body within a few hours after administration and nuclei preparing for mitosis at slightly later periods will not be labeled. Based on this information, more frequent [^{3}H] thymidine injections could have resulted in more labeled nuclei for better and more reliable data.

Although most of my data were not statistically significant, I believe that additional observations will add support to my interpretation of the effects of induced stretching on the orofacial musculature.

The findings of my study do have clinical implications for orthodontic treatment especially when dealing with stretching and changing the normal function of orofacial musculature. The orofacial muscles can respond to tension or change in functional

length quite rapidly. The response of cheek pouch muscle may not exactly be the same as that of other muscles in this area but close similarities must nonetheless exist. The longitudinal muscle of the cheek pouch is quite unique as it is a very thin muscle and is composed exclusively of type II A muscle fibers. The effect of stretching with the device that simulates a buccal shield showed progressive decrease in both diameter and number of muscle fibers. Thus, the longer the stretching persisted the more marked changes occurred in the muscle.

Tension from the stretching device stimulated mitosis of muscle and connective tissue cells. This was possibly coupled with new sarcomeres being added at the anterior end of the pouch as an adaptive mechanism in response to alteration in length of muscle fibers. The use of buccal shields to stretch the buccal musculature may have the same result.

After removal of the stretching device to allow the muscle to recover for seven days, the mean values of fiber diameters and number of muscle fibers returned to normal values. This indicates that muscle is not only a very highly adaptive tissue in response to the insertion of an appliance but also to its removal. Thus, there must be interaction between skeletal and muscle response to maintain treatment results. Otherwise, and as this study has shown, relapse will occur. Structural recovery of the muscle is quite rapid once the insult is removed. Skeletal chan-

ges as a result of treatment may be sufficient to sustain the alteration in the muscle. Through a possible feedback mechanism the muscle alteration will, in turn, help to maintain the skeletal change if the stretching appliance has been worn long enough to induce the addition of new sarcomeres in the buccinator muscle.

SUMMARY AND CONCLUSIONS

The aim of the present work was to investigate the response of subadult orofacial musculature to changes in its functional length due to mechanical stretching and to consider the clinical implications of such manipulation.

Twenty-eight male Golden hamsters, age 30 days, were used in the study. A stretching device was inserted into the left cheek pouch of all experimental animals and sutures were placed at the opening of the pouch. Sham-operated animals had the left pouch orifice sutured. The right cheek pouch served as control site in all animals. The experimental periods were one, two, three and four weeks. For the last group (four weeks), the stretching device was removed after 21 days to allow for a 7 day recovery period.

Data were obtained by muscle histochemistry and autoradiography. The animals in the autoradiographic part of the study received intraperitoneal injections of [^{3}H] thymidine in a dose of 1μ Ci/gm body weight every 7 days and 24 hours before they were killed.

Data on fiber type, fiber diameter and number of muscle fibers were obtained from the histochemical part of the study. Paired comparisons between the experimental and control sides within each group were made by means of the t-test. The data

did not yield statistical significance with regard to diameter and number of muscle fibers, except for the number of muscle fibers following seven days of stretching which showed a significant decrease. However, mean values of the experimental sides demonstrated a consistent tendency of progressive reduction in fiber diameters and number of muscle fibers with increased duration of stretching. After removal of the stretching device, rapid recovery of the muscle fibers was observed. Only one fiber type, type II A, was detected on both experimental and control sides in all animals. Thus, the stretching device did not induce fiber type transformation.

The autoradiographic part of the study showed that most labeled nuclei were located in the anterior parts of the experimental pouches This finding might suggest that sarcomeres in series were added at the anterior end of the pouch muscle fibers in order to adjust to the new functional length resulting from the mechanical stretching.

The clinical significance of these findings is that mechanical stretching has effects on the orofacial musculature and the recovery of the muscle after removal of stretching is very rapid. This may provide for a better understanding of functional appliances and the phenomenon of relapse after removal of therapeutic appliances in clinical orthodontics.

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