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Effect of age on the mechanism of induced hypertrophy in skeletal muscles of squirrel

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Abstract
There has been much interest in recent years in understanding the mechanism and effects of the exercise in aged people. However, it is not clear whether or not aging influence the ability of the muscle to respond to training. There have been reports of the decline in the activity of muscle proteins in aged animals due to hypertrophy. In the present study, the result confirms the hypothesis that the muscle activity declines with age. There is a decrease in the total proteins and glycogen in the older animals when compared to younger animals indicating reduced synthetic rate and increased catabolic rate of proteins. This is also attributed to the neural factor which may be one of the reasons for reduced hypertrophy in aging animals.

Keywords: compensatory hypertrophy, DNA, RNA, synergistic, soleus muscle and tenotomy

1. Introduction
Alteration in work demands are known to induce appreciable changes in the size of skeletal muscles, reduced activity leads to atrophy where as increased activity evokes compensatory growth. While it is relatively easy to achieve muscle hypertrophy in man with exercise and training, it is much more difficult in laboratory animals like mice and all the more difficult in wild animal like squirrel Even a most intensive training program causing alterations in the biochemical characteristics of different muscle types, Gutamann and Hajek (1972) [1]. For this reason, the model of compensatory hypertrophy was first described by Denny Brown (1960) [2] has become a suitable experimental approach for attaining a standard, rapid and considerably higher rates of hypertrophy in laboratory animals. This model is based on the fact that elimination of greater part of synergists in certain muscles group by tenotomy induces hypertrophy of remaining muscles. Skeletal muscles can undergo rapid growth in response to sudden increase in workload. In the case of rat soleus muscle increase in weight is by 40% within six days after tenotomy of synergistic muscles which involve the enlargement of muscles fibres and longitudinal splitting Goldberg *et al*., (1979) [3]. There are also several reports on time course, and on factors affecting this types of muscle hypertrophy. In most of the experiments, it has been shown by many authors that slow soleus muscle exhibited the remarkable gain in muscle weight, Mackova and Hnik *et al*., (1972) [4].The maximum hypertrophy was attained at 7 days in soleus muscle was 30%, Mackova Hnik *et al*., (1976) [5]. Compensatory hypertrophy with reference to age has been studied by many authors. It has been shown that prominent increase in hypertrophy observed in younger animals than in older ones. This difference is more in slow muscles than fast muscles. Since compensatory hypertrophy is non-neural factor produced tenotomising the synergists muscles, there appear to be many biochemical adaptations to increase mechanical tensions, Protein metabolism seems to be a key adaptation in compensatory hypertrophy because the increase in muscle bulk is a direct result of an increase in protein synthesis and a decrease in protein breakdown, Goldberg *et al*., (1972) [6].The difference in the increase in muscle bulk with reference to age may be attributed to decrease in hormonal activity.

The present study is taken up to understand, what is the mechanism which makes the difference with respect to age in hypertrophy, especially with reference to some energy yielding biochemical parameters in an active rodent like a squirrel. It is shown that age attenuate the hypertrophic response in exercising muscles. This present study also aimed to study whether muscle memory exists, in the light of recent study which suggests that exercising at a younger age induces a similar muscle memory that could help people to stay fit with lesser exercise in future as they grow old Kristein Gunderson, (2013) [7]. This study may help to understand the mechanism in sports person who has increased their muscle mass in early age due to exercise and will help to sustain it later in their lives.

2. Material and Methods
Animals were divided into two groups. In the first group, animals were tenotomised to assess the rate Hypertrophy at seven days in soleus muscle. These animals were used to analyze the change in wet weight and dry weight of hypertrophied muscles and also used to determine biochemical protocols like total proteins and glycogen estimation of DNA and RNA. In the second group of animals which are older than the previous group are kept for maintenance for nearly two months in cages and these were used to for hypertrophy the wet and dry weight was estimated at the 7th day of hypertrophy and biochemical profile like total protein, glycogen, DNA and RNA, they were also estimated. These animals were compared with respect to all parameters in young and old animals which represent two groups. In all the experiments ipsilateral limb was operated for experimental purpose and contra lateral was regarded as control. All the groups are sub grouped as A and B. A serve as younger and B as the older group of animals.

Separation of experimental tissue:
The experimental animals were maintained at aforesaid time periods and were sacrificed at appropriate time periods for various parameters. Animals were decapitated and

soleus muscle was excised with almost care and they were kept in an ice cube to maintain the optimum temperature. The tendons and connective tissue were freed and separated carefully from the muscle. Each muscle weighed on a digital balance and wet weight of each muscle was recorded in two groups of animals.

Rate of Hypertrophy

The rate of hypertrophy was determined by comparing the wet weight of experimentally muscles with their contra lateral muscles of the same animal and comparison also is done between the rate of hypertrophy of two groups between the younger and older animals and differences is noted.

Dry weight determination

The soleus muscle was separated and after taking the wet weight, they were kept in the desiccators containing phosphorus pent oxide a dehydrating reagent, and due to its hygroscopic action and overnight incubation at 60-70 C facilitate the muscle to get completely dry within 24 hours. On the following day, the dry weight is recorded on digital balance and difference in younger and older animals in hypertrophy is ascertained.

Total protein estimation:

Total protein in hypertrophied muscles was estimated using original method of Lowry *et al*., (1951) [8]. The soleus muscle was excised and 50 mg's muscle used for protein estimation. The muscles were homogenized in 2 ml of distilled water and 3 ml of 10% TCA. This was kept for 10 minutes to settle down thoroughly. The homogenate was centrifuged at 3000 rpm for about 30 minutes and the precipitate was collected in a separate test tube. This precipitate was dissolved in 10 ml of 0.1N Na OH. The soluble and structural proteins were estimated along with total proteins by following the, Lowry *et al*., (1951) method. About 5 mgs of muscle was homogenized in 2 ml of 0.25 M sucrose solution and centrifuged at 2000 rpm for 20 minutes. The precipitate and supernatant fractions were separated. Structural proteins were estimated using this precipitate fraction while the supernatant is utilized for estimation of soluble proteins. To each of this separated fraction 3 ml of 10%, TCA was added and allowed to settle for 30 minutes. The precipitate was dissolved in the known volume of 0.1N sodium hydroxide.

To 1 ml of 0.1 ml, N sodium hydroxide and 1ml of the sample 5ml of freshly prepared copper reagent was added followed by 0.5 ml of dilute Folin ciocaltean reagent (1:1 Folin and sodium hydroxide) after 10 minutes to all the test tubes. The test tubes were shaken thoroughly and allow standing for 20 minutes. The colour intensity was read at 540 nm and proteins were calculated from a standard graph of bovine albumin protein. The proteins were expressed as mg/mg of wet weight of muscles.

Glycogen Estimation

The Anthrone method of Klicpera *et al*., (1957) [9] was used to estimate glycogen content from the muscle freshly excised of weight 100 mgs. The muscle was kept in the test tube 1 ml of 30% potassium hydroxide was added and boiled for about an hour in hot water bath at 100o C. The test tubes were allowed to cool down to room temperature and 0.2 ml of 2% sodium sulphate is added followed by the addition of 6 ml of absolute alcohol. The samples were kept overnight and in the refrigerator. On the following day, these samples were centrifuged at 3000 rpm for 20 minutes. The supernatant was discarded and the precipitate was used for glycogen estimation. The sediment was dissolved in 5 ml of distilled water and centrifuged at 1000 rpm for 1 minute. The supernatant was collected and diluted with distilled water to known volumes. The Anthrone reagent was added (5 ml of 0.16% reagent) to 1 ml of the blank. The test tubes were thoroughly shaken and were kept on the ice tray. Later the test tubes were transferred to hot water bath and left for 10 minutes to boil. They were cooled down under running tap water for 5 minutes. The blue green complex formed was read at 610 nm and glycogen content was estimated from the standard graph of glucose and given as µg of glucose /mg of wet weight of tissue.

DNA estimation by Diphenyle method using Spectrophotometry

The concentration of DNA by diphenyle method involves the reaction of diphenyle with deoxyribose sugar producing blue colored complex. The DNA sample is boiled under the acidic condition which causes depurination of the DNA followed by the dehydration of the deoxiribose sugar into a highly reactive w-hyderoxylevulinyl aldehyde. The hypertrophic and control muscles are extracted and the assay was prepared. To each 1 ml of sample add 3 ml of diphenyle reagent and mix it well, place them in boiling water bath for 10 minutes. Take out all the test tubes from the water bath and allow them to return to room temperature. Now measure the absorbance of the tubes at 595 nm a glass cuvette against the reagent blank. The readings are taken as µg of DNA in µl of volume.

RNA Estimation by Orcinol method

The standard Orcinol RNA estimation method is a modified method of Almog, Shirey T I *et al*., (1978) [10]. In this method 0.4 ml homogenate is prepared in 2 ml 0.2 N sucrose solution and centrifuge at 3000 rpm.Take the supernatant and add 4 ml of Orcinol reagent and reaction between Orcinol and ribose sugar start and ribose is converted to furfurol to give green color on heating in boiling water.Then cool it and read he maximum absorbance at 665 nm on spectrophotometer.

Statistical Analysis: All the data were analyzed in triplicate and average was taken for each parameter and compared with control which is taken as percentage.

3. Results

Dry Weight

Both dry weight and wet weight results show an increase at 7th day of hypertrophy when compared with normal muscle weight. The increase was observed in both young and old animal group, however the increase is more in pronounced in younger animals than old animals. The increase is 50 % more in young animals than old animals. The dry weight in soleus muscle of younger animals is 30% at 7th day and 15% in old animals as shown in fig. 1. The results show that the water content is remarkably present in hypertrophied muscle.

Proteins: As shown in fig. 2 the hypertrophy induces rapid increase in the total protein in young animals than old animals. Total protein increases up to 25% in young where as 10% in old animals. Whereas the soluble proteins do not show any

increase but it decrease by 3% at 7th day of hypertrophy in soleus younger animals and 1% in older animals. But structural proteins have increased enormously to 27% in soleus at 7th day of hypertrophy and 9% in older animals as shown in fig.3 and 4.

Glycogen Content: Results of glycogen content in the hypertrophied soleus muscle as in fig.5 shows increase up to 40% in young animals. The glycogen content was found to increase up to 30% in hyper trorphied soleus muscle of old animals.

DNA and RNA Content: DNA content has increased in hypertrophy in soleus muscles at 7th day. The increase was with respect to proteins and it is more pronounced in younger than older animals. The increase in DNA content was 20% in young and only 10% in older animals shown in fig.6. Whereas RNA content increases to 25% in young group and older group of animals. The RNA result is same in both young and old animals as seen in fig.7.

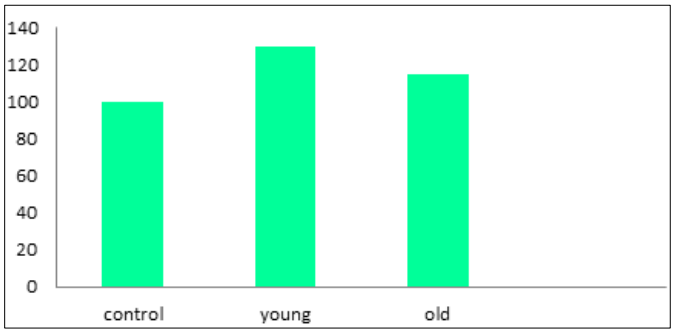

Fig 1: Changes in the soluble proteins in soleus muscle after 7 days of hyper trophy

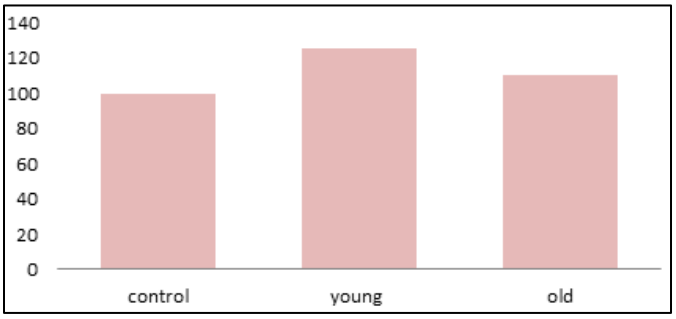

Fig 2: Total protein changes in soleus muscle after 7 days of induced hypertrophy

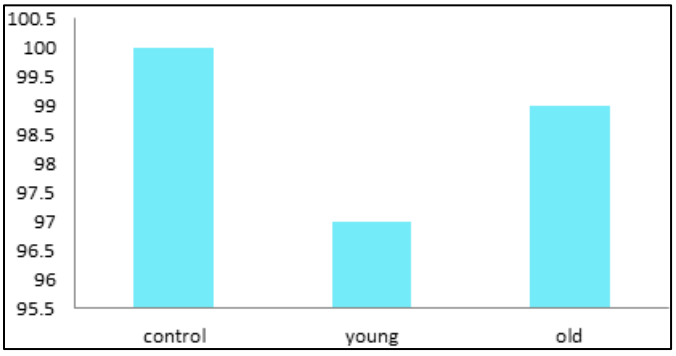

Fig 3: Changes in the soluble proteins in soleus muscle after 7 days of hyper trophy

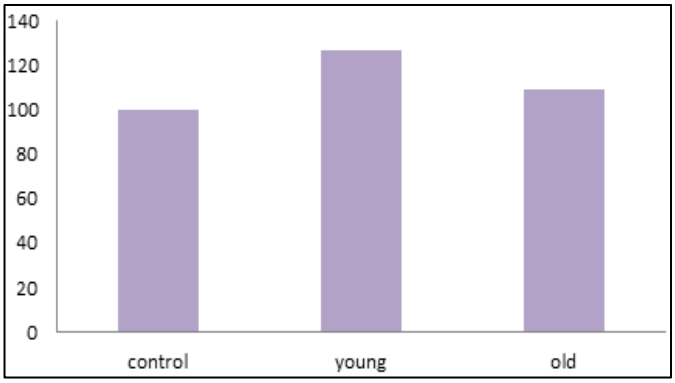

Fig 4: Changes in the structural proteins in soleus muscle after 7 days of hypertrophy

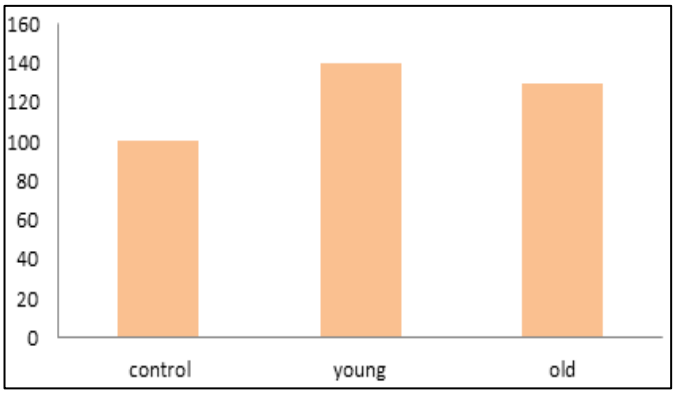

Fig 5: Increase in the glycogen content in soleus muscle after 7 days of hypertrophy

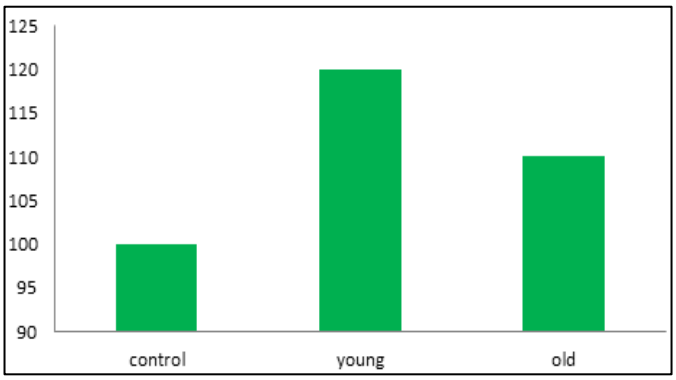

Fig 6: Changes in the DNA in soleus muscle after 7 days of hypertrophy

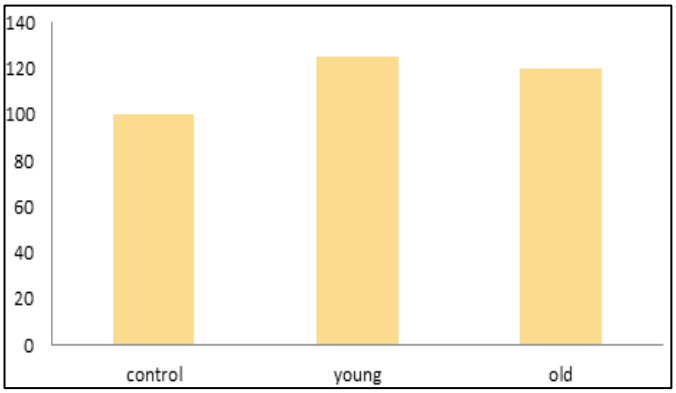

Fig 7: Increase in the total RNA content in soleus muscle after 7 days of hypertrophy

4. Discussion

Muscle hypertrophy involves an increase in size of skeletal muscle through an increase in the size of its component cells. A range of stimuli can increase the volume of muscle cells. These changes occur as an adaptive responsive to increase the ability to generate force skeletal muscle undergo rapid growth in response to sudden increase in workload which is induced by tenotomy of synergistic muscles, Goldberg 1978 [11], Gutmann Hnik *et al*., (1972). Which involve in enlargement of muscle fibers and longitudinal splitting, Goldberg (1972).

Muscle system in mammalian model composed of innervated twitch fibers which are of three types. Fast twitch glycolytic white, slow twitch oxidative red glycolytic fibers based on morphological structural, physiological and biochemical characteristics Close *et al*., (1972) [12]. Red fast muscles have high myoglobin content because of more blood supply compare to the other muscles, G.S. Herbison Barnuard (1979) [13].

It is relatively simple to achieve the muscle hypertrophy in man with training but it is much more difficult to induce in laboratory animals and more difficult in wild animals like squirrel. There are many methods of inducing hypertrophy in animals in experimental laboratory like by administrations of Vitamin C orally or by injecting anabolic androgenic steroids like testosterone, Gunderson *et al*., (1913). In the past year it has been observed that compensatory hypertrophy can be introduced by tentomising the synergistic muscle, Denny Brown (1960), Tomanck; woo (1970) [14], Goldberg A L *et al*.,(1980) [3], VK Reddy *et al*., (1985) [15].

The present project focus has been made on compensatory hypertrophy with reference to age. Several biologists worked on many factors such as age, nutrition, and sex which would affect the hypertrophy. It is observed that hypertrophy occurs at an increased rate in early age and stops at full growth in late teens. An adequate supply of amino acid is essential to produce muscle hypertrophy. As testosterone is one of the major growth hormone for an average man to get hypertrophy much easier. Taking anabolic steroid like testosterone will increase the hypertrophy, it is performance enhancing drugs and it is now a day's banned for competition in athletes and sports.

Compensatory hypertrophy with reference to age has been studied by many authors and it has been shown that prominent is hypertrophy is seen in younger animals than in aged ones. This is attributed to the fact that the growth hormone and its inactivation as age advances, Goldberg (1972) [6]. It is also shown that ageing can attenuate the hypertrophic response of muscle group to exercise training. The present study is of much importance in the light of recent studies made on mice, which says when exposed to performance enhancing drugs once in life time can be remembered by muscle when its exposure is withdrawn. This data suggest that exercising at a younger age induces a similar muscle memory that could help people stay fit with lesser exercise as they grow old and the ability to generate new myonuclei is impaired in adults when growth ceases, Gundergon Kristian (2013) [7].

In the present project interest is focused on the mechanism of induced hypertrophy and its variation in young and older animals. The biochemical protocol selected to study the mechanism mainly are energy generating components like total protein, structural and soluble proteins. Glycogen content, DNA, RNA activity which also is a indicator of protein synthesis during the hypertrophic changes. During the time course of hypertrophy the skeletal muscles remodel themselves to meet the challenge of increase functional load. There are several reports on time course and other factors affecting the type of muscle hypertrophy and in many experiments it has been shown that soleus muscle exhibit remarkable gain in muscle weight, Hnik *et al*., (1975,1986) [16,17] and the maximum hypertrophy is attained in seven day in soleus muscle, E. Mackova (1973, 1976). There is not much work done on wild animal like squirrel with respect to hyper trophy. The change response is due to many factors like difference in physiological, morphological and biochemical. This difference in response is seen in the present work is interpreted with possible reasons. This project main emphasis is on the effect of age on hypertrophy in a active animal like Squirrel. The difference may be due to position and usage of muscle fibers and is due to muscle protein fractions and blood supply, E. Mackova *et al*., (1973).

In spite of many school of thoughts little is clear about actual mechanism of this type of hypertrophy. The change in sarcoplasmic and contractile protein rises incorporation of amino acid into hypertrophying muscle has been increased, Goldspink (1983,1986) [18, 19] and protein synthesis in cell free system from hypertrophical muscles enhanced.

Protein metabolism in hypertrophying muscle is interesting subject, because it is speculated that increase in bulk during hypertrophy is due to increase in protein synthesis and decrease in protein catabolism. This type of adaptation is due to qualitative and quantitative change in muscle protein during hypertrophy in different muscles. The additional contractile protein appears to be incorporated into existing myofibrils. The hypertrophy results primarily from the growth of each muscle cell rather than increase in the number of cells. Skeletal muscle however is unique in having multiple nuclei to number of nuclei can increase during such activity. The soluble protein did not show much change which indicates that the mass increase of the muscle is due to only hypertrophy and due to addition of sarcoplasmic proteins which increased remarkably. There are number of reason to understand the factors that regulate rate of protein synthesis and degradation in young and old muscles we can possibly believe that there must be precise hormonal control other than the rate of hypertrophy which differs in different age group animals.

Glycogen metabolic reactibility of muscle to certain extrinsic factors finally reflects their trophic state. Any change in physiological state of muscles may be due to dramatic disturbance in their homeostasis. Consequently functional and structural alteration is due to varied reasons. It depends on energy levels which are derived from the various metabolic reactions. Exercise may cause rapid uptake of glucose by muscle, Goldspink (1985) [20] and exercise mimic the effect of insulin on the transport of glucose, Goldberg (1969) [21]. There are also reports of decrease activities of glycolytic and glycogreogenic enzymes during endurance training, Robert (1984) [22] and Rosenberg (1983) [23].

In the present study glycogen content increased both in young and old animals. Which shows that carbohydrate metabolism is almost similar to protein metabolism changes during the hypertrophy and its difference in young and old is similar. Muscles depend on muscle glycogen in emergency situations and it is a major source for glycolysis in the muscle. It is earlier reported that during compensatory hypertrophy increase in glycogen is predominant, Ianzzo and chen V, (1981) [24].

Increased glycogen reflects its increased energy usage during compensatory hypertrophy due to relative increase in the rate of muscle contraction. Its increased content also anticipates muscle dependency on carbohydrates during hypertrophy. The higher glycogen output is the direct result of increased glycogenesis probably with decreased glycogen catabolism and also depicts better trophic state of muscle during hypertrophy. The less increase in older animals is the result of low response to induced compensatory hypertrophy. Lower concentration of muscle glycogen in older animals is probably due to less activity in muscles, Clarl *et al*., (1983) [25].

DNA: The increase in DNA content in hypertrophied muscle indicates the greater muscles mass which needs to be utilized for increase protein synthesis. There is a relationship between DNA and protein synthesis, C J Brandl. S de Leon *et al*., (1987) [26]. The increase in the DNA content during functional overload with an increased in protein content during functional overload with an increased in protein content contribute to the hypertrophy response, possibly via the mobilization of satellite cells to provide increase in muscle DNA, Goldberg A L (1980) [3], Goldspink D F,(1985) [20].

Mechanism of work induced hypertrophy of skeletal muscles shows the significant increase in RNA and DNA synthesis linked to growth of muscles fibers Nuclear DNA synthesis increased observed by previous authors prove the fact that the increased protein synthesis cause DNA demand. The total RNA content and DNA demand. The total RNA content and DNA increases in hypertrophying muscles, S. C. Bodini, (2001) [27] due to increase oxidative capacity. My findings questions the ability of ground squirrel to respond to muscle loading challenges by increasing proteins, glycogen, DNA content and RNA content though the difference in age is predominantly noted in all parameters. The significant increase in RNA reveals the activity of muscle to enhance oxidative metabolism in the face of increased mechanical loading. The earlier authors claimed the increase in younger than older animals, M. Brown *et al*., (1992) [28] is due to the fact number of capillaries and blood circulation is more in younger than older animals and higher hormonal output seems to be also convincing reason in this project.

5. Conclusion

There is substantial evidence from various authors that activity levels decline with age. There is increasing in rodents and human that muscle mitochondrial function decreased. As seen by our study synthesis rates of several muscles proteins are responsive to both aerobic and resistance exercise programmes studies performed on rodents showed mitochondrial DNA numbers are lower in skeletal muscles in older rat that is, directly related to an oxidative capacity of the tissues.

The underlying mechanism of functional and structural changes in aging muscles are plenty in rodents looks to revolve around protein metabolism synthesis rate of sarcoplasmic proteins are relatively hyper in an older animal which suggest that the age related inhibition of muscle protein is not on all types of protein. But however, the reasons for the decrease in synthetic rates of certain proteins are to be determined. The interpretation of this individuals shows changes in muscle protein synthesis in response to physiological changes predicts in between protein synthesis and breakdown in older animals have a greater proportion of fibrous tissues in muscle mass and less metabolically active tissue with higher water content than do younger animals. These proteins results are further supplemented by increasing the rate of DNA and RNA concentration. Further regulation of transcription, translation of DNA and RNA is likely to provide the understanding of the molecular mechanism of aging muscle and also its memory protein during the process effects on the hypertrophy of young and old squirrels.

6. Acknowledgement

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