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BIOCHEMICAL CHANGES DURING REGENERATION OF TRANSPLANTED EXTENSOR DIGITORUM LONGUS MUSCLE IN SPRAGUE DAWLEY RATS*

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Abstract: Effect of transplantation on the DNA, RNA and protein contents of the regenerated Extensor Digitorum Longus (EDL) muscle in rats have been studied. The transplantation was found to cause a decrease, initially in the level of DNA, RNA, total protein, insoluble protein and soluble protein contents. Within 24 hours of transplantation, DNA decreased 33% and RNA 39%. Later on, with the initiation of regeneration, nucleic acids content started increasing and peak level of DNA was achieved on day 10 and RNA on day 15. Both these contents decreased afterward, but at the end of the experiment, these were still 2.34 x (DNA) and 1.48 x (RNA) more than that of control. The total protein content first showed decrease and then increase with the progress of regeneration. At the end of the transplantation it was only 34% lower than the control value. The insoluble protein content also followed the same pattern, so at the end of the experiment insoluble protein content was 32% less than that of control. The soluble protein content was comparatively stable component of the EDL muscle. Maximum decrease in soluble protein was obtained on day 15 of transplantation. While during the remaining days, the soluble protein content increased due to progressive regenerative activity.

Key words: Muscle transplantation, muscle nucleic acids content, muscle protein contents.

INTRODUCTION

It is well established that skeletal muscle can be grafted and the histological events of muscle transplantation conclude that muscle regeneration recapitulate the ontogenic events of normal muscle in that the regenerating myoblast arise from the original degenerating fibers. These myoblasts fuse with each other to form myotubes which then synthesizes myofibrils and become new muscle fibers (Carlson, 1978; Faulkner, *et al.*, 1989; Gill and Shakoori, 1995). A number of factors influence the success of muscle regeneration occurring within a graft. These include revascularization, hormones or growth factor, phagocytic activity and biochemical factors which support muscle regeneration.

The protein biosynthesis occurs more slowly in adult striated muscle as compared to secretory tissues like pancreas or liver. Early studies by Brachet (1941, 1950) and Davidson and Waymouth (1946) showed both the intensity of basophilia and the RNA content of muscle tissue are very low. The rate of protein biosynthesis as well as RNA content may vary considerably in different types of muscle cells, according to their specialized metabolic features and function. No systematic studies have so far been carried out to establish such a correlation. Indeed a few chemical analyses have been

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carried out on individual muscle, instead of groups of muscles. The values reported in the literature for the RNA content of muscle vary widely from author to author and according to the methods employed (Leslie, 1995; Muscatello, *et al.*, 1961; Perry and Zydowo, 1952; Schneider, 1945).

Winick and Noble (1965) for examining skeletal muscle growth frequently utilize DNA and protein/DNA as estimates of cell number and cell size, respectively. Because skeletal muscle is a multinucleated tissue, the DNA content (the number of nuclei) should not be equated with muscle cell number. Muscle fibers are multi nucleated, undergo structurally distinct growth in longitudinal and transverse dimensions appear capable of adding nuclei at any point along the fiber (Burleigh, 1974) and maintain the ability to add nuclei from satellite cells beyond the end of muscle growth (Young *et al.*, 1978). In addition to the rapid accumulation of nuclei during muscle regeneration, it will be demonstrated that changes in nuclear and sarcoplasmic basophilia precede the formation of structural proteins. These inter-related events offer favourable material for the study of cytological changes accompanying rapid protein synthesis. Results of a recent study, which examined the role of enzymes associated with glucose metabolism in regenerating free grafts of the extensor digitorum longus (EDL) muscle, were consistent with the interpretation that the grafts did not attain complete maturity, at least not in the biochemical sense (Wagner *et al.*, 1977; Schwartz *et al.*, 1985).

No quantitative data exist for the biochemical changes in free muscle grafts. So the objective of the present investigation was to determine the level of total muscle DNA, RNA and protein contents in regenerated EDL muscle following transplantation.

MATERIALS AND METHODS

The experiments were performed on male Sprague Dawley rats of 210-255 gms. The animals were kept in semi-controlled temperature conditions and were provided with tap water and commercially prepared food *ad libitum*.

Transplantation

The experiments were carried out under semi-sterile conditions. The EDL muscle was isolated from the anterior tibialis muscle and transplanted as described previously (Gill and Shakoori, 1995). The tendinous connections were securely stitched while the cut ends of the nerves and the blood vessels were left lying nearby. The operated legs were cleaned with 70% alcohol and acriflavin solution (5%) was applied over the wound to avoid infection.

After various time intervals, viz., 1, 3, 5, 7, 10, 15, 30 and 60 days, both right and left EDL muscles were dissected out and processed for biochemical analysis.

Biochemical analysis

For estimation of nucleic acids, the method described by Shakoori and Ahmad (1973) was adopted. DNA was extracted in 10% perchloric acid (PCA) at 65°C for 30 minutes, while RNA was extracted in 20% PCA at 4°C for 24 hours. The hot PCA

extract was used for the estimation of DNA by Diphenylamine method according to Schmidt and Thannhauser procedure as described by Schneider (1957), while cold PCA extract was used for the estimation of RNA by orcinol method. The standard curve of DNA was prepared from calf thymus gland, while that of RNA was prepared from pure yeast.

For estimation of protein contents, the weighed amount of muscle was crushed in 0.89% saline, centrifuged and supernatant was used for the estimation of soluble proteins according to Lowry *et al.* (1951). The pellet obtained above was digested in 6N NaOH at 36-40°C for 24 hours, heated at 45°C for ten minutes till muscle was dissolved completely. This was used for the estimation of insoluble protein content. For the estimation of total protein content muscle was dissolved in 5N NaOH. The O.D. obtained was caliberated against standard curve prepared from BSA.

RESULTS

Nucleic acids and protein contents

Figure 1 shows the changes in nucleic acids and protein contents. The control EDL muscle contained $0.64 \pm 0.03 \mu g$ DNA (n=5) and 1.15 ± 0.05 μg RNA/mg muscle weight (n=5). One day after transplantation, both the DNA and RNA content decreased 33% and 39%, respectively. While with the initiation of regeneration, nucleic acids content started increasing and maximum level of DNA was achieved on day 10 which was 4.65 x more than that of control, and that of RNA was 4.36 x, which was achieved on day 15 of transplantation. After attainment of peaks on the above days of observation, the nucleic acids content started decreasing. The values of DNA and RNA contents had decreased to 1.50 ± 0.03 μg (n=5) and 1.70 ± 0.08 μg/mg muscle weight (n=5), respectively, at the end of the experiment. These were still 2.34x (DNA) and 1.48x (RNA) higher when compared with the control levels. The RNA and DNA hold a ratio of 1.79 in the control muscle. After transplantation, the ratio between the RNA and DNA increased to 2.65 on day 7. While with the initiation of regeneration, ratio started decreasing and was 1.13 on day 60 of experiment (Fig. 2).

The total protein content of EDL muscle in control rat was 154.24 ± 2.72 μg/mg muscle weight (n=5). With the initiation of transplantation, total protein content started decreasing and continued to do so till day 10 when it was 60.92 ± 1.43 μg/mg muscle weight (n=5). During subsequent days of experiment *i.e.*, with the initiation of regenerative activity, total protein content started increasing and reached to 102.51 ± 2.81 μg/mg muscle weight (n=5) on day 60. The total protein content was 34% lower than the control value at the end of the transplantation period. The insoluble protein followed almost the same pattern. The insoluble protein component in control rat EDL muscle was 120.98 ± 2.96 μg/mg muscle weight (n=5) which decreased 61% on day 10 and 32% on day 60. The soluble protein was comparatively much stable component of the EDL muscle. The control EDL muscle had 33.34 ± 0.90 μg soluble protein/mg mucle weight (n=5). The soluble protein content showed maximum decrease of 67% on day 15 of transplantation. During the later days of the experiment, the soluble protein content increased, but these contents were still 39% less than the control values.

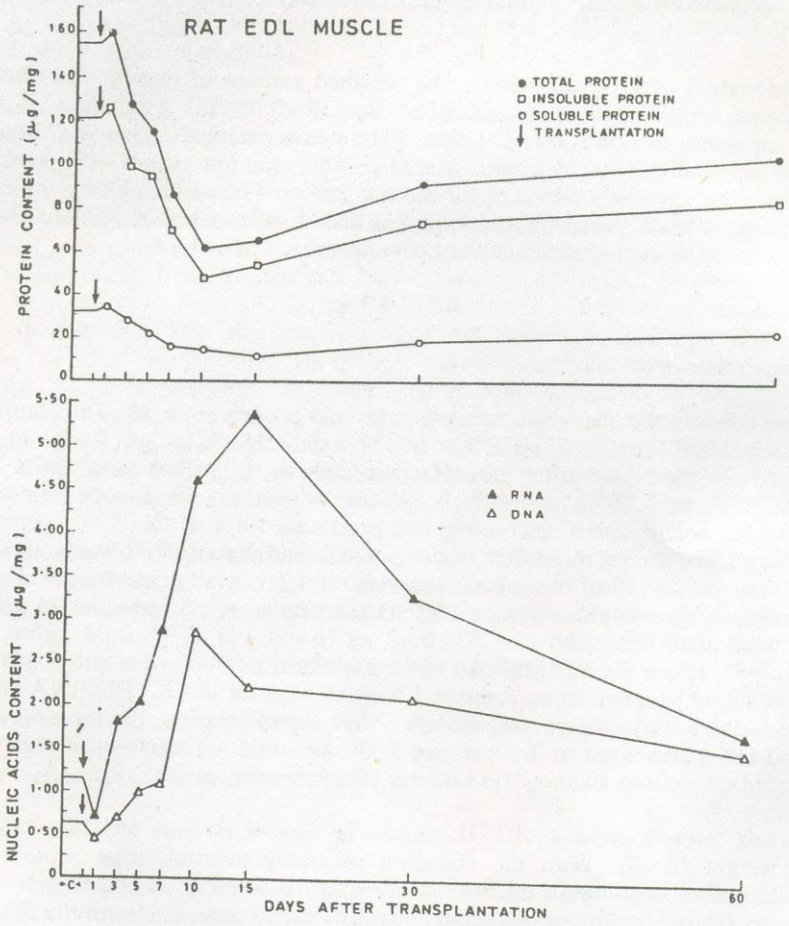

Fig. 1. Effect of transplantation on the nucleic acids and protein content of EDL muscle of rat.

The total protein hold a ratio of 4.62 with the soluble protein, the ratio reached to its minimum level on day 10, but then showed an increase afterward. The total protein and RNA content relationship showed some very distinct variations during the post operational period. This ratio in control was 134.12, which shot up to 226.57 within one day and then drastically reduced during the subsequent days (Fig. 2).

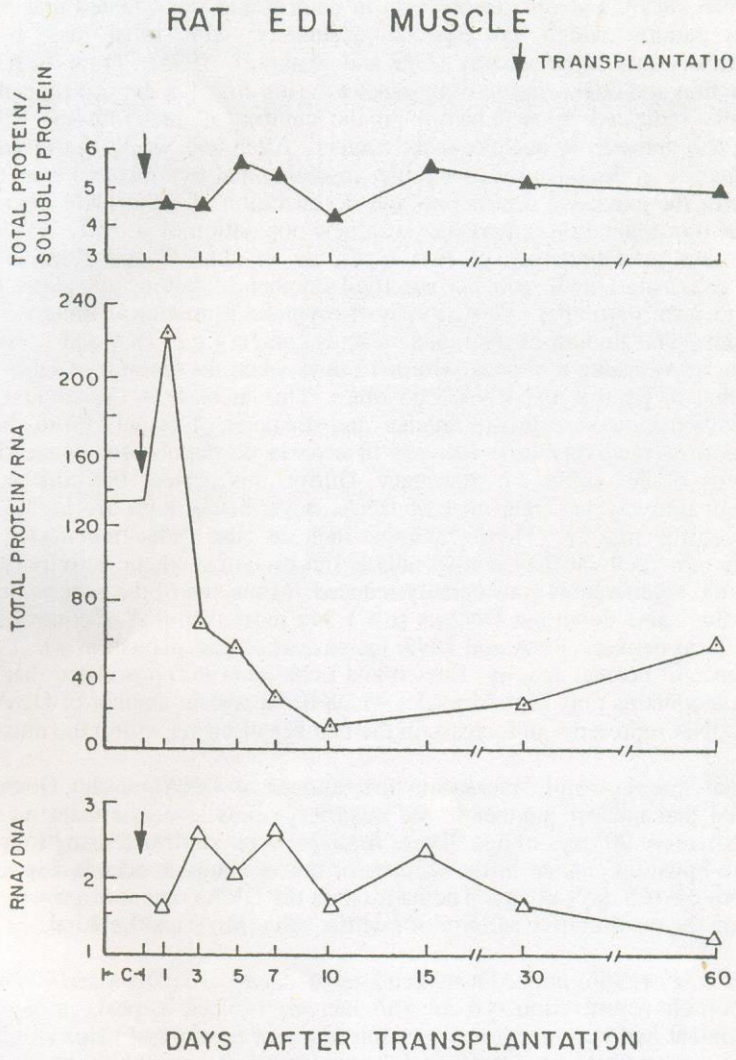

Fig. 2. Changes in ratios between RNA and DNA, total protein and RNA and total protein and soluble protein of rat EDL muscle after transplantation.

DISCUSSION

Transplantation produces alterations in morphological, functional and biochemical characteristic of muscle. This experiment shows that DNA, RNA and protein contents are more important determinants of the success of a free muscle graft. The observation

made on DNA, RNA and protein contents in control and transplanted muscle show a quantitative pattern which corresponds accurately with what has been seen morphometrically and histologically (Gill and Shakoori, 1995). There is observed a rapid destruction and degeneration of the nuclei during first 1-5 days of transplantation, which is duly indicated by a reduction in the number of nuclei/muscle fiber and a decrease in the amount of nucleic acids content. After one week of transplantation, there is reduction in the quantity of soluble, insoluble and total protein contents which coincides with the extensive destruction and degeneration of the muscle fibers. By the 7-15 days of transplantation, emergence of a new population of actively dividing cells. These cells are predominantly of two types, as myoblastic and fibroblastic. The myoblastic cells, after undergoing a specified number of mitotic divisions fuse with each other to form myotubes. This process of myotube formation is quite conspicuous by the 7th day. The amount of total nucleic acids content *i.e.*, DNA and RNA increase sharply from a low value to a peak within 15 days when the amount of DNA is 2.46x more than that of control and RNA 3.65 times. This increase in the amount of DNA coincides with the increase in the number and diameter of nuclei. From the 15 day onwards, there is relatively little increase in myoblastic population, connective tissue and tendinous tissue within the transplant. During this period, the concentration of RNA is comparatively less than that of DNA, nevertheless both are higher than the levels of control muscle. These findings indicate that older regenerate are still considerably more cellular than normal muscle but protein synthetic activity of the cells comprising the regenerate is considerably reduced. At the end of the experiment *i.e.*, 60 days, the value came down but DNA is still 1.34x more than that of control and RNA 0.47x. The ratio between RNA and DNA increase after transplantation which is due to the disturbance of normal activity. Enesco and Leblond (1962) postulated that a normal muscle tissue contains only diploid nuclei which has a costant amount of DNA and any increase in DNA represents an increase in the number of nuclei within the muscle.

In normal muscle, while measuring the amount of DNA/muscle, Gordon *et al.* (1966) found that nuclear number in the quadriceps muscle of the male rat increased until approximately 90 days of age. These findings were confirmed using four different muscles. No apparent change in the number of nuclei / muscle occurred in any of the muscles from 81-165 days of age. The increase in the DNA content of growing fibers is dependent on the proliferative activity of satellite cells (Moss and Leblond, 1970).

Gallucci *et al.* (1966) noticed a tremendous increase in the DNA and RNA contents of minced muscle regeneration system. This increase reached its peak on day 15, when the DNA content had become almost four times that of the control value and RNA 2½-4 time that of normal. At day 60, the value remained high but came down to three time that of normal value. Moss (1968) and Burleigh (1974) believe that postnatal growth is achieved both by nuclear (DNA) proliferation and increase in protein / DNA ratio which are measures of cell size.

Gorin *et al.* (1989) mentioned that the magnitude and time course of DNA synthesis can be compared with changes in RNA and protein content during regeneration. The DNA synthesis reflects satellite cell proliferation and was maximal on postgraft day 5. As a result of which significant changes in total RNA content, mRNA content and muscle protein content during later regenerative times. Martin *et al.* (1990) suggested

that recovery of up to 1 year was insufficient for the normalization of several connective tissue matrix components and biochemical properties of the grafts.

In the present study, muscle regeneration after transplantation is associated with marked decrements in protein synthesis. The total protein content decreased to a considerable extent after transplantation which coincide with the destruction and degeneration of the muscle fibers. At the end of the experiment, the total protein content is 34% lower than the control value. The insoluble protein content also followed the same pattern and is 32% less than that of the control. The soluble protein content is stable component of the EDL muscle, so after transplantation maximum loss is obtained on day 15 whereas that of total and insoluble protein content is obtained on day 10. The ratio between the total protein and soluble protein and total protein and RNA is also disturbed after transplantation. The ratio between the total protein and soluble protein reached its minimum level on day 10 which is due to distinct behaviour of the soluble protein after transplantation. The total protein and RNA ratio shoots up within 24 hours of transplantation, this abrupt change is due to disturbance of normal mitotic activity. The ratio reached its minimum level afterward which is due to regeneration of degenerated fibers.

Kelly *et al.* (1984) while working on pre- and post- natal growth and protein turnover in skeletal muscle of rat postulated that the fractional rates of protein synthesis (measured *in vivo*) and break down in each muscle declined with age, the change in the former correlates with decrease in the ribosomal capacity of the muscles. Wagner *et al.* (1977) and Schwartz *et al.* (1985) mentioned that the EDL muscle grafts did not attain complete maturity at least in the biochemical sense. It is well known that any treatment or disturbance effect the normal activity of the muscle. Howard *et al.* (1989) and Steffen *et al.* (1990) observed alteration in the pool of mRNAs in immature muscles within hours of the initiation of hind limb suspension. Changes in the levels of protein synthesis and degradation (Goldspink *et al.*, 1986; Loughna *et al.*, 1986; Loughna *et al.*, 1987; Thomason and Booth, 1990) occur within the first 3 days of hind limb unweighting.

It is concluded from all the above mentioned information that it is more important to determine the level of total muscle DNA, RNA and protein contents in regenerated EDL muscle after transplantation. All these information support the idea of regenerative capability of muscles.

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