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EFFECTS OF ANDROGENS ON NUCLEIC ACIDS AND PROTEIN CONTENTS IN REGENERATING EXTENSOR DIGITORUM LONGUS MUSCLE FOLLOWING ORTHOTOPIC TRANSPLANTATION IN RATS*

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Abstract: Effects of testosterone, on regeneration of muscle fibres within rat Extensor digitorum longus (EDL) muscle grafts were studied under various experimental conditions. It was discovered that the muscle grafts reacted negatively to lack of androgens. The RNA and total protein contents of the grafts in the gonadectomized rats were less than those found in controls. EDL muscle grafts in testosterone propionate replaced rats developed much better than those in the androgen deprived ones. These results suggest that mammalian skeletal muscle is, in general, sensitive to the presence or absence of androgens while regenerating following free transplantation.

Key words: Gonadectomized, testosterone propionate, muscle grafting.

INTRODUCTION

Free skeletal muscle transplantation has, extensively, been studied in various animals (Mufti *et al.*, 1977; Grim *et al.*, 1986; Roberts *et al.*, 1989; Qazi and Mufti, 1990) and used therapeutically in a number of cases involving different muscles (Hakelius, 1974a,b; Grotting *et al.*, 1990; Chuang *et al.*, 1994). Process of skeletal muscle fibre regeneration following the grafting, in general, enables the graft to achieve about 50% of its original structural and functional characteristics (Wagner *et al.*, 1977; Faulkner and Côte, 1986; Ontell, 1986). Various parameters have been elaborated to determine the success of a free muscle transplant, viz mass, number and diameter of regenerated muscle fibres, enzyme profiles and physiological characteristics (Faulkner *et al.*, 1981; Côte and Faulkner, 1984; Faulkner and Côte, 1986; Ontell, 1986; Carlson, 1988).

Among these measures, DNA, RNA and total protein contents of regenerating skeletal muscles have received practically little attention and only a few studies can be enlisted in this regard (Jones, 1983; Friedman *et al.*, 1986). However, recently Gill and

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Shakoori (1995) have reported that these biochemical parameters are more important determinants of the success of a free muscle graft.

Owing to the fact that a freely transplanted muscle regenerate remains deficient in its various characteristics, efforts have been made to improve quality of the regenerate through different postgrafting interventions (White *et al.*, 1981; Mufti and McNemar, 1986; Weiss and Oron, 1992; Bischoff and Heintz, 1994). In this connection Qazi and Mufti (1989, 1990) have initiated studies pertaining to the influence of some anabolic hormones on regeneration of skeletal muscle fibres following the grafting.

Effects of anabolic hormones on growth and maturation of skeletal muscles are well known (Gustafson *et al.*, 1986; Bates and Holder, 1988; Boissonneault *et al.*, 1989; Balon *et al.*, 1990). And among these hormones testosterone has been most thoroughly studied and discovered to be highly myotropic (Kumar *et al.*, 1981; Kuhn and Max, 1985). Griffin and Wilson (1985) have described that a major component of androgen-induced weight gain and nitrogen retention in hypogonadal man is an increase in skeletal and muscle mass. Application of this hormone results in an increase in the protein synthesis as well as a decrease in the protein degradation within the skeletal muscle (Goldberg *et al.*, 1975; Crist *et al.*, 1983). Testosterone and its derivatives have been applied clinically for a number of years as general anti-catabolic agents to counter negative nitrogen balance *e.g.*, during muscle wastage (Rothstein and Rose, 1982). Levator Ani (LA) has long been considered an index of the myotrophic activity of androgenic and/or anabolic hormones (Eisenberg and Gordon, 1950). A greater concentration of androgen receptors in the LA than other skeletal muscles (Rance and Max, 1984) could contribute to the importance of the androgenic response of the muscle. Rand and Breedlove (1992) have found that androgen exerts its anabolic effect by acting locally upon a cell population within or near the bulbocavernosus and levator ani muscles. LA has also been shown to retain its androgen sensitivity during its regeneration (Carlson *et al.*, 1979; Max *et al.*, 1981; Mufti, 1985).

Apart from effects of androgens on skeletal muscles, in general, and on regenerating LA muscle, there is little information, about their role on regenerating muscles which are not known specifically sensitive to these substances. The present work was, therefore, intended to study the effects of androgens on regenerating extensor digitorum longus muscle and this paper reports response of the muscle transplants in terms of DNA, RNA and total protein contents in gonadectomized and testosterone propionate replaced rats. This information is helpful in improving the regeneration of free skeletal muscle grafts in hypogonadal patients.

MATERIALS AND METHODS

Animals

Adult male rats (*Rattus norvegicus*, Sprague-Dawley strain), weighing 100-300 g were obtained from National Institute of Health (Islamabad) and kept in standard animal room facilities with roughly 12 hours dark/light cycle. They were fed commercially prepared food and given a constant supply of drinking water. The food contained 1)

poultry feed, 5 kg; 2) fish meal, 1 kg; 3) wheat flour, 2 kg; 4) molasses, 100 g; 5) water, 3 lit.

Surgical procedure

All surgeries were performed under disinfected conditions. The rats were anaesthetized with ether both at the time of transplantation and while recovering the grafts. The operated animals were supplied with 0.06% terramycin in their drinking water for 3-4 days postoperative. The operated animals were kept in cages and fed routinely.

Gonadectomy

Male rats were gonadectomized 4-days prior to the muscle transplantation, so as to eliminate residual testosterone within the blood. In this process the ventral aspect of scrotum of the anaesthetized animals was first shaven clean and wiped with 70% alcohol. A median incision was given along the raphe seen on ventral side of the scrotum. The internal fascia were then cut through to reach the testes which were then pulled out of tunica vaginalis, along with whole of epididymis and part of vas deferens. Since all this area is highly vascularized, a tight knot was applied proximal to the level at which the epididymis and vas deferens were severed, so as to avoid excessive bleeding during removal of the testis. Tunica vaginalis was sutured close and then various fascial sheaths were sutured with 6-0 silk and finally the two cut ends of the skin were sutured with 4-0 silk. The sutured area was wiped again with 70% alcohol.

Transplantation of extensor digitorum longus (EDL) muscle

This muscle was orthotopically transplanted, both in control and experimental animals. All experimental rats were castrated as described above. The EDL muscle was isolated from its surrounding associations such as neural and vascular connections and its proximal and distal tendons were cut leaving a small tendinous stump behind. The muscle was taken out, weighed and then grafted back in its original bed in proper orientation. Both proximal and distal tendons were sutured with respective stumps with a 6-O silk. This was followed by suturing the fascia, again by 6-O silk and finally the two cut ends of the skin were carefully and closely sutured with 4-O silk.

At each prescribed interval, ranging from 1 to 4 weeks postgrafting, the grafts were isolated, removed, weighed and processed for the biochemical analyses.

Effects of androgens

The EDL muscle grafts in gonadectomized (G) rats were compared with those of uncastrated control rats (C). Testosterone propionate anhydrous (Sigma) was dissolved in such a way that 0.1 ml of corn oil contained 0.1 and 1.0 mg of it. The drug was administered intra-peritoneally, each day starting from the day of muscle transplantation in orchidectomized rats. Following orthotopic transplantation of both EDL muscles, two groups of the rats received 0.2 mg/100 g body wt. (GTP-I) and 2.0 mg/100 g body wt.

(GTP-II) doses of testosterone propionate. EDL muscle grafts in vehicle injected gonadectomized rats (GVC) served controls for this series of experiments.

Biochemical analyses

Total protein contents of the EDL muscle were assessed by the methods of Lowry *et al.* (1951) as modified by Miller (1959) and Schacterle and Pollack (1973). DNA and RNA contents were estimated by methods of Wannemacher *et al.* (1965), Shibko *et al.* (1967) and Munro and Fleck (1969) as modified by Lone and Matty (1980). Purified calf thymus DNA, calf liver RNA and bovine serum albumin were used to prepare standard curves.

Statistical analyses

The mean values of experimental EDL muscle grafts were compared to respective controls by applying Student's "t" test and single-factor analysis of variance. Following the analysis of variance the detailed comparisons were made according to Campbell (1989). Standard curves were drawn after applying regression equations.

RESULTS

Weight of animals and EDL muscle grafts

No change was observed in pattern of body weight gain between control and gonadectomized rats and at end of the experiment the animals showed 17.45 and 17.97% gain in body weights, respectively. Concerning the weight of EDL muscle grafts a significant decrease in the gonadectomized rats was found in 3 week old grafts only. Percent recovery in weights of the regenerates (mg/100 g body wt.) at this stage was turned out as 48.21 ± 2.17 and 32.24 ± 4.72 for the control and gonadectomized rats, respectively.

In case of testosterone propionate replaced rats there had been no significant difference in % gain in body weights from that of GVC rats except at 3 week postgraft period where 1.17% weight loss was observed for GTP-II animals, while the GVC rats attain 8.13% gain in their weights. At 2-week stage % recovery in weights of the muscle regenerates was found as 40.67 ± 3.45, 44.38 ± 3.36 and 57.28 ± 2.77 for GTP-I, GTP-II and GVC rats, respectively, and the values for the testosterone propionate replaced series of rats are significantly less than that of GVC rats. However, values of the percent recoveries of 3-week GTP-I and 4-week GTP-II grafts were found significantly higher from respective figures of the GVC grafts. GVC grafts of 3- and 4-weeks could attain 33.04 ± 2.32 and 32.15 ± 3.46 % recoveries respectively while the figures turned out to be 43.04 (± 2.84) and 42.29 (± 2.25) for GTP-I and GTP-II grafts representing 3- and 4-week stages, respectively.

Nucleic acids and total protein contents

The average values of DNA, RNA and total protein contents within the control and gonadectomized grafts are shown in Table I. In first week, the DNA contents showed a significant decrease within the G grafts, as compared with the control grafts. Thereafter, from the 2nd week onwards there were no significant differences in the DNA contents of the experimental and control grafts (Table I). The EDL muscle transplants of G rats showed significantly less RNA contents than the respective control values throughout the study period. The total protein contents of the EDL grafts from the G rats also remained significantly lower than the values for the control regenerates (Table I).

Table I: **DNA, RNA (μg/100 mg of tissue) and total protein contents (mg/100 mg of tissue) of the EDL muscle grafts in the control and gonadectomized rats.**

Type of Graft	STAGES OF REGENERATION (Weeks)			
	1	2	3	4
Control-DNA	193.95[a] ±26.04 (3)	205.52 ±25.23 (4)	159.77 ±28.12 (3)	124.40 ±16.04 (3)
Gonadectomized-DNA	86.40* ±5.07 (3)	170.33 ±29.48 (3)	175.91 ±17.63 (3)	172.42 ±20.26 (4)
Control-RNA	1031.07 ±137.76 (3)	1568.62 ±99.71 (4)	1378.24 ±136.76 (3)	1255.41 ±104.72 (4)
Gonadectomized-RNA	545.51* ±56.37 (3)	1073.31* ±101.64 (3)	665.91** ±36.85 (3)	857.80* ±106.91 (4)
Control-protein	10.57 ±0.39 (3)	11.92 ±1.29 (4)	14.99 ±0.63 (3)	13.68 ±0.69 (4)
Gonadectomized-protein	8.52* ±0.26 (3)	7.24* ±0.96 (3)	7.31*** ±0.44 (4)	8.24** ±1.22 (4)

[a]Mean ± SEM; asterisks show significant difference. *=P<0.05; **=P<0.01; ***=P<0.001. Number in parenthesis indicates sample size (Student's 't' test).

Table II: **DNA, RNA (μg/100 mg of tissue) and total protein contents (mg/100 mg of tissue) of the EDL muscle grafts in the GVC, GTP-I and GTP-II rats.**

Type of Graft	STAGES OF REGENERATION (Weeks)			
	1	2	3	4
GVC/DNA	161.75[a] ±12.57 (4)	141.95 ±2.66 (3)	154.79 ±10.76 (3)	181.74 ±11.29 (3)
GTP-I/DNA	168.64 ±15.44 (3)	138.25 ±3.34 (3)	161.39 ±5.13 (3)	177.61 ±10.50 (3)
GTP-II/DNA	130.36 ±12.22 (5)	186.60**,†† ±9.46 (3)	160.63 ±8.08 (4)	117.55**,†† ±7.61 (4)
GVC/RNA	801.31 ±128.71 (3)	983.11 ±85.31 (3)	785.98 ±53.35 (3)	1046.00 ±95.92 (3)
GTP-I/RNA	568.10 ±73.16 (3)	1070.86 ±183.41 (3)	847.00 ±37.51 (4)	1415.96* ±101.80 (4)
GTP-II/RNA	571.66 ±35.59 (4)	1010.24 ±99.37 (5)	859.66 ±32.41 (4)	1446.33* ±110.00 (4)
GVC/protein	7.64 ±0.46 (4)	8.24 ±0.34 (3)	8.14 ±0.38 (4)	5.85 ±0.29 (3)
GTP-I/protein	6.18 ±0.41 (3)	8.57 ±0.64 (4)	8.73 ±0.48 (4)	14.37*** ±0.86 (4)
GTP-II/protein	6.77 ±0.45 (4)	7.97 ±0.27 (5)	8.03 ±0.71 (4)	10.28**,†† ±0.99 (4)

[a]Mean ± SEM; asterisks are significantly different from respective controls. Significant differences between values of GTP-I and GTP-II grafts are indicated by clubs: *=P<0.05; ††**=P<0.01; ***=P<0.001. Number in parenthesis indicates the sample size.

GVC= gonadectomized plus vehicle injected rats; GTP= gonadectomized and testosterone propionate administered rats.

DNA contents of GTP-I transplants remained statistically unaltered throughout the study period. However, 2-week old EDL muscle transplants in GTP-II rats showed a significant increase followed by a decrease at 4-week stage (Table II). A significant

elevation of about 35% and 38% in RNA contents was observed in GTP-I and GTP-II regenerates, respectively at 4-week stage (Table II). For remaining periods of the study there was no change between the RNA contents of GVC, GTP-I and GTP-II grafts. Regarding total protein contents the data resembles closely with RNA distribution-pattern. The protein contents remained, statistically speaking, unaltered in three types of the transplants for first three sample periods. At 4-week stage the hormone-treated EDL muscle grafts did show significant increase of about 146% and 75% for GTP-I and GTP-II types, respectively in their total protein contents compared to the androgen deprived grafts. The 4-week old GTP-I grafts also contained significantly higher amounts of total protein contents than the GTP-II transplants (Table II).

DISCUSSION

At the end of experimental period, lower % recovery in weight of muscle regenerates in gonadectomized and the higher values in case of testosterone propionate replaced rats as compared to uncastracted and androgen-deprived controls, respectively, indicated that the EDL muscle do respond to presence/ absence of the hormone while regenerating following orthotopic transplantation. The biochemical analyses of these regenerates confirmed this notion. In the absence of androgen the muscle grafts failed to develop the biochemical determinants to the level attained by transplants in uncastrated rats. Significantly lower DNA contents in the androgen-deprived grafts in the initial stage of regeneration is an indicative of lower proliferative activity of satellite cells. As satellite cells are the source of myoblastic cells that give rise to the formation of regenerated muscle fibres within an injured muscle (Snow, 1977a,b; Carlson and Faulkner, 1983; Schultz *et al.*, 1986; Roberts *et al.*, 1989; Schultz, 1989) and their proliferation has been reported to cause DNA increase in growing rats' muscles (Moss and Leblond, 1971; Beermann *et al.*, 1983). Situations of other parameters had not been different from the DNA pattern. These biochemical results support earlier histological and morphometric reports indicating that in the absence of androgens orthotopically transplanted EDL muscle grafts undergo considerable atrophy (Mufti and Qazi, 1987; Qazi and Mufti, 1989).

On the other hand, EDL muscle grafts, in the testosterone propionate replaced rats developed better than the androgen-deprived control animals (GVC), as envisaged by their significantly higher RNA and total protein contents at 4-week stage. Reduced amount of DNA contents in 4-week GTP-II grafts is attributable to the hypertrophied nature of the regenerated muscle fibres in these regenerates (Qazi, 1995), since less number of myonuclei per unit fresh weight of the tissue were sampled. Decline in DNA concentration in growing muscle has been shown to corresponds with the initiation of fibre hypertrophy (Wigmore and Stickland, 1983).

Previously only a select group of skeletal muscles was considered sensitive to androgens; during regeneration and development. The levator ani muscle in rats was an example of such muscles which retained its sensitivity to androgens while undergoing regeneration following various kinds of trauma such as mincing (Carlson *et al.*, 1979). Max *et al.* (1981) have shown that by day 3 in regenerating rat levator ani muscle, after a crush lesion, androgen binding decreased to 25% of control values. This decrease was

followed by a 4-5 times increase in hormone binding, which attained control values by day 7 after crush. These workers have concluded that synthesis of the androgen receptors may occur after the fusion of myoblasts and during the differentiation of myotubes into cross-striated muscle fibres. Later on, Mufti (1985) also showed that formation of androgen receptor ensues within 2-3 days post-crush in the regenerating myoblasts of rat levator ani muscle. The ligand-receptor complexes were found both within the nuclei and the sarcoplasm indicating their possible role in regulating nuclear activity. In conclusion, this information is new, in the sense that EDL muscle which is a fast contracting muscle, not known to be specifically sensitive to androgens, is seen to respond negatively to the absence of testosterone and shows a positive response when supplied with exogenous testosterone propionate, while regenerating following the transplantation. These observations suggest that most of the skeletal muscle in mammals may be sensitive to androgens rather than a few, considered previously. Many more muscles, especially different kinds of muscles, slow and fast, will have to be tested before to generalize the statement.

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