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**The Effect of Aging on Skeletal Muscle Regeneration**

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Supported By: National Institute of Health AR41995

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5-3-96

## **Abstract:**

Muscle atrophy and decreased strength are an accepted part of aging. While physical inactivity may account for some of these losses, physically active individuals still experience age related reduction in muscle mass and strength. The purpose of this study is to examine the effects that aging has on skeletal muscle regeneration. It is hypothesized that the recovery of muscle mass and force following muscular injury will decrease with age. The anesthetic bupivacaine, a potent myotoxic agent, was injected into the left tibialis anterior (TA) of young, adult, and senescent rats (3, 18, 31 months). The right leg served as an internal control. Following a 2, 3, or 4 week recovery period, the mass and isometric force of each TA were measured. In the young animals, the mass of the bupivacaine injected leg was 60% of the control value at two weeks post injection. By three weeks, the mass was fully recovered. In the middle age and senescent animals, mass of the injected TA was approximately 50% of the control at two weeks and remained at 50% of control following 3 and 4 weeks recovery. Muscle force measurements demonstrated parallel trends. These results indicate that skeletal muscle regeneration is impaired in middle age and senescent rats.

Recently, a family of four genes was identified that causes the expression of skeletal muscle specific proteins when activated

in precursor cells. One member of this family is myogenin. It is known that myogenin is expressed only during skeletal muscle regeneration, making it an excellent marker for this process. Myogenin mRNA levels were measured in the control and experimental TA muscles of the young , middle age, and senescent animals used in this experiment. All three age groups have elevated levels of myogenin mRNA in the bupivacaine-injected TA when compared to the control. In addition, the 31 month animals have significantly higher levels of myogenin mRNA in their control legs at all time periods than those found in the other age groups. It is known that the expression of myogenin is down-regulated by electrical activity. The high levels found in the control leg indicate reinnervation problems during regeneration in the old animals.

## Introduction:

Webster's Compact Dictionary defines disease as, " a condition of the body that impairs its normal functioning." Skeletal muscle atrophy is prevalent among the elderly and is a leading cause of impaired mobility in the aging members of our population. (Fitarrone et al., 1994). Such a loss of mobility leads to dependence and greatly decreases the quality of life in the later years. In addition, it was estimated that \$ 10 billion are spent yearly to treat fall related fractures in the elderly that occur as a direct result of insufficient muscle strength and endurance (Tinetti et al., 1994). While these decreases in muscular strength and mobility are often attributed to a sedentary lifestyle, trained athletes who show higher strength and power than normal individuals, still experience similar declines in structural and functional properties throughout their lifetime (Brooks and Faulkner, 1994). Despite the obvious impairment of normal functions caused by muscle atrophy with aging, the medical field has done little to investigate the causes behind this loss.

Quetelet (1835) originally correlated impaired skeletal muscle function with age over 150 years ago. Since that time, a significant amount of information has been gathered on the subject. Today it is known that human skeletal muscle begins to

atrophy at 25 years of age in men. Muscle mass decreases 10% over the next 25 years without a corresponding loss in strength (Lexell et al. 1986). After the age of 50, however, there is a 30% loss of muscle mass by the age of 80. Closely associated with this is a 22% loss of muscle strength that occurs between the ages of 45-50 and 60-65 years of age (Larsson et al., 1978). Similar losses of mass and strength occur a few years later in women. While other possibilities have been proposed, an increased susceptibility to injury coupled with a decreased ability to recover from injury may be a major cause of muscle atrophy and weakness seen in old animals and the elderly.

Skeletal muscle is a dynamic tissue. It is constantly injured and repaired throughout the course of a normal day due to the contractions that allow for movement. Such damage is generally focal in nature, with 20 - 30% of muscle fibers being damaged during eccentric exercise. When studying regeneration, it is often advantageous to observe wide spread muscular damage. As a result, various methods including pinching, freezing, and anesthetic injections have been used to induce the degeneration / regeneration process in laboratory animals. The ability of skeletal muscle to regenerate following such injury is well documented, although the extent and success of regeneration varies

with the extent and cause of injury (Grounds, 1991).

Following extreme damage, muscles lose their ability to contract, but regain some degree of function during regeneration. Of particular interest, is the recovery of contractile properties following a localized injection of the anesthetic bupivacaine. Following grafting, pinching, or freezing, contractile properties are only partially restored (Carlson et al., 1983). After a bupivacaine injection, however, muscle mass and contractile properties are completely restored within 3-4 weeks (Rosenblatt, 1992). Only 15 minutes after the injection of bupivacaine, the sarcolemma is disrupted, the sarcoplasmic reticulum dilates, myofibrils hypercontract, and the myonuclei are disrupted (Bradley, 1979). Over the next 12 hours, the myofibrils rupture, and the sarcolemma dissolves resulting in cytoplasm fragmentation (Hall-Craggs 1974). Macrophages begin to engulf necrotic fibers at 12 hours post injury and infiltrate the tissue by 24 hours (Nonaka et. al., 1983). Myoblasts are observed after the first day and fuse to form mature myotubes within 4 weeks (Rosenblatt, 1992).

The fast action and complete recovery are due to the selective action of the drug on myofibrils and lack of damage to the muscle's satellite cell population (Hall-Craggs, 1980b), basal

lamina (Hall-Craggs, 1980a), vascular supply (Grim et al., 1983) and intramuscular nerves (Tomasi Ferre et al., 1989). The other methods selectively damage one or more of these 3 elements, greatly limiting regenerative possibilities. Thus, bupivacaine provides an excellent opportunity for studying skeletal muscle regeneration in an ideal environment.

The regenerative process involves revascularization, invasion of phagocytic cells, removal of damaged tissue, proliferation and fusion of precursor cells, and reinnervation (Grounds, 1991). Each of these steps is necessary. Without their ordered occurrence regeneration is not successfully completed. The following is a brief discussion of the events that occur during regeneration.

Revascularization is extremely important to the regeneration of skeletal muscle. Upon removal of the vascular supply, the majority of the myotubes, except for a few at the periphery, degenerate. The peripheral fibers are kept alive by the diffusion of nutrients and gases from vessels in neighboring tissues (Carlson, 1981). In general, regeneration trails behind revascularization because blood supplies the oxygen and nutrients necessary for new tissue formation. The mechanisms behind revascularization are poorly understood. It is known that the new



vascular tissue comes from small neighboring vessels rather than a major vessel. This indicates that angiogenic factors released from the area of injury may cause local blood vessels to branch towards the damaged tissue. Such factors have yet to be identified.

Slightly before revascularization, a few macrophages enter the damaged tissue and begin removing debris (Hansen-Smith et al., 1979 and Roberts et al., 1990). Shortly following revascularization, a wave of leukocytes enters the tissue and phagocytizes the debris. This process is important because regeneration is inhibited by persisting necrotic tissue. Removal of dead tissue creates room for new myotubes to form.

The normal growth and repair of muscle fibers is carried out by satellite cells. These are undifferentiated muscle precursor cells located between the sarcolemma and basal lamina of a muscle fiber. They cause normal growth and repair by either fusing with existing fibers (hypertrophy) or forming new myotubes (hyperplasia) (Schultz, 1990). In addition, satellite cell proliferation is responsible for the addition of new myonuclei in regenerating muscles (Snow, 1977). Satellite cells are normally quiescent and are activated following injury. The factors that directly or indirectly control their proliferation and fusion are

unknown due to the complexity of the situation. It is known, however, that a decrease in either satellite cell number or activity would decrease regenerative capabilities.

The establishment of a functional neuromuscular synapse is critical for the maturation of regenerating muscle fibers. Without neural activity, the regenerating tissue dies. Motor axons generally make contact with regenerating tissue in the same area as the original endplate. Although neighboring axons can sprout and innervate the regenerating tissue, the same axon that innervated the fibers before injury generally innervates the regenerated fibers (Fowler, 1994). It is also known that nerve growth factor (NGF) acts as a chemotactic agent and is responsible for the sprouting of axons.

An extremely important step during regeneration is myogenesis. Myogenesis is characterized by the proliferation and fusion of satellite cells. Under normal conditions, satellite cells are inactive. Following injury, however, they are activated. Such activation occurs via a recently identified family of genes. This family consists of myogenin (Edmonson and Olson, 1989), Myo D1 (Davis et al., 1987), myf 5 (Braun et al., 1989), and herculin/MRF 4 (Miner and Wold, 1990). All four genes have homologous sequences, including an area which codes for a

series of basic amino acids that is responsible for the DNA binding ability of the proteins (Davis et al., 1990). Their expression ultimately leads to the expression of muscle specific proteins. Recent studies report that predominantly quiescent satellite cells do not express any members of the myogenic family (Grounds et al., 1992 and Fuchtbauer & Westphal, 1992). In addition, Grounds (1991) reported that the expression of myo D1 and myogenin was apparent six hours following crush injury to skeletal muscle. Thus, myogenin is only expressed in regenerating skeletal muscle, making it an excellent marker for this process.

In light of this information, the purpose of this study is to examine the effects of aging on skeletal muscle regeneration. More specifically, the temporal relationship of muscle regeneration, recovery of muscle force, and myogenin mRNA expression in young, adult, and aged rat skeletal muscle are investigated. The following hypotheses are tested:

- 1) Recovery of muscle mass and force generating capacity following injury occurs later with age.
- 2) The expression of myogenin mRNA will differ in the regenerating muscle of young, adult, and aged rats.

Results of this experiment indicate that skeletal muscle regeneration is impaired with age, pointing to it as a probable cause of age-related atrophy.

**Methods:**

*Animals:* The rats used in this experiment were male Brown Norway-Fischer 344 cross. Fifteen 3 month, 18 month, and 33 month rats were purchased from the NIH Aging program in Indianapolis. Upon arrival they were placed in temperature (21°C) and light (12:12 hr. light/dark) controlled quarters in the University of Texas-Houston Medical Center animal care facility. The rats were housed two per cage and isolated from other animals. All food and water was sterilized by autoclave. Protocols were approved by the Institutional Animal Welfare Committee.

*Bupivacaine Injection:* The animals were anesthetized with an intramuscular injection (0.84 ml/kg) of ketamine(54 mg/ml), xylazine (2.2 mg/ml), and acepromazine (3.5 mg/ml). An incision was made over the distal third of the left tibialis anterior (TA). A 25 gauge 5/8" needle (0.5x16 mm) needle was inserted along the longitudinal axis of the muscle. While the needle was slowly withdrawn, 0.5 ml of 0.75% bupivacaine was injected into the

muscle. A similar injection was administered at a 15° angle, 3 mm away from the original injection. The right leg was undisturbed and served as an internal control. Once the animals recovered, they were returned to their cages.

*Force Measurements:* Following two, three, or four weeks recovery the rats were anesthetized with an intramuscular injection of the ketamine cocktail described earlier. An incision was made through the quadriceps femoris, exposing the deep peroneal nerve. Two electrodes were sutured to the nerve. The nerve was severed to prevent stimulation of the lower limb via the central nervous system. The rats were placed in a heated box. Anesthetic causes body temperature to drop. Any significant change in body temperature would have affected force measurements. A stimulator was wired to the electrodes. The tendon of the TA was attached to a force transducer. Computer programs were used to determine optimal muscle length and current for force measurement. Force measurements were taken at 50, 75, 100, 150, 200, 250, and 300 HZ. Average force was calculated for each leg using the frequency that caused tetany.

*Muscle Sampling:* Immediately following force measurements the rats were anesthetized and the TA was removed from each limb. They

were frozen with liquid nitrogen-cooled Wollenberger tongs. Muscle samples were frozen at  $-80^{\circ}\text{C}$  until further analysis. Rats were sacrificed by cervical dislocation while under anesthesia.

*Total RNA Isolation and Quantification:* Tibialis anterior muscles were individually powdered under liquid nitrogen using a mortar and pestle. Total RNA was extracted from 150-300 mg samples of each muscle using the guanidine thiocyanate method of Chomczynski and Sacchi (1987) with Trisolve (Biotecx Laboratories). The RNA was dissolved in diethylpyrocarbonate treated water and quantified spectrophotometrically at 260 nm. RNA integrity was confirmed by examining an ethidiumbromide stained gel of 18S and 28S rRNA prior to its use in Northern Blots.

*Myogenin mRNA determination:* Northern blot analysis was used to assess the concentration of myogenin in all muscles. Separate 15 ug samples of RNA were loaded onto 1% agarose / 6.7% formaldehyde gels and electrophoresed at 5 V/cm for 2.5 hours. The RNA was transferred to nylon membranes via capillary action and ultraviolet cross-linked to the membrane (Carson et.al.,1995).

Full length cDNA for rat myogenin provided by Dr. Victor K. Lin was subcloned into the plasmid vector pBluescript (Wright et al., 1989). A cDNA insert was cut from the plasmid (EcoRI) and random primed. The RNA containing membrane was prehybridized with 12 ml of hybridization buffer (Quick Hyb, Stratagene) for 30 minutes at 68°C. An aliquot of random primed myogenin cDNA probe was mixed with the hybridization buffer and incubated for 2 hours at 68°C. The membrane was then washed twice in 2x sodium chloride - sodium citrate (SSC) - 0.1% sodium dodecylsulfate (SDS) for 15 minutes at 20°C. It was washed again with 0.1 SSC - 0.1 SDS for 30 minutes at 55°C. The membrane was autoradiographed (-80°C, 30 min.) and the band corresponding to myogenin mRNA was quantified by densitometric scanning (Bioimage) and normalized to ethidium bromide stained 28s rRNA.

*Statistical Analysis:* All statistical analysis was done using a standard statistical package on a Macintosh computer. The software used was Gopher.

## Results:

*Muscle Mass and Force:* The mass of the individual control and bupivacaine injected TA muscles are compared for each age group (Fig. 1). At two weeks post-injection, the mass of the injected TA (3 mo. rats) was 77 % of control values. At three weeks it was 105% of the control value. It remained at this level at four weeks post-injection. In the 18 month animals, injected muscle mass was 53%, 64%, and 64% of control values following 2,3, and 4 weeks recovery respectfully. The results from the 31 month animals parallel those from the 18 month animals. Experimental muscle mass for the 31 month animals was 60%, 63%, and 67% of the control values at 2, 3, and 4 weeks following the bupivacaine injection.

Results of the force measurements (Fig. 2) are similar to those for muscle mass. In the 3 month animals, the force produced by the bupivacaine injected leg was 58% of the control at 2 weeks, 95 % of the control at 3 weeks, and 105% of the control at four weeks post-injection. Force measurements for the adult animals were 34% of the control at 2 weeks, 30% of control at 3 weeks, and 46% of control at 4 weeks post injection. In the aged animals, the force produced by the bupivacaine injected TA was 37% of the control muscle. This did not increase significantly at 3 or 4



weeks.

Statistical analysis of this data by the Student - Newman - Keuls test and the Turkey - Kramer test indicated significant differences between young and adult animals and young and old animals for both muscle mass and force. The difference between the adult and old animals, however, was not significant. In addition, a strong correlation existed between the recovery of muscle mass and force production.

*Myogenin mRNA*: The presence of myogenin is a powerful indicator of muscle damage. In all three age groups and at all three time periods, myogenin mRNA expression in the bupivacaine injected legs was greater than 100 % of control values (Fig. 3). Myogenin mRNA decreased slightly between 14, 21, and 28 days in the young rats. It remained elevated at all three time periods in the adult rats. It increased significantly from 14 -28 days in the old rats. In addition, the control leg of the old rats had significantly higher myogenin mRNA levels at all three time periods than the control legs of the young or adult rats (Fig.4).

## Discussion:

This experiment supports the general proposal that skeletal muscle regeneration is inhibited with age. The hypothesis that the recovery of muscle mass and force generating capacity following injury occurs later with age is supported by the present set of experiments. As seen in figures 1 and 2, the young rats recover from injury significantly sooner than the adult and old rats. The lack of a significant difference between the adult and old rats, however, is surprising. A qualitative experiment done by Menachem Sadeh (1988) found that both degeneration and regeneration were most active in young animals, decrease slightly in middle age, and are markedly impaired in senescence. Similar results are expected here, but are not observed. One explanation for this observation could be the age of the adult rats. In most cases, an adult rat is considered to be 9-12 months of age. However, due to the long life span of the Brown Norway-Fischer 344 cross, 18 months is considered middle age. Although this breed of rat has a longer life span than others, it is possible that the aging process begins at the same time. If this is the case, then an 18 month rat is considered old and regeneration is already inhibited.

The results of the myogenin mRNA analysis are very interesting. The mRNA levels in the control leg are slightly higher than normal. This is because experimental legs were useless due to the bupivacaine injection, causing the control legs to rely upon more heavily than normal. This extra use caused slight damage to fibers, resulting in satellite cell activation. As expected, myogenin mRNA levels in the experimental legs are significantly higher than in the control legs for all age groups and at each time period. This indicates a higher than normal degree of satellite cell activation, which directly correlates to increased regeneration.

Myogenin mRNA levels in both the control and bupivacaine legs of the old animals are significantly higher than those found in the young or adult animals. It is known that myogenin is down regulated by electrical activity (Eftimie et. al. 1991). High levels indicate a lack of electrical activity. This could be due to poor reinnervation of newly formed myotubes in the old rats. Without proper innervation, muscle fibers die and the regenerative capacity of old rats is decreased. A loss of alpha motor neurons and motor units has been well documented (Ansved and Larson, 1990). The exact cause of this loss, however, is unknown.

This result indicates that it is not the regenerative capacity of muscle cells, but changes in the environment which inhibits regeneration with age. This result is supported by a previous study which found that old animals show a decreased regenerative capacity following free whole muscle transplantation (Foster, 1980). Under these circumstances, the whole muscle degenerates as a result of the ischemic injury. It was found that muscles from young or old rats transplanted into young animals regenerated equally well, but muscles from young or old rats transplanted into old rats regenerated equally poorly (Carlson and Faulkner, 1989). It is not the regenerative capacity of the cell, but the environment in which the regeneration occurs that impairs this process in aging animals.

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## **Acknowledgments:**

I would like to thank the University of Texas-Houston for the scholarship which allowed my participation in the summer research program. I would also like to thank Dr. Frank Booth and Dr. Dan Marsh for allowing me to work with them on this experiment. Dr. Booth heads the lab in which I worked. Dr. Marsh is doing post doctorate work in Dr. Booth's lab and designed the experiment that I assisted with.

Finally, thank you Dr. Thurman for opening the door to the UT-Houston Summer Research Program and for the the time you spent helping me prepare this project.

Figure 1. Relative percent changes in the mass of bupivacaine-injected TA's of young (3 months), middle age (18 months), and old rats (31 months) compared to the contralateral control at 2, 3, and 4 weeks after the injection.

### Muscle Mass

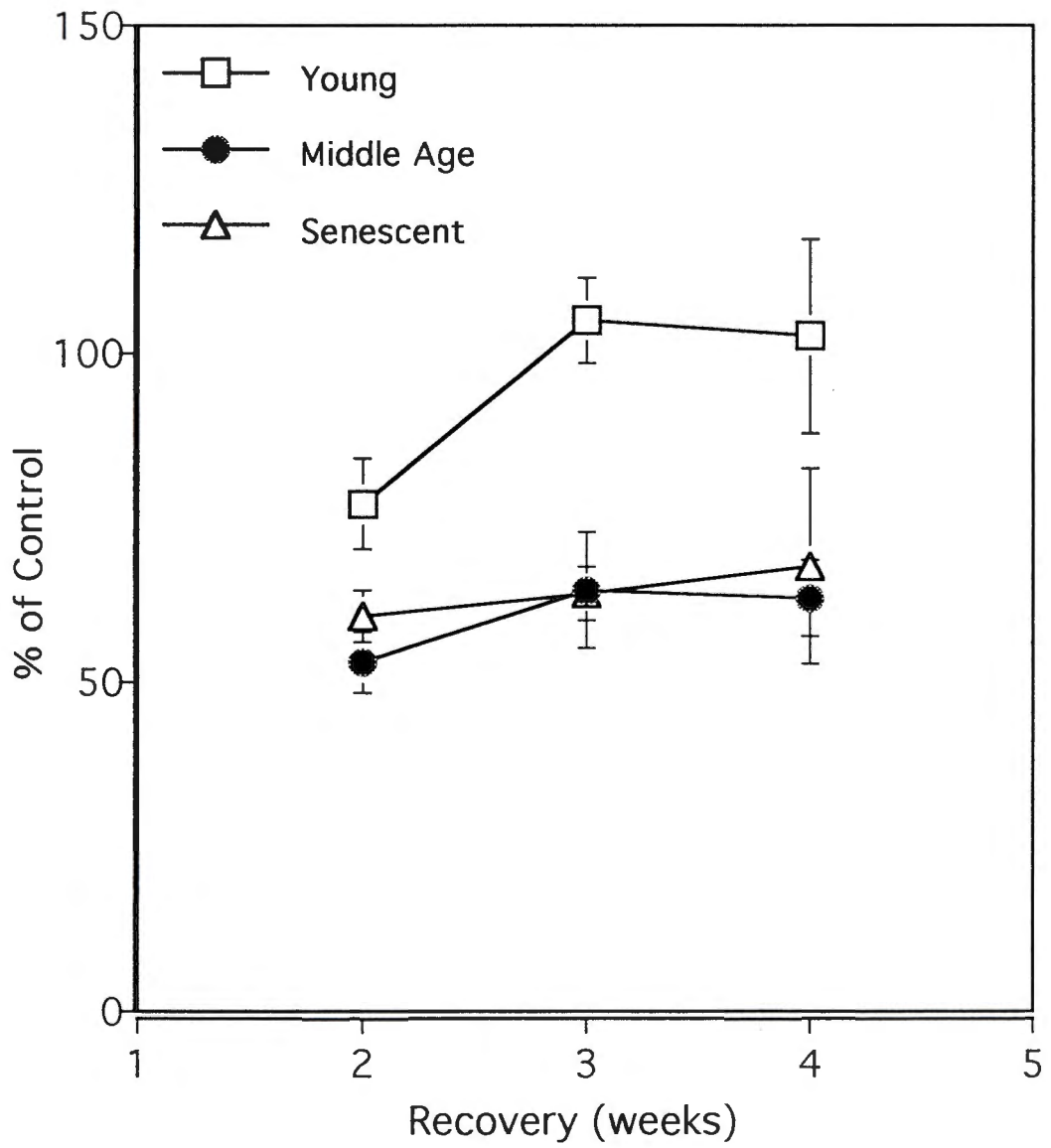
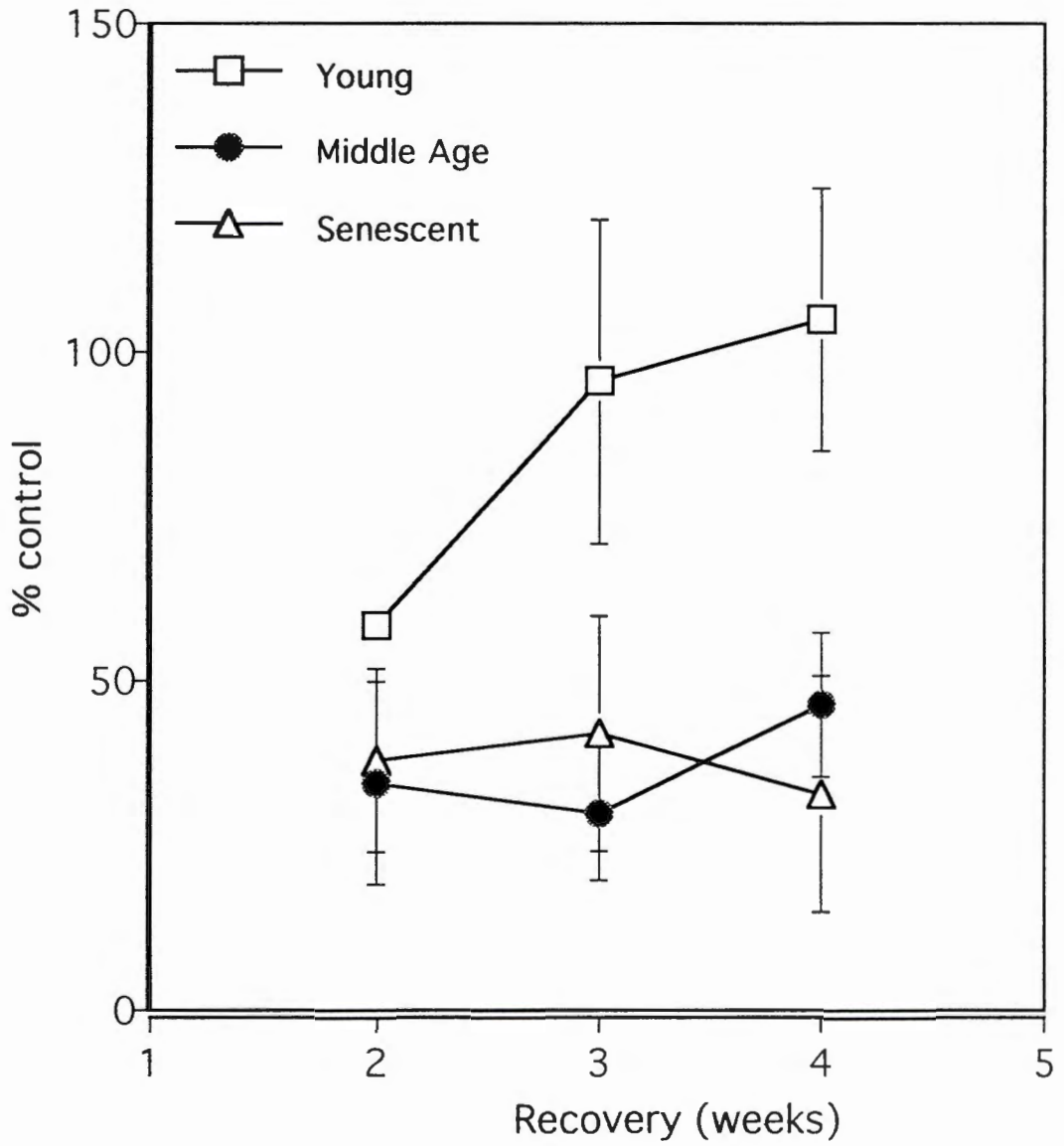


Figure 2. Relative percent changes in force production by the bupivacaine-injected TA compared to the contralateral control 2, 3, and 4 weeks after injection in young (3 months), middle age (18 months), and old (31 months) rats. Plotted values are means  $\pm$  SE.

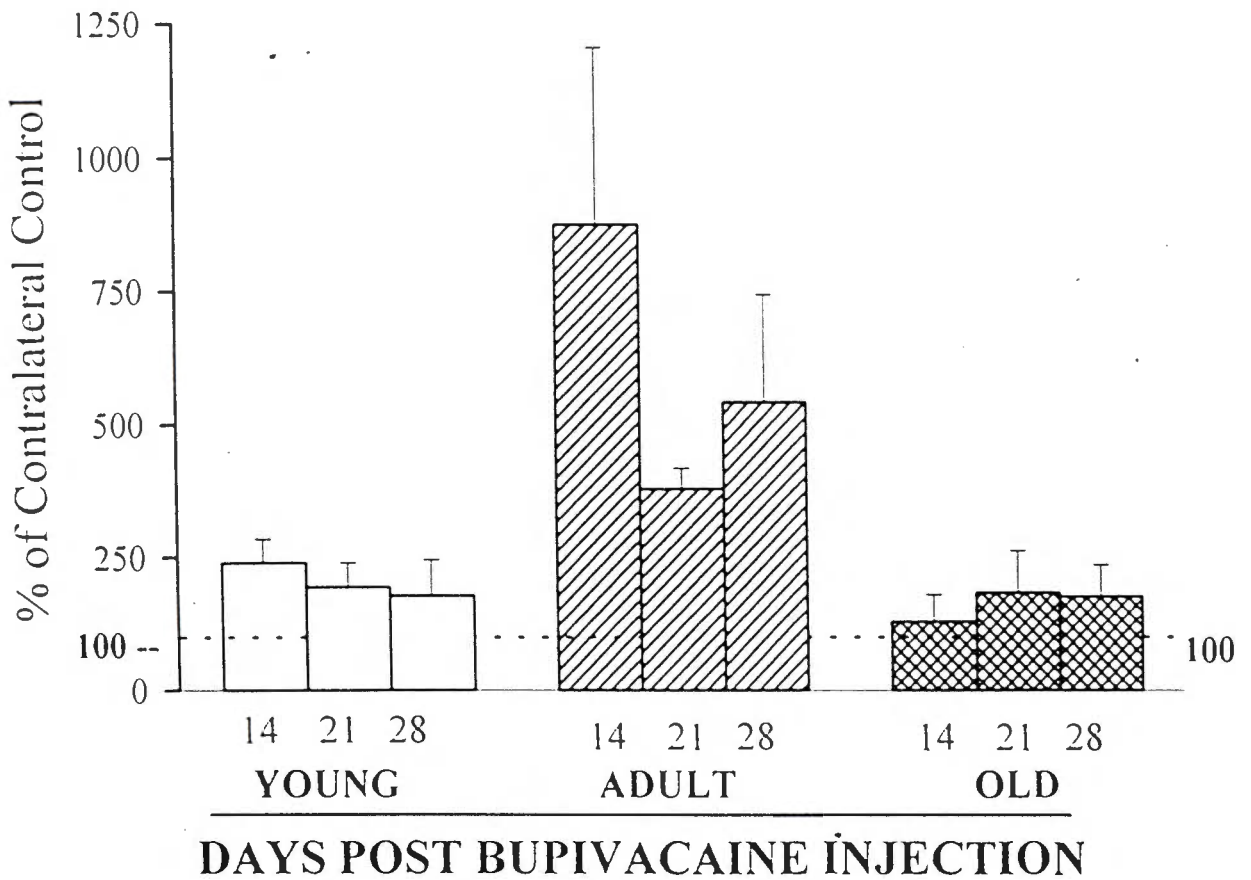
### Force (N) Recovery



**Figure 3.** Relative percent changes in myogenin mRNA content in the bupivacaine-injected TA compared to the contralateral control 2, 3, and 4 weeks after injection in young ( 3 months), middle age (18 months), and old (31 months) rats.



# MYOGENIN mRNA



**Figure 4.** Myogenin mRNA levels in the control and bupivacaine injected legs of young (3 months), middle age (18 months), and old (31 months) rats 2, 3, and 4 weeks post-injection.

# MYOGENIN mRNA

