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Development of Muscle Disuse Model in Rat: Effect of Denervation and Tenotomization on the Skeletal Muscle Mechanics

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Abstract: Denervation refers to the condition that represents loss of nerve connection with the muscle. The term tenotomization or tenotomy refers to the condition where the tendon of the skeletal muscle is damaged. Both these conditions lead towards muscle disuse and then deterioration. It may be caused by some diseases, chemical toxicity, physical injury or intentional surgical interruption. To observe the effects of denervation and tenotomy, it is important to develop an animal model with such pathological conditions for a better understanding and investigation of a possible cure. Current study was designed to develop an animal model in rat for denervation and tenotomy. The objectives were to optimize the anaesthetic dose for rats, to develop muscle disuse models in rats including denervation and tenotomization and to determine the mechanical and physiological properties of the gastrocnemius muscle of the animal model for muscle disuse. Gastrocnemius muscle of the rat was targeted for study. Models were developed by surgical procedures. We succeeded in developing the rat model for both conditions and it was verified by observing the changes in the physiological properties of muscles.

Keywords: Muscle disuse, Tenotomy, Denervation, Rat, Skeletal.

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INTRODUCTION:

Loss of nerve supply is called denervation. Causes of denervation may include; diseases, chemical toxicity, physical injury or intentional surgical interruption of a nerve. The mechanical behaviour of the denervated muscles has been studied by a number of workers to demonstrate the effect of disuse on their contractile apparatus [1, 2). Denervated muscles have shown the ability to survive after different periods of denervation.

Takagi and co-authors [1] reported atrophy with over-expression of certain enzymes in correlation with the denervation in skeletal muscles. Previously, it has been reported [3] that the effects of denervation are correlated with the fibre types. After the long term denervation of sciatic nerve they have reported atrophy of Dark and Intermediate fibres but no changes were found with Light fibres. Mazher and co-authors [4] reported major changes, seen in the muscle weight of (SOLEUS) slow muscle and (PLANTARIS) fast muscle after denervation. However, the recovery period of slow muscle was 2 weeks while in the case of fast muscle the time duration was 3 weeks. In a study [5], prolong isometric twitch contraction was reported in skeletal muscle after 2 - 6 days of denervation and rate of relaxation was delayed with increased rate of rise in muscle tension.

The term tenotomy is used to represent a condition when the distal tendon of the muscle is cut off and the muscle becomes deteriorated. However physiologically, the term tenotomy illustrates a condition where the muscle loses the incoming proprioception impulses. It causes the muscle to lose its passive tension, making it unable to exert effective force. However, the integrity of the nervous system is not obstructed. Various studies [6, 7, and 8] have reported the changes in physiological properties of skeletal muscles after tenotomy.

One study [9] reported that the anterior latissimus dorsi (ALD) of chick decreased in weight by 20% after tenotomy, whereas, posterior latissimus dorsi (PLD) decreased by 30%. Similarly, the fibre diameter of ALD and PLD muscles also decreased about 20% and 30% respectively. The electron microscopic studies by the same authors further demonstrated that the muscles denervated and tenotomised simultaneously show atrophy of the muscle fibres because of the loss of contractile proteins.

A few studies [10, 11] confirmed that following tenotomy, there is a sudden decrease in the ability of soleus muscle of rabbit to develop tension. A slight shortening of twitch contraction time was followed by a significant reduction in the half relaxation time. Buller [11] further suggested that these changes were the result of rapid atrophy of the slowly contracting fibres with in a muscle that contain fibres of different contractile speeds, rather than a change in the speed of the contractile elements themselves.

Following objective were set for the present study:

- To optimize the anaesthetic dose for rats.
- To develop muscle disuse models in rats including denervation and tenotomization.

- To determine the mechanical and physiological properties of the gastrocnemius muscle of the animal model for muscle disuse.
  Following mechanical properties were studied before and after fatigue in normal and tenotomized gastrocnemius muscles obtained from rats:
  - The rate of rise (Kg/cm^2)
  - The rate of relaxation (Kg/cm^2)
  - The relaxation time (sec) and
  - The tension (gm)

Materials And Methods

Animals:

Rats (Wistar), weighing in between 200 to 300gms, were purchased from the animal house of ICCBS, University of Karachi and kept in the animal house of the Department of Physiology University of Karachi during the study. All animals' procedures were carried out in accordance with the international guidelines for the care and use of laboratory animals and after approval by the local ethical committee of the Faculty of Science, University of Karachi. The animals were housed individually into the cages and provided with sterile water and food *ad libitum*.

Reagents and Buffer:

All the reagents used were obtained from BDH. Kreb's Henseliet solution was used to maintain the isotonic environment for muscles during mechanical recordings.

Anaesthesia:

Before starting the procedure all the instruments/tools and surfaces were cleaned and sterilized by ethyl alcohol. Rats were anesthetized with an intra-peritoneal (i.p.) injection of ketamine hydrochloride (50 mg/ml) and xylazine hydrochloride (100 mg/ml). Different doses were given to the rats and finally it was optimized as 60 mg/kg and 7mg/kg respectively.

Development of Animal model for Tenotomization:

A small incision was made over the skin at Achilles tendon in right leg of the anesthetized rat. After opening the area the Achilles tendon was cut by the scissor and the skin was sutured back to close the wound. The animal was kept in the lab until it became conscious again. Finally the animal was kept in animal house under observation for one week.

Development of Animal Model for Denervation:

A small incision was made over the skin below the hip joint. The muscles of the leg were cut opened to locate the sciatic nerve. After recognizing the sciatic nerve a smaller piece of the nerve (around 1 mm) was cut and removed. Immediately the muscles and skin were sutured back to close the wound. The animal was kept in the lab until it became conscious again. Finally the animal was kept in animal house under observation for one week.

Isolation of Gastrocnemius Muscle:

One week post surgery animals were sacrificed via decapitation. The gastrocnemius muscle was dissected out. For this purpose, a large incision was made in the skin, from the knee joint to the phalanges of hind limb, on the ventral side of the animal body. The skin was then removed carefully with the help of scalpel. The gastrocnemius muscle was than dissected out with the knee joint. The muscles were kept moist with buffer solution during dissection. Immediately after dissection the muscle was transferred to the muscle chamber.

Experimental Setup and Protocol:

The muscle chamber was a horizontal perplex chamber provided with thermostatically controlled water jacket (outer chamber) through which the temperature of the buffer solution, placed in the inner chamber, is maintained at the desired value. The proximal end of the muscle having knee joint was fixed in the chamber. The tendon of insertion was tied with a silk thread that passes under a pulley and attached with the isometric transducer (MLT 500/d, ADInstruments, Australia). Both the pulley and transducer were fixed through laboratory clamp and macro-manipulator (MLA41, ADInstruments, Australia) respectively.

A square waved pulse stimulator (Harvard 6002) was used. Its front panel provides the variable frequency (0.1-100HZ), width (0.1-5ms) and output voltage (1-50V). Two modes of stimulation were provided: the single pulse at 1Hz and continuous high frequency stimulation at 80Hz (50V strength and 0.5ms of duration). Muscle was subjected to fatiguing protocol by providing 100 stimuli at the frequency of 1Hz.

Mechanical activities of treated and control muscles were recorded with the help of two separate isometric force transducers. These transducers are suitable for the recording of 0-500gm of force. Length of the muscle was adjusted at optimal level (resting length) through macro-manipulator to record optimal mechanical activity of muscle. Force transducers compatible with data acquisition system were calibrated and units were converted as standard protocol described in the instruction manual of Power Lab.

Power Lab (Data Acquisition System):
Transducers were connected with 4 channel powerlab data acquisition system (ML856 AD Instruments, Australia) through bridge amplifier (ML221 AD Instruments, Australia).

Measurements & Calculations:
Once the unit of recorded data was set to grams, all of the data was converted in the standardized units of Kg/cm^2 with arithmetic panel.

Statistical Analysis:
Statistical analysis was done by using Microsoft Excel. Student's t-test was applied for the comparison of data and significance level was determined taking the * $p<0.05$, ** $p<0.005$ and *** $p<0.001$ as standards for "significant", "more significant" and "highly significant".

RESULTS
Rate of Rise
Comparison Before And After Fatigue:
When compared statistically the values before fatigue (Bf) and after fatigue (Af) were found significantly different (*$P<0.05$) as shown in Fig1a and 1b.

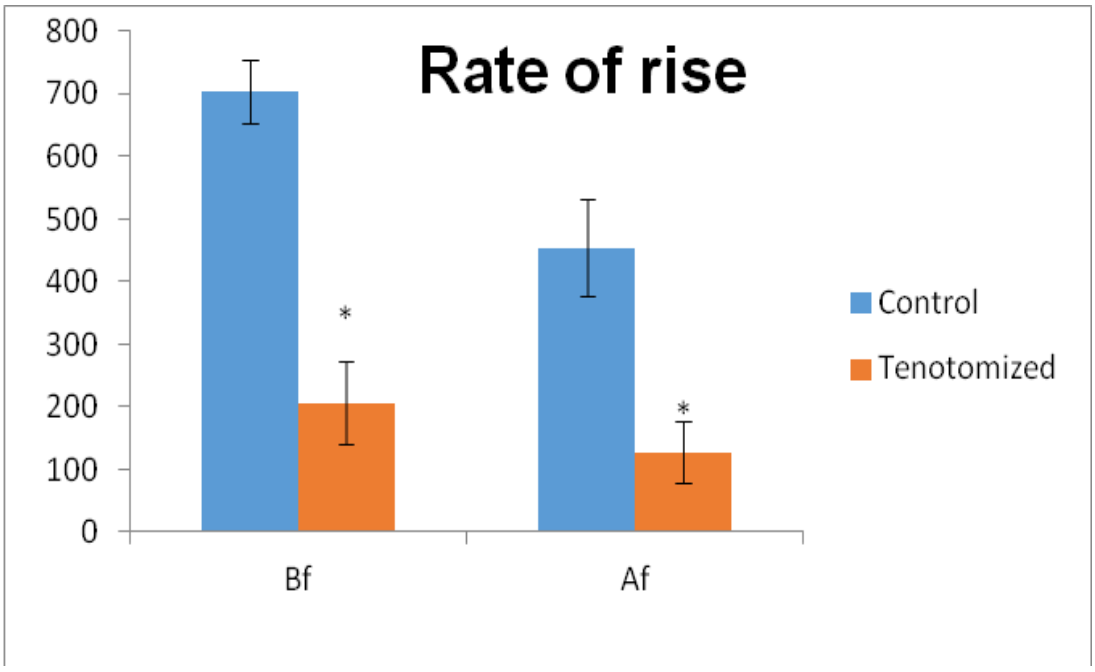

Fig 1a:
Bar diagram showing comparison between rate of rise before and after fatigue obtained from skeletal muscles of control and tenotomized animals.

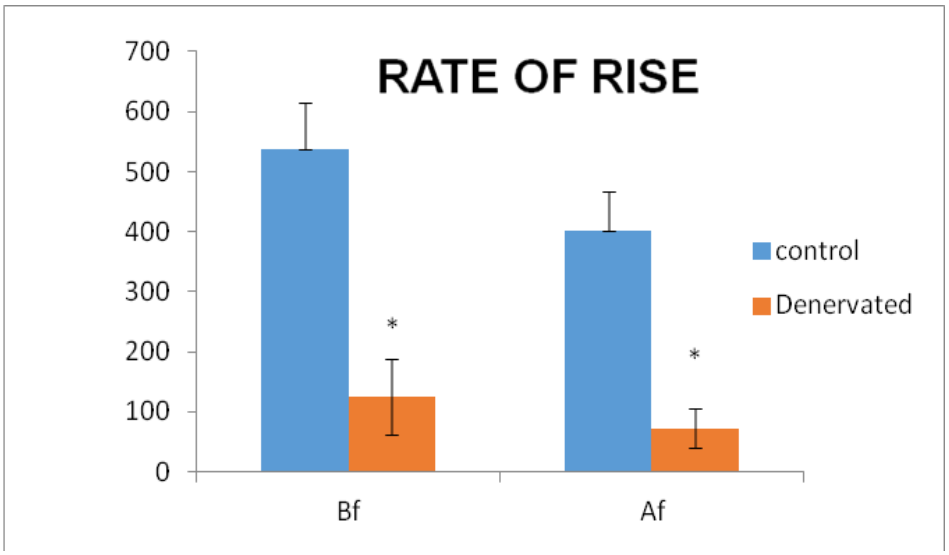

Fig 1b:
Bar diagram showing comparison between rate of rise before and after fatigue obtained from skeletal muscle of control and denervated animals.

Comparison Between Control And Disused Muscles:
When compared statistically the values of control and disused muscles were found significantly different (*P<0.05) before and after fatigue as shown in Fig 1c and 1d.

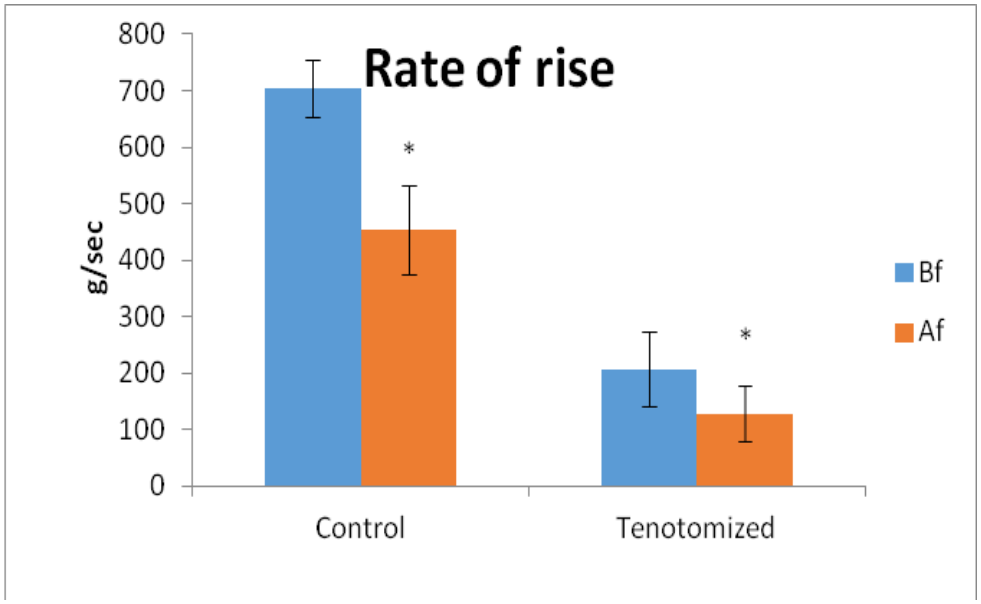

Fig 1c:
Bar diagram showing comparison between rate of rise obtained from skeletal muscle of control and tenotomized animals.

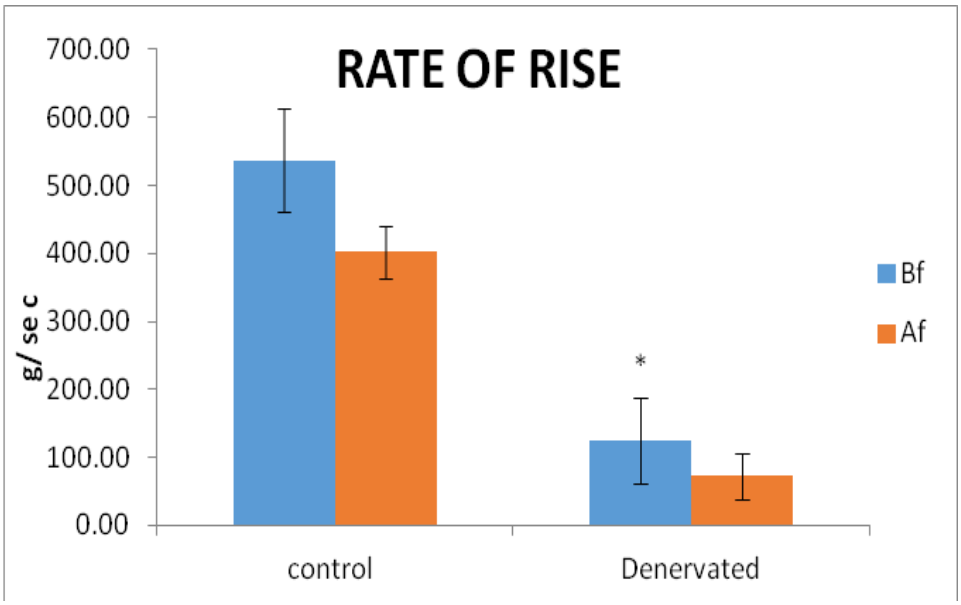

Fig 1d:
Bar diagram showing comparison between rates of rise obtained from skeletal muscle of control and denervated animals.

Rate Of Relaxation
Comparison Before And After Fatigue:
When compared statistically the mean value before and after fatigue were found significantly different (*p<0.05) in both control and disused muscles as shown in fig 2a and 2b.

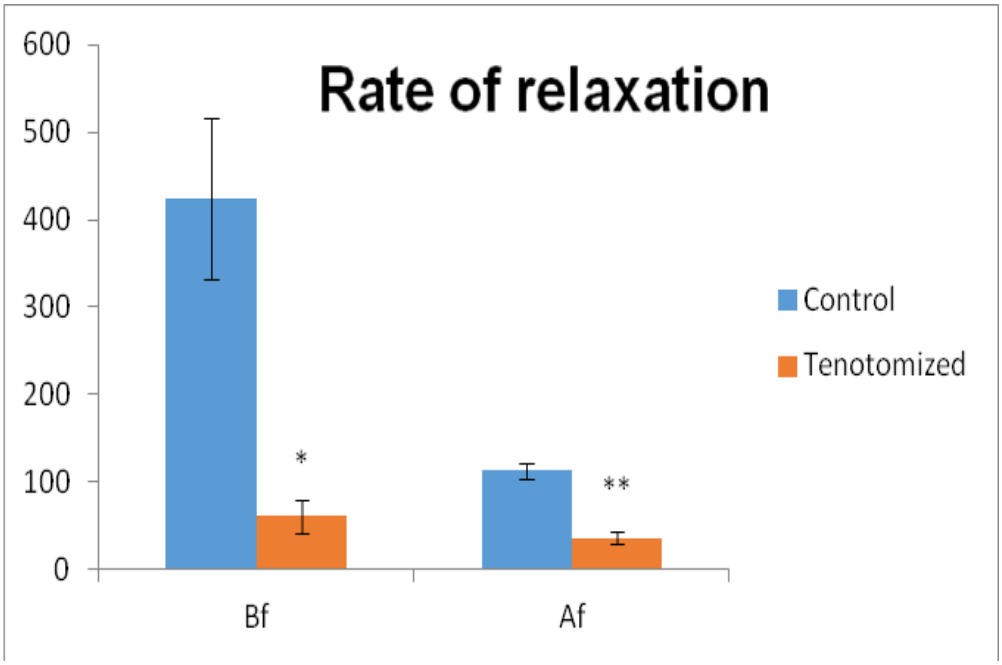

Fig 2a:
Bar diagram showing comparison between rate of relaxation before and after fatigue obtained from skeletal muscle of control and tenotomized animals.

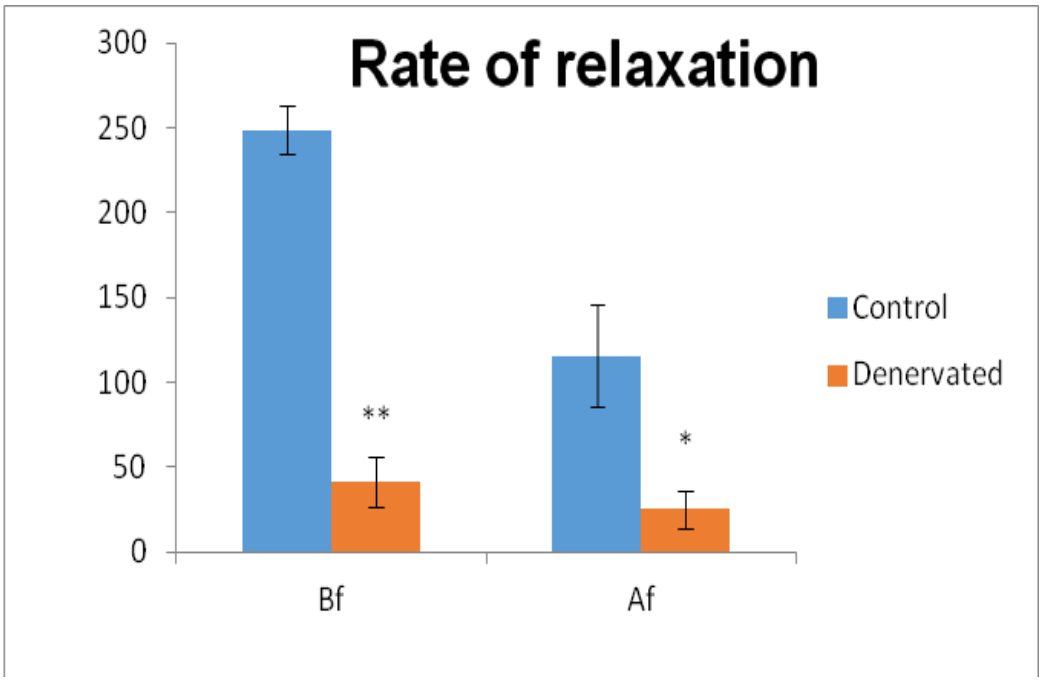

Fig 2b:
Bar diagram showing comparison between rates of relaxation before and after fatigue obtained from skeletal muscle of control and denervated animals.

Comparison between control and disused muscle
When compared statistically the value of control and disused muscles were found significantly different (*p<0.05) before and after fatigue as shown in fig 2c and 2d.

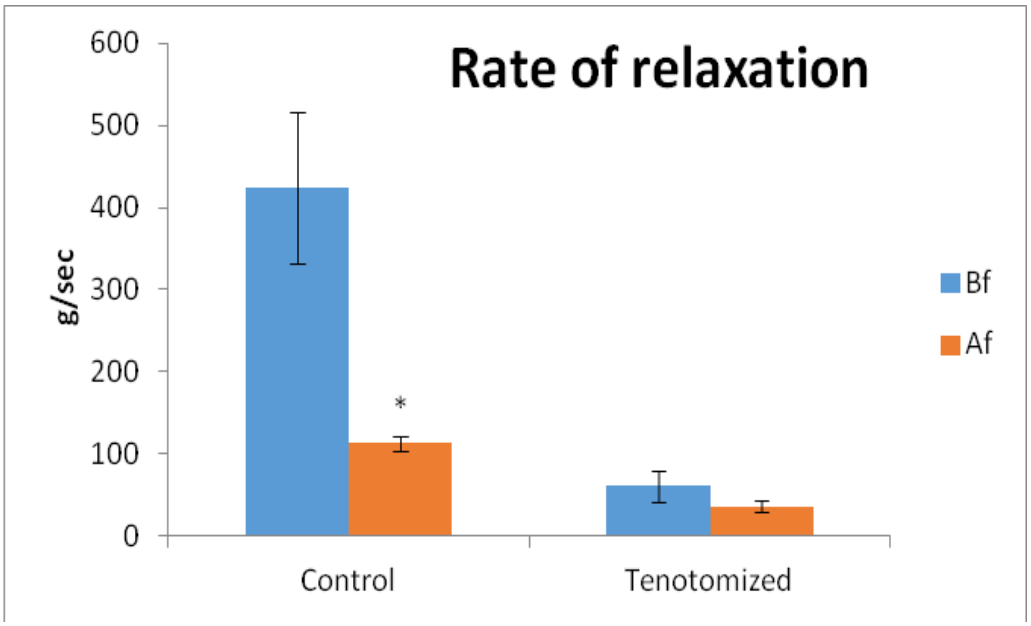

Fig 2c:
Bar diagram showing comparison between rate of relaxation obtained from skeletal muscle of control and tenotomized animals.

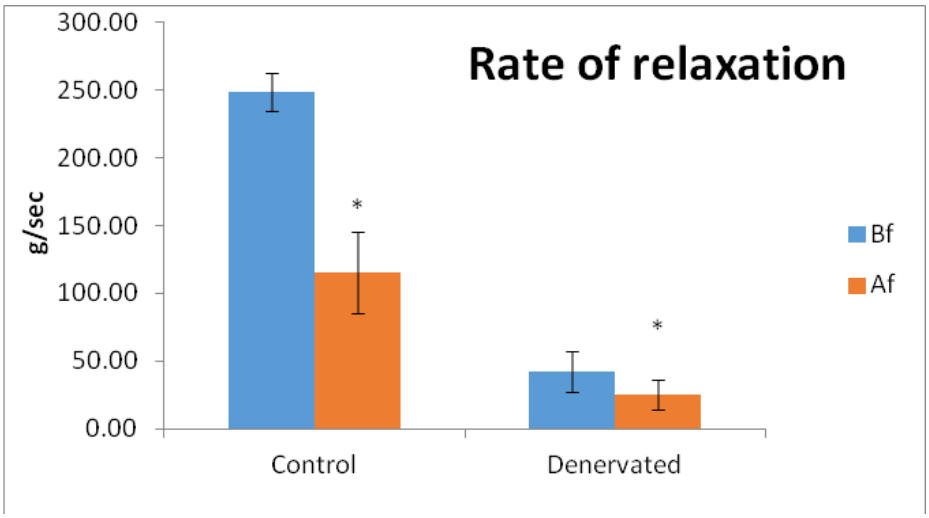

Fig 2d:
Bar diagram showing comparison between rates of relaxation obtained from skeletal muscles of control and denervated animals.

Relaxation Time
Comparison Before And After Fatigue:
When compared statistically the mean value of before and after fatigue were found significantly different (*p<0.05) in both type muscles as shown in fig 3a and 3b.

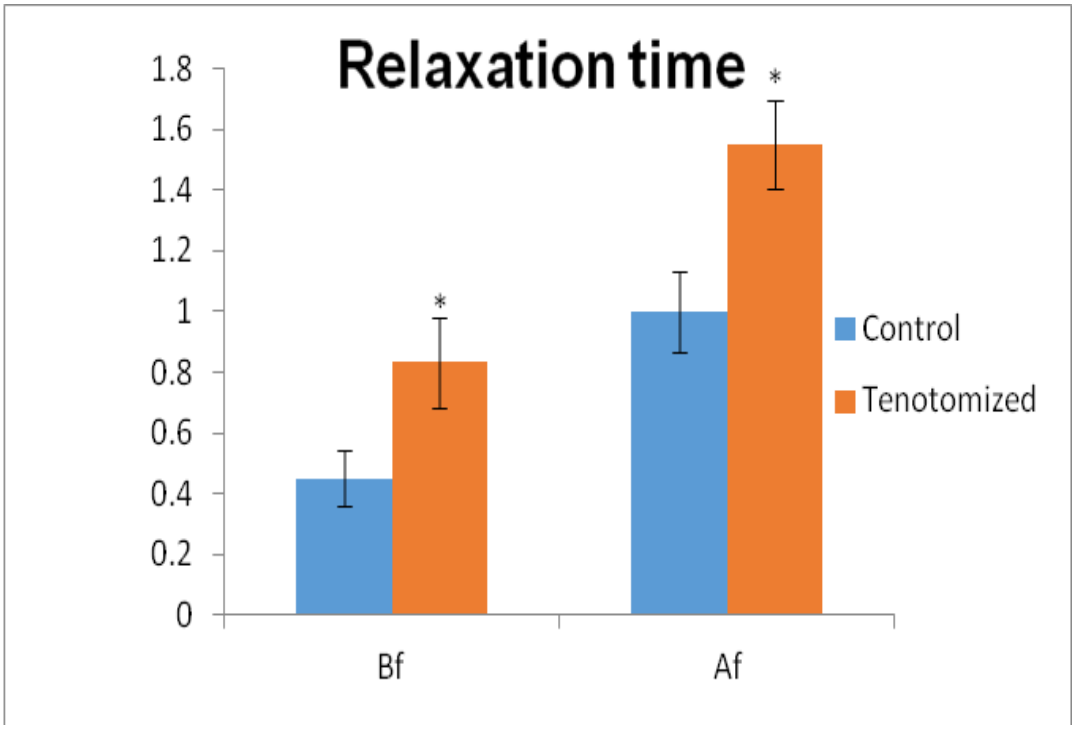

Fig 3a:
Bar diagram showing comparison between relaxation time before and after fatigue obtained from skeletal muscle of control and tenotomized animals.

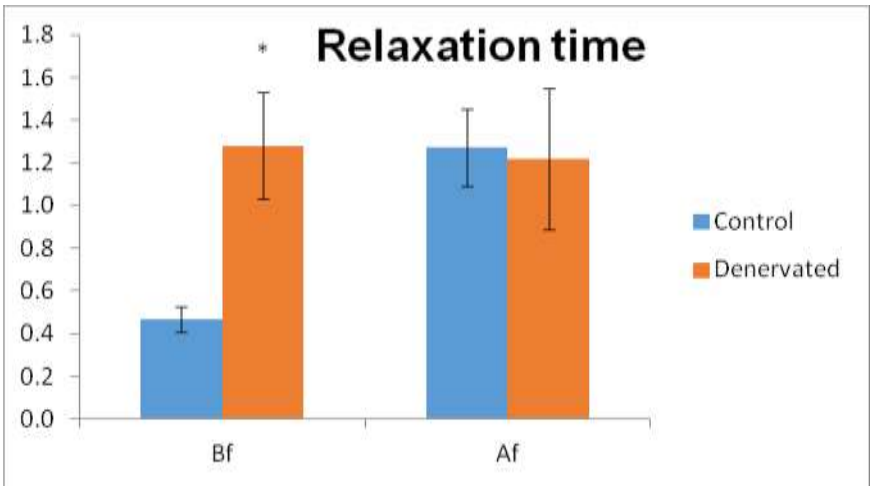

Fig 3b:
Bar diagram showing comparison between relaxation time before and after fatigue obtained from skeletal muscle of control and denervated animals.

Comparison Between Control And Disused Muscle

When compared statistically the values of control muscle were found significantly different (*p<0.05) as shown in fig 3c and 3d.

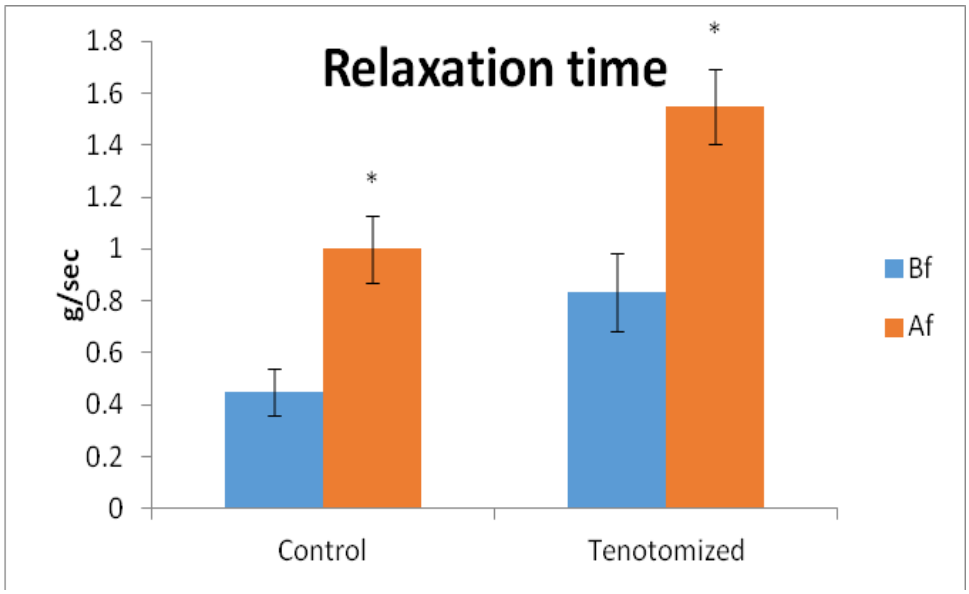

Fig 3c:
Bar diagram showing comparison between relaxation time obtained from skeletal muscles of control and tenotomized animals.

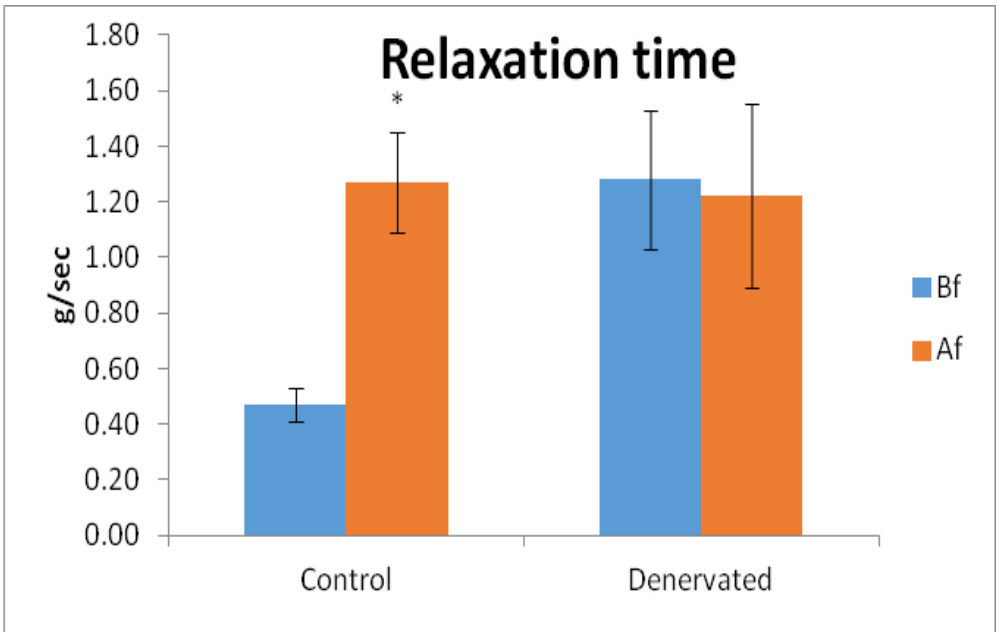

Fig 3d:
Bar diagram showing comparison between relaxation time obtained from skeletal muscle of control and denervated animals.

Tension

Comparison Before And After Fatigue

When compared statistically the value of before and after fatigue were found significantly different (*p<0.05) as shown in fig 4a and 4b.

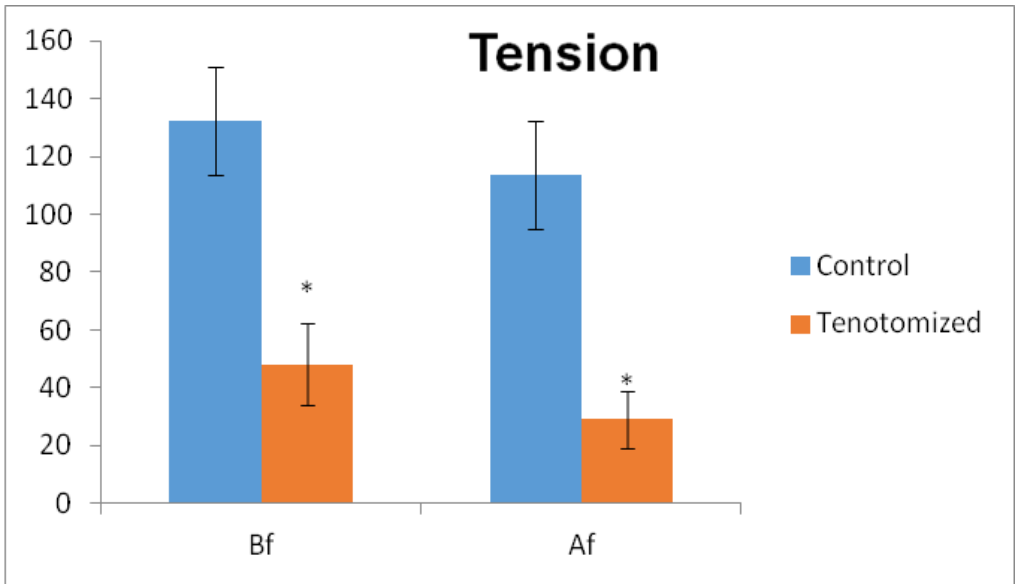

Fig 4a:
Bar diagram showing comparison of Tension before and after fatigue obtained from skeletal muscle of control and tenotomized animals.

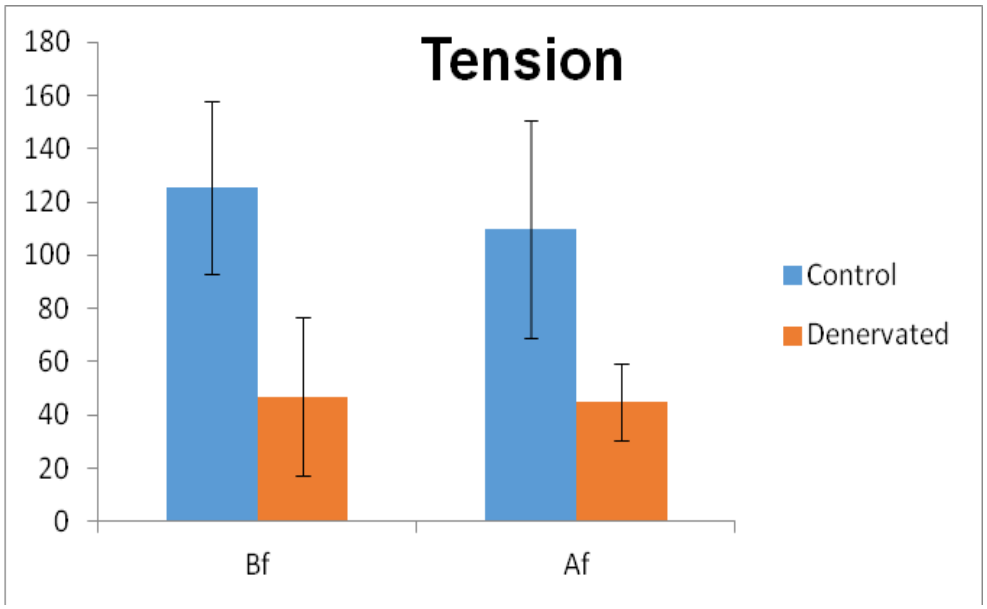

Fig 4b:
Bar diagram showing comparison of Tension before and after fatigue obtained from skeletal muscle of control and denervated animals.

Comparison Between Control And Disusedmuscle
When compared statistically the mean value both muscles were found significantly different (*p<0.05) as shown in fig 4c and 4d.

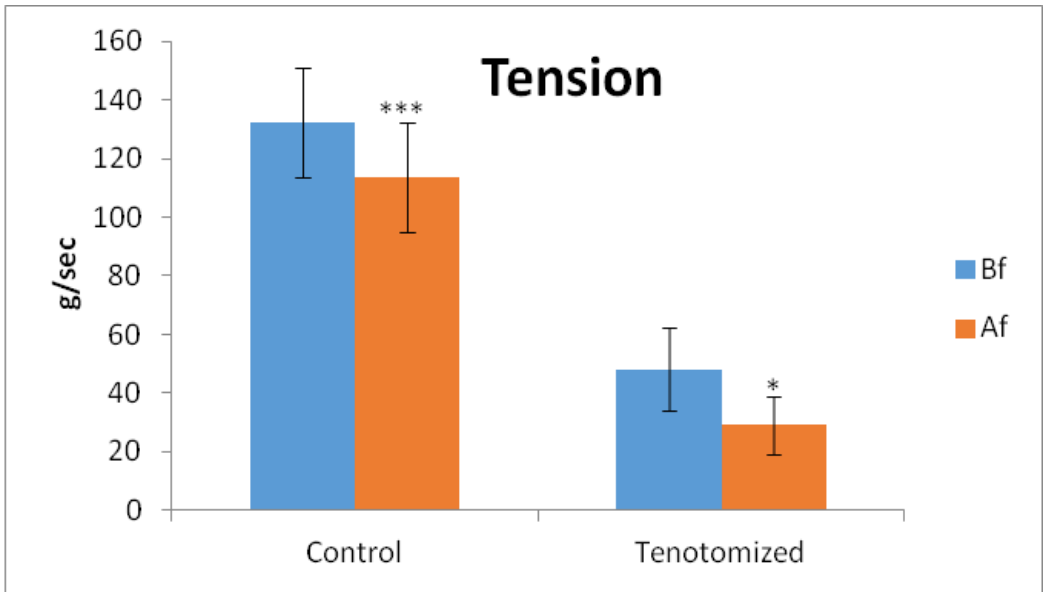

Fig 4c:
Bar diagram showing comparison between Tension obtained from skeletal muscle of control and tenotomized animals.

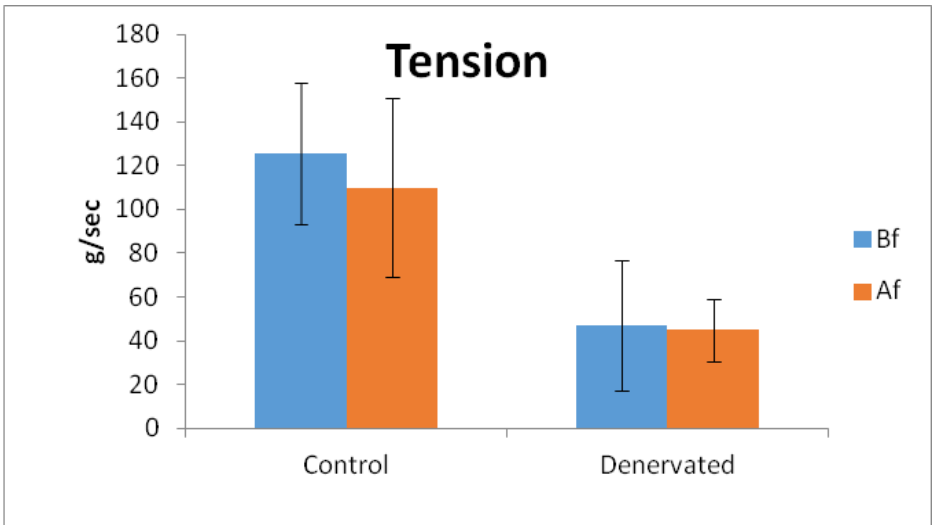

Fig 4d:
Bar diagram showing comparison of Tension obtained from skeletal muscles of control and denervated animals.

DISCUSSION:

Loss of nerve supply to an organ, tissue or any part of the body is called denervation as mentioned before in the introduction. There are many different reasons for denervation, sometimes it may be due to the interruption of nerves, injury of nerves or physical damage. On the other hand, tenotomy denotes the condition in which the muscle tendon is damaged or injured. In our experiment rats were divided into two groups. Gastrocnemius muscle of one group was denervated by cutting the Sciatic nerve, and same muscle of the other group was tenotomised by cutting the Achilles tendon. Mechanical properties of the disused and control muscles were recorded and compared with the fatiguing process.

Skeletal muscle mass is controlled by the balance between protein synthesis and degradation. Muscle atrophy is caused by both increasing protein degradation and decreasing protein synthesis [1]. Many different theories have been published that were based on the phenomena of denervation in connection with the protein degradation. David and co-authors [12] explained the phenomena of protein turnover after the denervation. According to the authors, increased protein breakdown was observed after 24 hrs of denervation that leads to decrease muscle weight.

In control and denervated rat skeletal muscle, the rate of rise before fatigue and after fatigue was found significantly different. Interestingly, the rate of relaxation in control muscle before and after fatigue was significantly different but in case of denervated muscle it was non-significant. However, when the comparison was made for rate of relaxation between the control and denervated muscles before and after fatigue, the difference was statistically significant. According to a study [13] nerves have the ability to regenerate at a slower rate and side-to-side conjunctions in the nerve bridge helps to repair the damaged area or damaged nerve. Alessandro et al., [13] have proposed a theory that based on the re-innervation of the nerves. In that article Peripheral nervous system (PNS) was prominently described. According to their theory PNS has an intrinsic ability to repair and regenerate. Their experimental strategies were based on the regeneration or re-innervation. It was believed that an alternative and novel approach involved to combine the repaired nerves and also to enhance the regeneration of axons. That theory also mentioned the natural cell therapy through the complex events that followed the nerve injury and leads to cause regeneration of nerve. Under the other circumstances significantly different values in our results were the identification of some specific molecules and single targets to stimulate the response for the repair of nerve.

In our experiment, Tetanic Tension, when compared in control and denervated muscles before and after fatigue, was found non-significantly different. On the other hand some of the reasons for the non-significant results have shown the active duration state of both control muscle and denervated muscle. The decreased duration of active state were found unable to fall the sarcoplasmic concentration of calcium (ca^{+2}) to a limit which leads to cause a fall in isometric force. Similar result was demonstrated by a study [14] in rats. They performed their experiments on the diaphragm. Another reason for the non-significant result was also observed due to the extensibility and stiffness of the muscle on the tendon region after the denervation that may result in the loss of muscle fibres. In the case of relaxation time the result for both control and denervated musles before fatigue was significantly different. When the gastrocnemius muscle of rat was deprived of its tendon of insertion, the contractile behaviour of muscle was changed from its normal pattern, representing a decreased isometric twitch

tension. These findings are similar to some of the studies [10, 11, 15 and 16]. In our results, the disuse period of 7 days has decreased the tension significantly. Tenotomization induced shortening also resulted in the decrease in resting lengths of the experimental muscles.

Our results have shown significant difference, between control and 7 days of tenotomised muscles before and after fatigue. Since, the twitch is a measure of elasticity in muscle; therefore it is possible that the tenotomization affects the elasticity of gastrocnemius muscles of rat. The results regarding the rate of rise in tension twitch represent lesser value in the tenotomised muscle. This result further indicated that tenotomization has affected the degree of cross bridge interactions along with the rate with which the cross bridge interacts in the presence of calcium ions. Buller and colleague [11] in their experiment on the tenotomised muscles of rabbit showed decrease rate of rise in twitch tension of tenotomised muscle. The duration of active state was found to be significantly different between control and tenotomised muscles before and after fatigue. It means that tenotomization has produced some effect on the total quantity of calcium ions release for the contractile process. The higher value of peak duration in twitch found in the tenotomised muscle along with decrease rate of rise showed that calcium is released slowly from the sarcoplasmic reticulum as compared to control muscle. The time dependent parameter such as rate of relaxation and relaxation time were reportedly decreased in tenotomised muscle. Our results showed significant difference between control and tenotomised muscle before and after fatigue. We concluded that tenotomy affects the interaction between the cross bridge and their detachment during relaxation time as well as rate of relaxation as compared to control muscle. Our results also indicate that the resting lengths of the tenotomised muscle were decreased significantly than their control. It showed a direct relation between resting length and the tension produced. Stretched and shortened muscle lengths were decreased in tenotomised muscle indicating that tenotomization directly influences the physiology of intra-fusal fibres in the skeletal muscles.

Attempts to develop a suitable model for muscle disuse has been continuously made by various scientists [17, 18] and most of the time the method for the development of model remained expensive and less result oriented. We have shown a very economical and simplest method to develop a rat model of muscle disuse like tenotomy and denervation.

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