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OF MUSCLE FIBER TYPES IN THE PLANTARIS
OF 1 - 36 DAY OLD RATS

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THE DIFFERENTIATION AND DEVELOPMENT OF MUSCLE FIBER TYPES IN THE PLANTARIS OF 1-36 DAY OLD RATS

By

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ABSTRACT

THE DIFFERENTIATION AND DEVELOPMENT
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The purpose of this study was to determine the differentiation and development patterns of the various muscle fiber types in the plantaris muscle of the rat, from one to thirty-six days of age. Forty-eight Sprague-Dawley rats were sacrificed at five-day intervals. The gastrocnemius, soleus, and plantaris complex was excised and prepared via fast freezing, for sectioning on a cryostat microtome. The muscle sections were stained with haematoxylin and eosin to study morphology. For histochemical analysis, the sections were stained with myosin adenosine triphosphatase (pH 9.4) with alkali (pH 10.4) preincubation, and succinate dehydrogenase. Fiber-to-fiber analysis was performed on the two histochemical stains, and classified using the nomenclature of Brooke and Kaiser (1970).

There were three major findings of the study. First is that muscle fibers are basically undifferentiated at birth. Secondly, that the differentiation process of the plantaris muscle follows a smooth and continuous pattern. Last is that the differentiation process appears to be associated with the aging process.

Dedication is made to my husband, Michael, for his love, support, and unending confidence in my ability. And to my parents, Dr. and Mrs. Wayne D. Van Huss, and my sisters, Terry, Randie, Trudy, and Amy, whose patience and love gave me added purpose and encouragement to complete the task.

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INTRODUCTION TO THE PROBLEM

It is well established that skeletal muscle fibers have been classified into types according to their morphological, physiological, biochemical and histochemical characteristics. The fiber types in different mature muscles have been described for several species (Ariano et al., 1973; Peter et al., 1972). However, the muscle fiber composition profile of mature muscle is not static (Goldspink and Ward, 1979; Rowe and Goldspink, 1969; White et al., 1978) and can change throughout life.

It has been shown that cross reinnervation of denervated muscles can alter the histochemical and biochemical composition of muscle (Brooke et al., 1971). Further evidence is accumulating that different types of physiological stress on skeletal muscle alters fiber composition and size (Goldspink and Ward, 1979; Gonyea et al., 1978; Maxwell et al., 1973). Goldspink and Ward (1979) and Gonyea et al. (1978) studied the response of the different fiber types to high intensity exercise (i.e., weightlifting). This type of exercise resulted in hypertrophy of all the fiber types; however, the extent of the response varied according to the fiber type. Fibers high in myosin adenosine triphosphatase activity (type II A, type II B) (Brooke and Kaiser, 1970) were found to hypertrophy more readily than fibers that were low in myosin adenosine triphosphatase (type I). Distinction was made between type II A and type II B, according to the amount of hypertrophy, where type II B fibers were found to hypertrophy more than type II A. The difference between these two high myosin ATPase fibers is in their metabolic enzymes, where type II B is more glycolytic and type II A is more oxidative.

Maxwell et al. (1973) found that with endurance training the plantaris muscles of guinea pigs at six weeks old, had a greater proportion of type II A fibers and a lower proportion of type II B fibers than did the muscles of control animals at the same age. They also found that there was no difference between the control and exercised animals in the proportion of type I fibers, or in the size of the various fiber types.

It has been shown that undernutrition can change the proportions of the fiber types (Goldspink and Ward, 1979; Haltia et al., 1978). Haltia et al. (1978) found that in rats, undernourished both pre- and postnatally, histochemical differentiation was retarded in the extensor digitorum longus. When pregnant rats were undernourished, their growth was severely retarded and only myotubes were found in the newborn skeletal muscles. With proper nutrition postnatally, large type I and type II B fibers were seen at five days, and the histochemical maturation proceeded almost normally at later stages. However, at all stages there was a proportional reduction in fiber and body size.

Goldspink and Ward (1979) also found a decrease in size of the fiber types with partial starvation in guinea pigs and mice. Of the fiber types, they found that type II B showed the largest decrease in size. They also determined that the effects of undernutrition on the fiber types were completely reversible. Starvation did not have an effect on the total number of fibers or the numbers of any fiber type.

There are great similarities and differences in the development and growth of muscles, not only between individual muscles, but also between species (Ariano et al., 1973; Colling-Saltin, 1980; Brooke et al., 1971; Rowe and Goldspink, 1969). The main difference is in the time course for development and differentiation. In rats it is believed that muscles are basically undifferentiated into fiber types at birth (Curless and Nelson, 1976; Riley, 1977; Shafiq et al., 1972) and that the fiber population found in the adult rat is determined by the postnatal differentiation process (Haltia et al., 1978; Curless and Nelson, 1976; Riley, 1977; Shafiq et al., 1972; Goldspink, 1970). Three stages of development are known to occur prenatally: the immature myotube, the mature myotube, and the young myofibril. The mature myofibrils occur postnatally (Platzer, 1978; Colling-Saltin, 1980). The young rat is born with a high proportion of mature myotubes to young myofibrils (Haltia et al., 1978;

Curless and Nelson, 1976; Shafiq et al., 1972). However, this proportion rapidly changes. Curless and Nelson (1976) found that in the first day of life sixty to eighty percent of the fibers in the soleus were in the myotube stage. By the third day only fifteen to thirty percent were in that stage. Brooke et al. (1971) found two types of fibers, type I and type II, in the soleus of the rat at two days of age. This investigator states that the type II fiber found is a type II C, which is a precursor to all other fiber types. Again, the percentages of the fiber types changed drastically at the early stages.

It has been shown that neonatal neurectomy will halt the differentiation process and the fibers will not develop from the myotube stage (Riley, 1977; Shafiq et al., 1972; Engel and Karpati, 1968). The results of these studies are discussed in more detail in the literature review. In the present investigation, only muscles with normal innervation were studied.

The problem is complex and the reports vary between investigators, species and muscles. One possible reason for the difference in percentages is that many investigators used random sampling of fields when classifying muscles. It is the intent of the present investigation to add information regarding the differentiation patterns of the entire plantaris muscle. It is hoped that the extensive nature of the present study will serve to reduce the confusion of the fiber type populations in early life, as previously reported for the plantaris (Maxwell *et al.*, 1973).

Many investigators have studied muscle and its dynamic properties (Close, 1964), but the major questions still exist regarding the mechanisms responsible for these changes. It would appear that to initiate study of these changes one must first attempt to determine how the various fiber types differentiate early in life. Therefore, the purpose of this study was to determine the differentiation and development patterns of the various fiber types from birth to thirty-six days of age in the plantaris muscle of the rat using histochemical techniques. To avoid sampling error, all individual muscle fibers across the entire muscle section of the plantaris were classified. The histochemical nomenclature of Brooke and Kaiser (1970) was used to classify the fibers.

Statement of the Problem

The purpose of the problem was to determine the differentiation and development patterns of the various muscle fiber types in the plantaris muscle of the rat from one to thirty-six days of age using histochemical techniques.

Importance of the Study

The importance of the study is to add information regarding the differentiation and development patterns of the entire plantaris muscle. Hopefully, this study will reduce the confusion of previously reported percentages of fiber types during postnatal growth by eliminating the error of tissue sampling.

Limitations of the Study

1. The results of this study are applicable only to the plantaris muscle of the rat under thirty-six days of age.
2. The results of this study are based primarily on fiber-to-fiber analysis using only two histochemical staining techniques: myosin adenosine triphosphatase (pH 9.4), with a preincubation solution of pH 10.4 and succinate dehydrogenase.
3. The results of this study are based only on histochemical analysis of muscle fibers, and not biochemical analysis of muscle fibers.

Definition of Terms

Type I fiber. According to the histochemical classification of Brooke and Kaiser (1970), the reactivity of a type I fiber using a myosin adenosine triphosphatase (myosin ATPase, pH 9.4) stain with the preincubation solution at a pH of 10.4 would be inhibited and, therefore, would yield a very light stain (cream color). Type I fibers contain high amounts of oxidative enzymes and stain dark with an oxidative enzyme stain. The succinate dehydrogenase (SDH) stain was used for the fiber-to-fiber analysis in this study. The type I fiber is classified as slow-oxidative (S.O.) by Peter et al. (1972).

Type II A fiber. A type II A fiber has an intermediate to high reaction (staining dark brown) when a myosin ATPase (pH 9.4) stain with a preincubation solution at pH 10.4 is used. This fiber will have a very strong reaction with the SDH stain and will stain even darker than a type I fiber. Peter et al.

(1972) refer to this fiber type as fast-oxidative-glycolytic (F.O.G.).

Type II B fiber. The type II B fiber demonstrates the strongest reaction to the myosin ATPase (pH 9.4), with a preincubation solution of pH 10.4, staining a very dark brown color. On the other hand, this fiber demonstrates a weak reaction (stains very light) to oxidative enzyme stains. Peter et al. (1972) have classified this fiber type as fast-glycolytic (F.G.).

Type II C fiber. This fiber stains dark brown with a myosin ATPase (pH 9.4) stain, with a preincubation solution at a pH of 10.4, and reacts strongly to oxidative enzyme stains such as SDH. Generally, this fiber cannot be distinguished using just these two stains and usually is classified as type II A. Brooke and Kaiser (1970) state that a type II C fiber is a precursor for the other three types of fibers. This fiber will not be classified in the present investigation.

Unidentified fiber. Some fibers cannot be classified via fiber-to-fiber analysis using succinate dehydrogenase (SDH) and myosin adenosine triphosphatase (myosin ATPase, pH 9.4), with an alkali preincubation solution. In the present investigation, these few fibers are listed as unidentified.

REVIEW OF THE LITERATURE

The purpose of this study was to determine the differentiation and development patterns of the various muscle fiber types in the plantaris muscle of the rat, from one to thirty-six days of age, using histochemical techniques. For this review of the literature, three categories of studies will be examined: histochemical techniques used for classifying muscle fiber types, muscle fiber types in the plantaris muscle of the adult rat, and differentiation of fiber types in developing muscles of the rat.

Classification Techniques

Early studies on skeletal muscle categorized fiber types by color (i.e. red or white) and/or speed of contraction (i.e. fast or slow). More recent investigations, using various histochemical techniques, have demonstrated that fiber types cannot be characterized simply on color or speed of contraction.

In 1955, Padykula and Herman developed a method to analyze myosin adenosine triphosphatase (myosin ATPase) activity in skeletal, smooth, and cardiac muscle. This investigation concentrated on ATPase activity in the myofibrils of skeletal muscle and not the ATPase activity present in mitochondria. Guth and Samaha (1969) reported that differences could be determined in the type of ATPase activity found in skeletal muscle fibers by altering the pH of the preincubation solution. Myosin ATPase activity is stable in an alkaline preincubation solution and is labile in an acid preincubation solution. Guth and Samaha (1969) found that there were no fibers stable in both acid and alkaline preincubation solutions.

Brooke and Kaiser (1970) used varying alkali and acid preincubation solutions to characterize the various types of fibers for myosin ATPase. They reported two major types, type I and type II, with type II having three subgroups: type II A, type II B and type II C. Type I fibers are labile in an alkali pH and, therefore, demonstrate a weak reaction when stained for myosin ATPase with a preincubation solution at pH 10.4. Type II fibers are stable in alkali solutions and demonstrate a strong reaction to the myosin ATPase stain, with the preincubation solution at pH 10.4. Type II fibers show different degrees of lability in various acid solutions and, therefore, are categorized into three subgroups. The reaction for type II A fibers is inhibited in a solution of pH 4.5. For type II B fibers, inhibition is demonstrated at pH 4.3 and below. Type II C fibers are labile in a solution of pH 3.9.

The type II C fiber was described further by Brooke _et al_. (1971) as the "primitive" fiber. This fiber is believed to be the precursor fiber to all the other fiber types.

Brooke _et al_. (1971) used the same nomenclature to differentiate fibers in the rat from birth to adulthood. Fiber-to-fiber analysis was performed using the myosin ATPase stain with alkali and acid preincubation solutions and an oxidative staining technique. The type II C fiber, when analyzed histographically for diameter size (Brooke and Kaiser, 1970) and histochemically via fiber-to-fiber analysis (Brooke _et al_., 1971), is similar to the type II A fiber. Therefore, these investigators state that the type II C fiber usually is included with the type II A fiber when muscle is analyzed.

Three fiber types, A, B, and C, were reported by Stein and Padykula (1962) based on the cytochemical distribution of succinate dehydrogenase (SDH) in the muscle fiber. SDH is an enzyme which is tightly bound to mitochondria. Fiber-to-fiber analysis of serial sections of the gastrocnemius and soleus were performed using SDH, "fixed" and "unfixed" ATPase, glycogen and other enzyme stains (e.g., non-specific esterase). These investigators found that the type A fiber contained few mitochondria and lacked SDH activity in the subsarcolemma. Type B had more SDH activity and more mitochondria than type A. Type C demonstrated the highest amount of SDH activity and, therefore, the most mitochondria. This investigation was performed using male and female albino rats, weighing between 175 and 200 grams. The age of the animals and the strain of rat were not reported.

Peter *et al*. (1972) classified fiber types into three categories based on the following criteria: the contraction time of the fiber relative to the other fibers in the same muscle, the glycolytic capacity of the fiber, and the oxidative capacity of the fiber. The three categories are fast-glycolytic (FG), fast-oxidative-glycolytic (FOG), and slow-oxidative (SO). Fast-glycolytic fibers are high in glycolytic enzymes and are fast in contraction. Fast-oxidative-glycolytic fibers are reported to have the highest concentration of oxidative enzymes with moderate to high amounts of glycolytic enzymes and to be fast in contraction. Slow-oxidative fibers have a moderate to high concentration of oxidative enzymes and are slow in contraction time. These investigators compared the previously reported nomenclatures of Brooke and Kaiser (1970), Stein and Padykula (1962) and others with the nomenclature of FG, FOG, and SO.

The classification of the various types of muscle fibers has been reported by several authors, each using a different nomenclature based on different histochemical techniques. The nomenclature of Stein and Padykula (1962) is not clearly defined for fiber typing in muscles of rats at an early age. The nomenclature of Peter *et al*. (1972) involves contraction time of fibers which is not applicable to this investigation. Therefore, the nomenclature of Brooke and Kaiser (1970) was used to classify muscle fiber types in this investigation.

Fiber Types in the Plantaris Muscle of the Adult Rat

Ariano et al. (1973) reported the fiber type populations for the hindlimb muscles of five mammals. Fiber populations were estimated histochemically and classified as fast-oxidative-glycolytic (FOG), fast-glycolytic (FG), and slow-oxidative (SO) (Peter et al., 1972). This nomenclature corresponds to that of Brooke and Kaiser (1970) as type II A, type II B and type I, respectively. Random sampling of muscle fibers of serial transverse sections was performed by fiber-to-fiber analysis of the myosin adenosine triphosphatase and reduced nicotinamide dinucleotide diaphorase procedures. For the adult Sprague-Dawley rat, the fiber type profile in the plantaris muscle was reported as fifty-three percent FOG, forty-one percent FG, and six percent SO. This profile was based on a sample size of three rats and a fiber sampling of 381 fibers.

The entire plantaris muscle of six Sprague-Dawley rats, at 140 days of age, were analyzed histochemically by Ho et al. in 1980. Fiber-to-fiber analysis was performed using the myosin adenosine triphosphatase (pH 9.4) with the alkali (pH 10.4) preincubation solution, and the NADH-tetrazolium reductase stains. These investigators reported a fiber type composition of forty-seven percent type II A, forty-three percent type II B and ten percent type I fibers. This fiber type population agrees reasonably well with the profile reported by Ariano et al. (1973). Total number of fibers in the plantaris muscle was not reported.

Differentiation of Fiber Types in the Developing Muscle of The Rat

Many investigators have agreed and reported that muscle fibers, in the rat, are basically undifferentiated at birth (Engel and Karpati, 1968; Brooke et al., 1971; Shafiq et al., 1972; Curless and Nelson, 1976; Riley, 1977; Haltia et al., 1978; Ho et al., 1980). Reports have varied, however, on the percentage of fibers in the myotube stage and the ability to distinguish the fibers at birth. Engel and Karpati (1968) found that 90 percent of the fibers in the soleus muscle were in the myotube stage and that the remaining ten percent could not be differentiated. Shafiq et al. (1972) agreed with these findings and found this to be true for the gastrocnemius and extensor digitorum longus muscle also. In both investigations, the rats were sacrificed within twelve hours after birth.

Haltia et al. (1978) report, for one day old animals, the extensor digitorum longus muscle has 90 percent of the fibers in the myotube stage, and ten percent are large type I fibers. Ho et al. (1980) report similar results for the plantaris and soleus muscles at the same age. Haltia et al. (1978) further define the myotubes as being small fetal-type "F" fibers. They state that the myotubes exhibit similar staining characteristics to the type II C fibers, which have been described as the "primitive" fibers by Brooke et al. (1971). These staining characteristics are positive reactions with both alkali and acid preincubation procedures for the myosin ATPase (pH 9.4) technique.

Brooke _et al_. (1971), using the aforesaid histochemical techniques, report that myotubes, type I, and type II C fibers are present at birth in the soleus muscle. Curless and Nelson (1976) support the finding that fiber differentiation is evident in one-day-old animals when the myosin ATPase stain is used with the preincubation solution at pH 4.35. However, these investigators state that the standard myosin ATPase procedure fails to reveal any distinct differentiation. They report that in any one microscopic field 60 to 80 percent of the fibers may be in the myotube stage. These investigators report differentiation of the myotube fibers with 90 percent of these fibers being type I and type II C fibers and the remaining ten percent being of an undetermined fiber type.

Curless and Nelson (1976) also report that the percentage of type I fibers in the rat soleus remains constant, at approximately 60 percent (range 53 to 65 percent), from one to twenty-nine days of age. Even though these investigators describe the percentage of type I fibers as basically unchanging with age, they report a dynamic pattern of differentiation for the other fiber types. For example, they report that the percentage of myotubes per field drops from 60 to 80 percent at one day of age to 15 to 30 percent at three days of age. The ten percent of undetermined fibers reported at one day of age are recognized as type II C fibers at seven days of age. Besides type I fibers, many type II C fibers are reported to be present until seventeen days of age.

Between seventeen and twenty-one days of age, type II C fibers drop to less than two percent with the type II A fibers emerging and increasing to thirty-three percent. At twenty-nine days of age, the fiber profile for the soleus muscle is reported as 65 percent type I, 33 percent type II A, and less than two percent type II C.

Several investigators have reported a dramatic decrease, from birth to between six and ten days of age, in the amount of myotubes present in muscle regardless of the muscle studied (Brooke et al., 1971; Engel and Karpati, 1968; Shafiq et al., 1972; Curless and Nelson, 1976; Riley, 1977; Haltia et al., 1978; Ho et al., 1980). Engel and Karpati (1968) and Haltia et al. (1978) reported a total absence of myotubes at twenty-one days of age. The latter investigators found a constant change in fiber composition of the extensor digitorum longus muscle to 60 days of age.

Brooke et al. (1971) and Curless and Nelson (1976) found that, in the soleus muscle, adult fiber profiles are attained by four weeks of age. These data are in contrast to the results of Ho et al. (1980) and Ariano et al. (1973). Ho et al. (1980) found 75 percent type II A and 25 percent type I fibers at six and eleven days of age. These percentages changed dramatically between eleven and sixteen days of age, and this rapid shift continued until thirty-one days of age. The fiber distribution then was reported as 25 percent type II A and 75 percent type I. These researchers also reported that the fiber composition continues to change until 140 days of age when there is sixteen percent type II A fibers and 84 percent type I fibers. This study is in disagreement with the previously mentioned study of Curless and Nelson (1976) in which no significant variation in the percentage of type I fibers from one to twenty-nine days of age was observed. One possible reason for this discrepancy in reported data, is that Ho et al. (1980) differentiated the entire soleus muscle; whereas, Curless and Nelson (1976) chose random fields for sampling.

Ho et al. (1980) also described a gradual change in the percentages of the various fiber types, for the plantaris muscle, from one to thirty-six days of age. At thirty-six days of age, there were 80 percent type II A, 14 percent type II B, and six percent type I fibers. Fiber composition continued to change until it became 47 percent type II A, 43 percent type II B, and ten percent type I fibers at 140 days of age. This fiber profile is supported by the work of Ariano et al. (1973).

It has been shown that fiber type differentiation is dependent upon neural stimuli (Engel and Karpati, 1968; Shafiq et al., 1972). Engel and Karpati (1968) observed that following neonatal neurectomy of the sciatic nerve type II fibers remained in the myotube stage but that type I fibers did differentiate histochemically. These authors described the type I fibers as being less dependent on neuronal "trophic" influences than type II fibers. Shafiq et al. (1972) presented evidence, using both histochemical and electron microscopic techniques, that fiber types are well differentiated in normal gastrocnemius, extensor digitorum longus, and soleus muscles two to three weeks after birth but not in denervated muscles.

Brooke et al. (1971) demonstrated that with cross re-innervation of denervated muscles homogenous groups of fibers are produced. They reported that these uniform groups of fibers are typical of the innervation of the "foreign" nerve. Therefore, they concluded that the nerve supply is responsible for the determination of the fiber type in any given motor unit.

Riley (1977) reported that nearly all muscle fibers of the soleus are in the myotube stage and are polyneuronally innervated at birth. He found that the conversion of myotubes to fiber types I and II is dependent on the transition from polyneuronal to unineuronal innervation. Riley (1977) also agrees that postnatal denervation halts the differentiation process of muscle fibers.

METHODS AND MATERIALS

Muscles were obtained from forty-eight male albino rats, Sprague-Dawley*, which were killed at one, six, eleven, sixteen, twenty-one, twenty-six, thirty-one, and thirty-six days of age. There were six animals per age group. At sacrifice, the animals were anesthetized by an intraperitoneal injection of sodium pentobarbital. The skin was stripped from foot to thigh and the gastrocnemius, plantaris, and soleus complex of the right leg was surgically separated from the rest of the leg. The proximal and distal attachments of these muscles were held with a hemostat to eliminate excess twitching. Using scissors, the muscles were detached and then immediately prepared for freezing.

The muscle complex was attached to a piece of cork, using gum tragacanth, with the proximal end up. This was placed in an isopentane solution, precooled to -170^{o}C with liquid nitrogen, for quick freezing. The muscle complex remained in this solution for approximately one to two minutes. It then was removed from the solution and placed on a precooled (with liquid nitrogen) aluminum foil square which was folded for storage. The muscles were stored in a

*Spartan Research Animal, Inc., Lansing, Michigan freezer at -70°C until they could be cut with a cryostat microtome and stained histochemically. The same procedure was followed for the left gastrocnemius, plantaris, and soleus complex.

Serial transverse sections (8μm) were cut with a cryostate microtome from the middle of the muscle belly and placed on coverslips. All sections were rapidly thawed by placing a warm finger on the underneath side of the coverslips. Each section was placed in a different coverslip jar that was labeled with its prospective stain. All sections were air dried in front of an electric fan for one hour before staining.

Sections stained with haematoxylin and eosin (H & E) were used to study fiber morphology. Fiber type differentiation was examined by histochemical staining for myosin adenosine triphosphatase (myosin ATPase, pH 9.4) (Padykula and Herman, 1955) with an alkaline (pH 10.4) preincubation solution (Guth and Samaha, 1969), and succinate dehydrogenase (SDH) (Stein and Padykula, 1962). After all staining procedures were performed, the coverslips were mounted on glass slides using protex for the myosin ATPase and H & E stains, and glycerine jelly for the SDH stain.

The series of histochemically stained slides of the plantaris muscle were projected with a Prado Universal Microprojector (Ernest Leitz Gmbh Wetzlar) in a darkroom. The myosin ATPase (pH 9.4) stain with alkali preincubation was used first to designate fibers. The stain intensity of each individual muscle fiber on the muscle section was subjectively evaluated using a rating scale of dark, medium, and light (Peter et al., 1972). This stain was projected on white paper, and the fibers staining dark were encircled with a blue felt-tip pen. The medium staining fibers were noted with a green felt-tip pen, and the light staining fibers were designated with a red felt-tip pen. All landmarks (blood vessels, muscle spindles, etc.) were drawn in with a number two pencil before proceeding with the fiber-to-fiber analysis. The entire plantaris muscle was outlined using this procedure.

The SDH stained slide then was used for fiber-to-fiber analysis. The landmarks noted from the myosin ATPase (pH 9.4) and H & E stained slides were found and the appropriate fibers to be used for serial analysis were identified. Staining intensity again was evaluated as dark, medium, and light (Peter et al., 1972) and designated by making a mark in the middle of the circled fiber. Dark staining fibers were designated with a blue mark, medium staining fibers with a green mark, and light staining fibers with a red mark. The entire muscle was analyzed using this procedure.

The completed muscle picture was labeled with the animal number, age, muscle name, date started, date completed, lens power, and right or left leg determination. The completed picture of the plantaris muscle ranged from four feet by six feet to six feet by eight feet in size, depending upon the age of the animal and the subsequent lens power used.

The completed picture then was analyzed and the fiber categories counted. Each of the individual fibers having a complete rating scale was typed using the classification system of Brooke and Kaiser (1970). The following table illustrates the rating scales and classification.

TABLE 1.

CLASSIFICATION OF FIBER TYPES

Fiber Types	Myosin ATPase Circle Color	SDH Mark Color
Type I	Light Red	Medium Green
Type II A	Medium to Dark Green to Blue	Dark Blue
Type II B	Dark Blue	Light Red

Each muscle had a very small percentage of "unknown" fibers that did not fit the classification of Brooke and Kaiser (1970). This percentage was trivial and therefore not recorded.

Mean values of the total numbers and percentages of the different fiber types were calculated for each muscle in each age group.

PRESENTATION OF DATA

The purpose of this investigation was to study the differentiation and development patterns of muscle fiber types, in the plantaris muscle of rats, at five-day intervals, from one to thirty-six days of age. See Appendix A for presentation of raw data.

In one-day-old rats, two histochemical fiber populations could be identified in the plantaris muscle. The majority of the fibers were in the myotube stage, but rare type I fibers were observed using the myosin ATPase (pH 9.4) stain (Brooke and Kaiser, 1970). Quite often, there was little to no reaction observed within the myotubes when they were stained for myosin ATPase activity following alkali preincubation. However, when there was a reaction present in the myotubes with the myosin ATPase (pH 9.4) stain, the color of the reaction was slightly darker than the color seen within the type I fibers. There was no reaction present when staining for SDH activity at this age. Therefore, exact percentages of myotubes and type I fibers cannot be presented due to the difficulty in staining for histochemical reactions in muscle tissue at one day of age. Consequently, when referring to Figure 1., the abcissa which represents age is a broken line, and the first age for reported data is at six days.

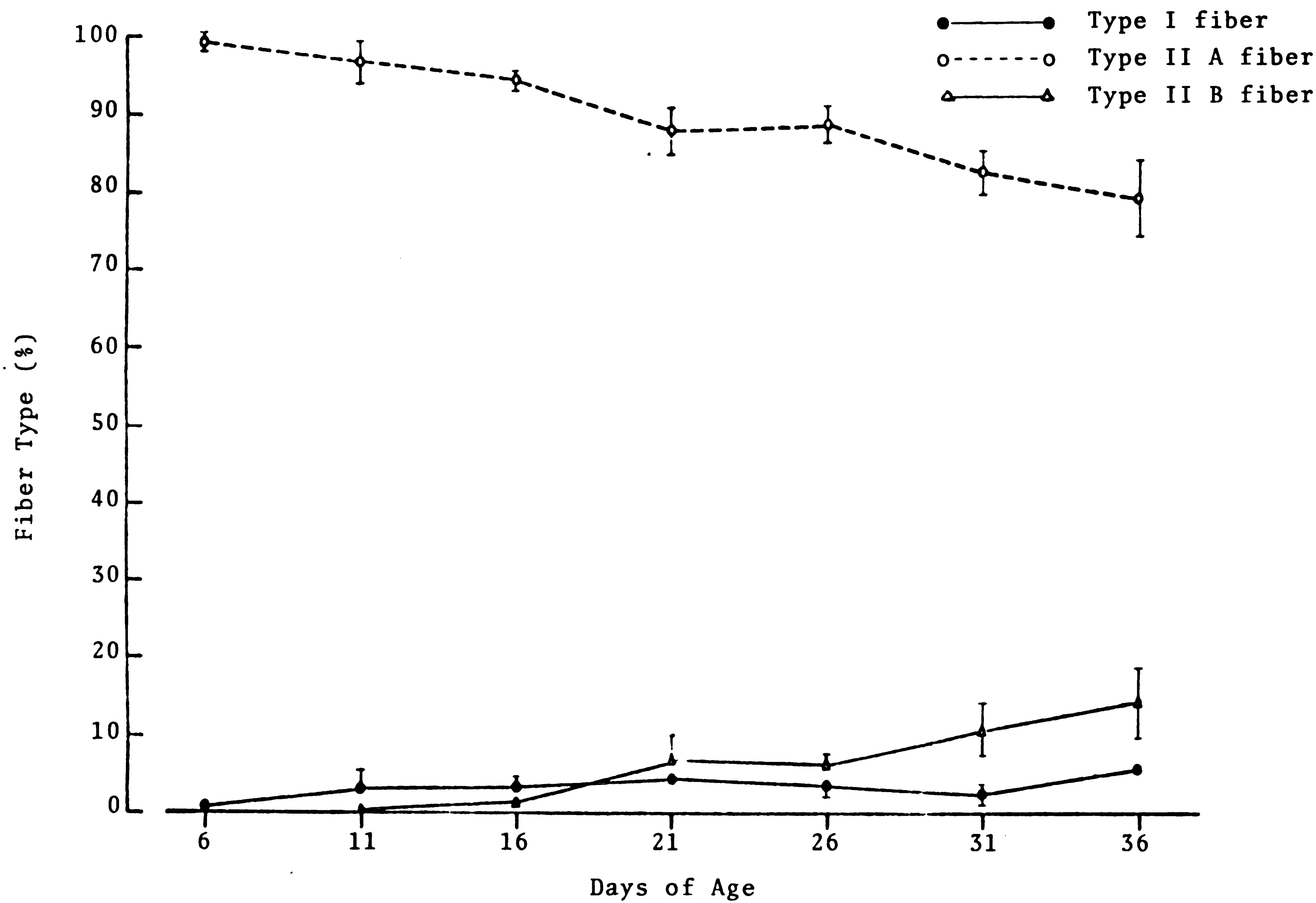

Figure 1. Fiber type composition of the plantaris muscle as a function of age. Each point represents the mean and standard deviation of six rats. (Unshown standard deviations < 1%)

At six days of age, 99.2 percent of the fibers in the plantaris stained positively with both the myosin ATPase (pH 9.4) and the SDH stains. Less than one percent (0.7%) of the fibers failed to demonstrate a positive reaction with the myosin ATPase (pH 9.4) stain and demonstrated a medium reaction with the SDH stain. Therefore, these fibers were classified as type II A and type I, respectively (Brooke and Kaiser, 1970). Single myotubes were found in the periphery of fascicles. These amounted to 0.1 percent of the fibers across the entire muscle. The reactions and staining intensities for both the myosin ATPase (pH 9.4) stain and the SDH stain were not as strong as they were in muscles of animals at older ages.

The staining intensities of the myosin ATPase (pH 9.4), and SDH reactions were stronger by eleven days of age, and there were four fiber populations present (Figure 1.). Only 0.44 percent of the fibers were present in the myotube stage. Ninety-seven percent were type II A fibers which reacted strongly with both the myosin ATPase (pH 9.4) and the SDH stains (Brooke and Kaiser, 1970). This strong reaction resulted in a medium to dark color shown with the myosin ATPase (pH 9.4) stain and the darkest color with the SDH stain (Peter <u>et al</u>., 1972). Approximately 3.1 percent of the fibers were found to be type I because they did not react with the myosin ATPase (pH 9.4) stain and demonstrated a medium reaction with the SDH stain. The fourth fiber type noted at eleven days of age was the type II B fiber. Due to an increase in the strength of the histochemical reactions at this age, it was clearly evident that they were present. The type II B fiber reacts strongly with the myosin ATPase (pH 9.4) stain giving the appearance of a dark color, and there is little to no reaction with the SDH stain. There was 0.11 percent type II B fibers found.

At sixteen days of age, there was a complete absence of fibers in the myotube stage, and only three fiber types were found. The fiber population was 94.5 percent type II A, 3.1 percent type I, and 1.1 percent type II B. Unfortunately, 1.4 percent of the fibers at this age could not be identified via the Brooke and Kaiser (1970) nomenclature and are therefore referred to as unidentified fibers.

Between sixteen and twenty-one days of age, the percentage of type II A fibers dropped from 94.5 percent to 87.1 percent. The type I fibers increased slightly from 3.1 percent to 4.3 percent. There was a sharp increase, from 1.1 percent to 6.7 percent, in the type II B fibers found. Less than one percent (0.95%) of the fibers could not be classified at this age.

It is evident in Figure 1. that no appreciable changes in percentages were detectable between twenty-one and twenty-six days of age. Types I, II A, and II B were 3.4, 88.9, and 6.2 percent, respectively, with 1.6 percent unidentified fibers.

Between twenty-six and thirty-one days of age, there was a decline in the percentage of type II A fibers from 88.9

percent to 82.9 percent. There also was an increase, from 6.2 percent to 10.7 percent, in type II B fibers. There was a slight decrease, from 3.4 percent to 2.3 percent, in the percentage of type I fibers. Only 4.2 percent of the fibers were unidentified at this age.

At thirty-six days of age, 79.5 percent type II A fibers, 14.2 percent type II B fibers and 5.6 percent type I fibers were found. There were 0.76 percent unidentified fibers.

The histochemical staining reactions for the various fiber types identified in this investigation are in agreement with the nomenclature of Brooke and Kaiser (1970). The color of the staining intensities were subjectively rated using the system of Peter et al. (1972). Table 1. presented in Methods and Materials was followed for the fiber-to-fiber analysis of the muscle tissue.

The results of this investigation support the observation that fiber types are basically undifferentiated at birth (Riley, 1977; Haltia et al., 1978; Shafiq et al., 1972; Engel and Karpati, 1968). Generally, the myotubes failed to show any selective activity towards the histochemical staining techniques used. However, in one-day-old animals, a small proportion of the myotubes demonstrated a slightly darker reaction with the myosin ATPase (pH 9.4) stain than did the type I fibers. These results are similar to the staining reaction found with the type II fibers. However, these fibers cannot be reported as a type II A or type II B fibers with certainty because they failed to show any reaction with the SDH technique at this age.

Several investigators have claimed that there is a fiber present during the early developmental stages in rat muscle which is a "precursor" to all of the other fiber types. This fiber is the type II C (or type F) fiber (Curless and Nelson, 1976; Haltia et al., 1978; Brooke and Kaiser, 1970; Ariano et al., 1973; Brooke et al., 1971). For certain identification of the type II C fiber one must use the myosin ATPase stain with acid (pH 4.35) preincubation, but this was not used in the present investigation. The large fiber population (99.2%) that demonstrated positive reactions with both the myosin ATPase (pH 9.4) and SDH stains, at six days of age, may well stem from this transitional fiber type II C. Although positive reactions of both stains are highly suggestive of the type II C fiber, due to not using the myosin ATPase (pH 4.35) stain for the fiber-to-fiber analysis this population is reported here as type II A.

The emergence of the type II B fiber was noted at eleven days of age. The differentiation patterns exhibited distinct changes starting at sixteen days of age and continued to thirty-six days of age. The developmental patterns followed a course which showed a decline in the percentage of type II A fibers and a steady rise in the percentage of type II B, with the type I fibers reaching a plateau. Ariano et al. (1973) reports that in the adult Sprague-Dawley rat the fiber type population is fifty-three percent type II A, forty-one percent type II B, and six percent type I. Ho et al. (1980) reports that for rats at 140 days of age the fiber type population is 47.4 percent type II A, 43.4 percent type II B, and 9.6 percent type I. Therefore, the differentiation pattern after thirty-six days of age must be similar to the pattern established from sixteen to thirty-six days of age as was found in this investigation. The same general differentiation pattern was observed by Maxwell et al. (1973) in the plantaris muscle of the guinea pig.

The oxidative enzyme capacity of the plantaris, measured by the SDH stain, is not apparent and is essentially insignificant at birth. However, oxidative enzyme activity quickly develops between one and six days of age, and at six days some SDH activity is demonstrated. The SDH reaction continues to become stronger with an increase in age. Goldspink (1969) found that, in muscle tissue of mice, some SDH activity was noted in larger fibers at birth, but that distinct staining of the enzyme's activity started at one week of age. Goldspink also states that differences in staining intensities are related not only to difference in contents, but also to changes in growth with age. Maxwell et al. (1973) found that fibers are relatively undifferentiated on the basis of SDH activity in the plantaris muscle of guinea pigs at one to two weeks of age. Colling-Saltin (1980) also reports that there is no oxidative enzyme differentiation in human muscle at birth and that this development occurs postnatally.

Morphologically, many of the myotubes at one and six days of age appeared to be horse-shoe shaped. At eleven days old, the myotubes appeared round. Curless and Nelson (1976) found in the soleus muscle of rats the myotubes appeared horse-shoe shaped until nine days of age. The large type I fibers noted at birth, and the type II A and type II B fibers that appeared later, all demonstrated centrally located nuclei. By twenty-one days of age, the nuclei appeared to be located at the periphery of the muscle fiber. Muscle spindles were first noted at six days of age. The H & E stain was used for morphological examination of the muscles. These findings are supported by several investigators (Curless and Nelson, 1976; Goldspink, 1970; Engel and Karpati, 1968).

It is evident that although myotubes can be observed at one day of age, it is not possible to classify fiber types because they are basically undifferentiated. From six through thirty-six days of age, there is a progressive decrease in type II A fibers (from $\overline{X}$ = 99.2 to $\overline{X}$ = 79.5 percent) along with progressive increases in type II B fibers (from zero to $\overline{X}$ = 14.2 percent) and type I fibers (from $\overline{X}$ = 0.7 to $\overline{X}$ = 5.6 percent). Differentiation, however, is only partially completed by thirty-six days of age as at 140 days of age the following means are observed: type II A - 47.4%, type II B - 43.4%, and type I - 9.6% (Ho et al., 1980). The differentiation process appears to be associated with the aging process. The stimuli for producing differentiation cannot be identified from the present study.

SUMMARY, CONCLUSIONS AND RECOMMENDATIONS

Summary

The purpose of this study was to determine the differentiation and development patterns of the various muscle fiber types in the plantaris muscle of the rat, from one to thirty-six days of age, using histochemical techniques. Previous investigators have reported dissimilar results, also using histochemical techniques, in either rat muscle or the plantaris muscle of a different animal. One reason these reports vary is that many investigators have used random sampling of the microscopic field when classifying fiber types. To reduce confusion this investigation classified the entire plantaris muscle. The intent of this study was to add information concerning muscle fiber differentiation patterns during the early life of the rat.

Muscles were obtained from forty-eight Sprague-Dawley rats at one, six, eleven, sixteen, twenty-one, twenty-six, thirty-one, and thirty-six days of age. There were six rats per age group. The gastrocnemius, soleus, and plantaris complex was excised from the leg and prepared via fast freezing, for sectioning on a cryostat microtome. Serial transverse sections were cut at 8 μm in thickness from the middle of the muscle belly. The sections were stained with haematoxylin and eosin (H & E) to study fiber morphology. Muscle fiber type differentiation was examined by histochemical staining for myosin adenosine triphosphatase (myosin ATPase, pH 9.4) with alkali (pH 10.4) preincubation solution, and succinate dehydrogenase (SDH).

Fiber-to-fiber analysis was performed on the plantaris muscle using the myosin ATPase (pH 9.4) stain with alkali preincubation and the SDH stain. When classifying the muscle fiber types, the nomenclature of Brooke and Kaiser (1970) was used with the rating system of Peter et al. (1972) (Table 1.). The entire plantaris muscle was classified at each age into myotubes, type I, type II A, and type II B fibers (Figure 1.). The type II C fiber was not classified in this investigation. Any muscle fibers which did not fit the nomenclature of Brooke and Kaiser (1970) were classified as "unidentified" (Appendix A -- Raw Data).

Conclusion

Muscle fiber types in the plantaris muscle are basically undifferentiated at birth, and the differentiation process occurs postnatally. However, this process is not complete by thirty-six days of age. The fiber populations continue to change until adulthood.

The development process seems to follow a smooth pattern of differentiation. Myotubes and a few large type I fibers are seen at one day of age. From six to thirty-six days of age, there is a progressive increase in type I fibers (from

$\overline{X}$ = 0.7 to $\overline{X}$ = 5.6 percent) and type II B fibers (from zero to $\overline{X}$ = 14.2 percent) with a progressive decrease in type II A fibers (from $\overline{X}$ = 99.2 to $\overline{X}$ = 79.5 percent). The differentiation process appears to be associated with the aging process.

Recommendations

In the future more stains should be performed.

In the future more sections should be cut and stained following the same histochemical procedures.

In future studies a fiber-to-fiber-to-fiber analysis, using the myosin ATPase with the acid preincubation solution should be performed, so as to find type II C fibers.

In future studies all fibers should be counted at birth, so as to add information about hyperplasia.

APPENDICES

APPENDIX A

RAW DATA -- NUMBER OF FIBERS PER FIBER TYPE

RAW DATA -- NUMBER OF FIBERS PER FIBER TYPE

ANIMAL NUMBER	TYPE I	TYPE II A	TYPE II B	MYOTUBES	UNIDEN-TIFIED	TOTAL
LG-1	N/C*			N/C		1298**
H-1	N/C			N/C		1163**
I-1	N/C			N/C		1818**
K-1	N/C			N/C		1238**
LK-1	N/C			N/C		1104**
L-1	N/C			N/C		2158**
G-6	95	4558	1	32	0	4686
H-6	26	3661	0	N/C	0	3687
I-6	26	3544	0	N/C	0	3570
J-6	0	3398	0	N/C	0	3398
K-6	13	4531	0	N/C	0	4549
L-6	12	4629	0	N/C	0	4641
A-11	13	2881	0	0	0	2894
C-11	507	6289	39	16	0	6851
E-11	102	5419	0	0	0	5521
G-11	62	3715	0	0	0	3777
H-11	106	3892	5	13	0	4016
I-11	182	4621	0	0	0	4203
A-16	193	4344	12	0	1	4550
B-16	216	4584	25	0	2	4827
C-16	105	4683	87	0	129	5004
D-16	124	4335	60	0	102	4621
E-16	168	5323	26	0	35	5552
F-16	101	4733	108	0	136	5078
A-21	155	4179	368	0	36	5038
B-21	165	4034	118	0	95	4412
C-21	149	2568	291	0	4	3012
D-21	278	5305	244	0	18	5845
E-21	119	2535	156	0	58	2868
F-21	205	3425	438	0	15	4083
B-26	186	4618	259	0	25	5088
C-26	191	3359	256	0	23	3829
D-26	179	3794	401	0	71	4445
E-26	132	3421	206	0	112	3871
F-26	148	3865	224	0	86	4323
G-26	22	2020	124	0	39	2205

*Myotubes and type I present throughout but not counted except as total.

**Estimated number, counting type I fibers and myotubes.

RAW DATA -- NUMBER OF FIBERS PER FIBER TYPE

ANIMAL NUMBER	TYPE I	TYPE II A	TYPE II B	MYOTUBES	UNIDEN-TIFIED	TOTAL
A-31	114	3134	401	0	150	3799
B-31	165	3251	381	0	81	3878
C-31	92	3867	259	0	215	4433
D-31	105	3466	382	0	193	4146
E-31	62	4137	780	0	249	5228
F-31	49	4316	724	0	251	5340
A-36	214	3165	238	0	11	3628
B-36	238	3892	547	0	13	4690
C-36	263	4089	763	0	20	5135
D-36	235	3511	814	0	39	4599
E-36	265	3713	895	0	26	4899
F-36	159	1768	377	0	53	2357

APPENDIX B

PERCENTAGES OF FIBERS PER FIBER TYPE

PERCENTAGES OF FIBERS PER FIBER TYPE

ANIMAL NUMBER	TYPE I	TYPE II A	TYPE II B	MYOTUBES	UNIDENTIFIED
G-6	2.02	97.3	0.0*	0.68	0.0
H-6	0.71	99.3	0.0	0.0	0.0
I-6	0.73	99.3	0.0	0.0	0.0
J-6	0.0	100.0	0.0	0.0	0.0
K-6	0.29	99.7	0.0	0.0	0.0
L-6	0.26	99.7	0.0	0.0	0.0
$\bar{X}$ 6 day	0.67	99.2	0.0	0.1	0.0
A-11	0.45	99.6	0.0	0.0	0.0
C-11	7.40	91.8	0.57	2.34	0.0
E-11	1.85	98.2	0.0	0.0	0.0
G-11	1.64	98.4	0.0	0.0	0.0
H-11	2.63	96.9	0.1	0.3	0.0
I-11	4.33	95.7	0.0	0.0	0.0
$\bar{X}$ 11 day	3.05	96.7	0.1	0.44	0.0
A-16	4.24	95.5	0.26	0.0	0.0
B-16	4.47	95.0	0.52	0.0	0.0*
C-16	2.10	93.6	1.74	0.0	2.58
D-16	2.68	93.8	1.30	0.0	2.21
E-16	3.03	95.9	0.47	0.0	0.63
F-16	1.99	93.2	2.63	0.0	2.13
$\bar{X}$ 16 day	3.10	94.5	1.07	0.0	1.35
A-21	3.08	83.0	7.30	0.0	0.71
B-21	3.74	91.4	2.67	0.0	2.15
C-21	4.95	85.3	9.66	0.0	0.13
D-21	4.76	90.8	4.47	0.0	0.31
E-21	4.15	88.4	5.44	0.0	2.02
F-21	5.02	83.9	10.72	0.0	0.37
$\bar{X}$ 21 day	4.28	87.1	6.66	0.0	0.95
B-26	3.66	90.8	5.09	0.0	0.49
C-26	4.99	87.7	6.69	0.0	0.60
D-26	4.03	85.4	9.02	0.0	1.60
E-26	3.41	88.3	5.32	0.0	2.89
F-26	3.42	89.4	5.18	0.0	1.99
G-26	1.00	91.6	5.62	0.0	1.77
$\bar{X}$ 26 day	3.42	88.9	6.15	0.0	1.56

*Less than 0.01 percent.

PERCENTAGES OF FIBERS PER FIBER TYPE

ANIMAL NUMBER	TYPE I	TYPE II A	TYPE II B	MYOTUBES	UNIDENTIFIED
A-31	3.00	82.5	10.55	0.0	3.95
B-31	4.25	83.8	9.82	0.0	2.09
C-31	2.08	87.2	5.84	0.0	4.85
D-31	2.53	83.6	9.21	0.0	4.66
E-31	1.19	79.1	14.92	0.0	4.76
F-31	0.92	80.8	13.56	0.0	4.70
$\bar{X}$ 31 day	2.33	82.9	10.65	0.0	4.17
A-36	5.89	87.2	6.56	0.0	0.30
B-36	5.07	83.0	11.66	0.0	0.28
C-36	5.12	79.6	14.86	0.0	0.39
D-36	5.11	76.3	17.70	0.0	0.85
E-36	5.41	75.8	18.27	0.0	0.53
F-36	6.75	75.0	16.00	0.0	2.25
$\bar{X}$ 36 day	5.56	79.5	14.17	0.0	0.76

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