Regular Paper

EFFECT OF TESTOSTERONE ON SKELETAL MUSCLE OF YOUNG AND OLD MALE RATS

Ricardo Noboro Isayama[1], Jorge Oishi[2], Valéria Helena Alves Cagnon[1] and Gerson Eduardo Rocha Campos[1]

[1]Department of Anatomy, Institute of Biology, State University of Campinas (UNICAMP), Campinas, SP, and
[2]Department of Statistics, Federal University of São Carlos (UFSCar), São Carlos, SP, Brazil.

ABSTRACT

The effects of testosterone on skeletal muscle were assessed in adult male *Wistar* rats aged 80 days and 1 year. The animals were divided into 4 groups: young testosterone (YT), old testosterone (OT), young control (YC), and old control (OC) groups. The YT and OT groups received 15 applications of testosterone cypionate (5 mg/kg) on alternate days and the controls received injections containing sterile oil alone. After 30 days the animals were sacrificed and the soleus (SOL) and extensor digitorum longus (EDL) muscles were analyzed using mATPase histochemistry. After treatment, YT group gained less body weight than YC group and OT group decreased body weight, differently from the body weight gain observed in the OC group. Testosterone treatment did not show significant changes in both relative muscle weight and muscle fiber composition profile. However, in the YT group we observed an increase in the cross-sectional area of type I fibers in the SOL muscle, and type I and IIAD fibers in the EDL muscle. In the OT group, the cross sectional area of type I was decreased in the EDL muscle. These results reveal that testosterone did not cause a shift in muscle fiber type, but the cross-sectional area had fiber type-specific changes.

Key words: EDL, fiber type, mATPase, soleus

INTRODUCTION

Skeletal muscle consists of various fiber types with different metabolic and contractile properties. Myosin is one of the main muscle proteins and its isoform expression pattern is used to classify the various fiber types. The slow isoform is known as myosin heavy chain I (MHCI) and the fast ones are known as MHCIIa, MHCIId and MHCIIb. Some fibers are called hybrid, which express two or more myosin isoforms: IC, IIC, IIAC, IIAD, IIDA, IIDB and IIBD. This diversity permits the muscle to adjust continuously to functional needs, conferring considerable plasticity to it [36-38,44,45]. Several factors such as physical exercise, electrical stimulation, denervation and reinnervation, aging, and hormones can affect the phenotype of the skeletal muscle of mammals [3,36,38,44].

In males, testosterone is produced by Leydig cells of the testes (95%) and by the cortex of the adrenal gland (5%). Testosterone has an anabolic effect and stimulates the growth of muscle [48]. Several studies have confirmed that testosterone increases muscle mass [20], strength and endurance [48], a fact that has led elite athletes to use it in their physical training regimens, although in a questionable manner [21]. Other studies have reported even more specific effects of testosterone in skeletal muscle [10,11,32,33,42]. The levator ani muscle, for example, disappears during the development of female rats, but it can be maintained with testosterone´s administration [18]. The expression of the tonic isoform of myosin (MHCton) in the laryngeal musculature of frogs is controlled by androgenic factors [10]. Testosterone also causes a reduction in the percentage of type I fibers in the gastrocnemius, *extensor digitorum longus* (EDL) and soleus (SOL) muscles of female rats [22]. In female rabbits, this hormone causes fiber hypertrophy and leads to a decreased oxidative metabolism in the *tibialis anterioris* muscle, but not

Correspondence to: Ricardo Noboro Isayama, MSc.
Departamento de Anatomia, Instituto de Biologia, CP 6109, Universidade Estadual de Campinas (UNICAMP), CEP: 13083-970, Campinas, SP, Brazil. Tel: (55) (19) 3788 - 6104. E-mail: noboroanato@ufrj.br / ricardonoboro@hotmail.com

in the EDL, *plantaris* or SOL muscles [41]. The emphasis on gender is due to the fact that males are not commonly used in experiments involving testosterone, or the effects are absent. According to Noirez and Ferry [34], anabolic/androgenic steroid treatment in young male rats did not alter myosin heavy chain expression in hindlimb muscles.

Aging is another factor related to changes in the skeletal muscle system [2,8,14,25,26]. Normally, aging is accompanied by a marked loss of muscle mass, reduction of muscle strength and increased muscle fatigability [30], which are events clinically known as sarcopenia [31]. The apparent increase in the number of slow-twitch fibers observed in aging humans is due to a reduction in the number of fast-twitch fibers [1,37]. Testosterone, considered to be a powerful anabolic agent, has been administered to elderly individuals to block or retard the muscle loss due to sarcopenia [4,31,43].

In the present study we investigated the effects of testosterone administration on fiber type composition and morphometry in the SOL and EDL muscles of young and old male rats. The aim was to determine whether treatment with high doses of this hormone would increase the percentage of fast-twitch fibers, as reported in sexually [16,37] and non-sexually dimorphic muscles [22,41], and whether this treatment would reverse the aparent fast-to-slow fiber type transition observed in elderly individuals.

MATERIALS AND METHODS

Animals and treatment

Wistar rats aged 80 days or 1 year old were obtained from the Central Animal House at UNICAMP. Animal handling and manipulation were approved by the Ethics Committee of UNICAMP. The animals were divided into 4 groups: young testosterone (YT, n=6), old testosterone (OT, n=6), young control (YC, n=4) and old control (OC, n=4). The YT and OT groups received 15 subcutaneous injections of testosterone cypionate (Deposteron, Novaquímica, 5 mg/kg) on alternate days, whereas the control groups received only sterile peanut oil as the vehicle. The animals were weighed before each injection and kept in a controlled 12h dark/light cycle and temperature between 18- 22ºC, with food and water *ad libitum*.

Surgical procedures

At the end of treatment the animals were anesthetized intraperitoneally with 10% chloral hydrate (2.5 ml/kg), their SOL and EDL muscles were removed, weighed and the animals were sacrificed with an overdose of the anesthetic.

Determination of fiber type

The middle portion of the muscle was separated, oriented in a mixture of gum tragacanth (Sigma-G1128) and TBS tissue freezing medium (Triangle Biomedical Sciences), immediately frozen in isopentane cooled to –156ºC in liquid nitrogen, and stored at -70ºC until ready to use. Transverse 12 μm sections were obtained in a cryostat, collected on coverslips and stored frozen at –40ºC until all samples were processed.

Fiber type and subtype were identified using the histochemical myofibrillar adenosine triphosphatase (mATPase) technique [45] after preincubation at pH 4.3, 4.5 [7] and 10.3 [17] (Fig. 1). The muscle section obtained at pH 4.5 was photographed in its entirety and mounted photographically as a plate. This plate was used as a guide to identify all the different fiber types (I, IC, IIC, IIA, IIAD, IID, IIDB and IIB) from sections obtained at pHs 4.3, 4.5 and 10.3 in two random fields for each animal. For the morphometric analysis, the cross-sectional areas of up to 50 fibers of each type per sample were calculated using the Image-Pro software (version 4.0).

Statistical analysis

Data were analyzed statistically by ANOVA and by the Kruskal-Wallis test for group comparison and by the Duncan post hoc test, with the level of significance set at 5% ($p < 0.05$). Data concerning muscle fiber subtypes (IC, IIC, IIAD and IIDB) were submitted to statistical analysis only when these subtypes were present in all animals.

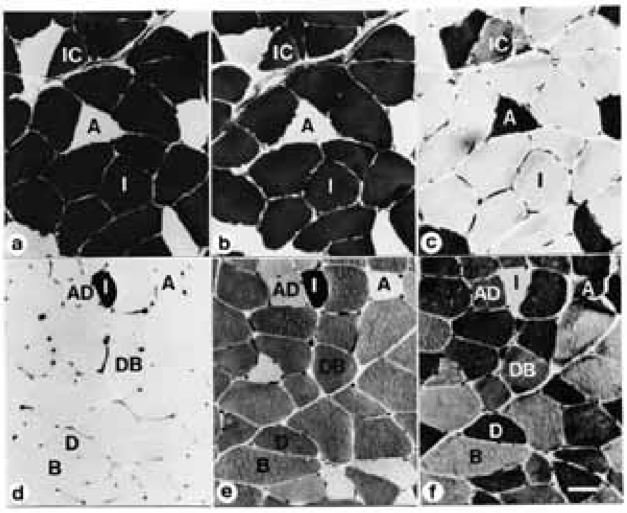

Figure 1. Histochemical mATPase reaction at pH 4.3 (a, d), 4.5 (b, e), and 10.3 (c, f) in serial sections of the SOL (a, b, c) and EDL (d, e, f) muscles of *Wistar* rat. I – type I fiber, IC –IC type, A – IIA type, AD – IIAD type, D – IID type, DB - IIDB type, and B – IIB type. 50 μm.

RESULTS

Animal and muscle weight

Old rats were significantly heavier than young ones at the beginning of treatment, i.e., 468.7±29.2g (n=10) and 302.1±22.5g (n=10), respectively. After the experimental period, the YT group presented a lower body weight gain (p=0.006) compared to its control (Fig. 2). In contrast, the OT group presented a significant fall in body weight (p=0.035) compared to OC group (Fig. 2).

Absolute muscle weight values were significantly higher in OC group than in YC group, i.e., 170±21mg and 132±5mg for the SOL muscle and 222±10mg and 147±9mg for the EDL muscle, respectively. Relative muscle weight (mg/g) was calculated to account for differences in body weight and we found no statistical difference between all the groups (Table 1).

Muscle fiber type

The muscle fiber types (I, IC, IIC, IIA, IIAD, IID, IIDB and IIB) were determined (Fig. 1) and their percentages obtained by counting the number of fibers. Electrophoresis was also performed to ratify distinct phenotypes between type IIAD and IIC fibers content (data not shown). As expected, type I fibers comprised more than 95% of the total number of fibers in the SOL muscle. Fiber types IC, IIC, IIA and IIAD were identified in the SOL muscle, but type IIAD was observed only in YT and OT groups representing 0.34% and 0.21%, respectively (Fig. 3A).

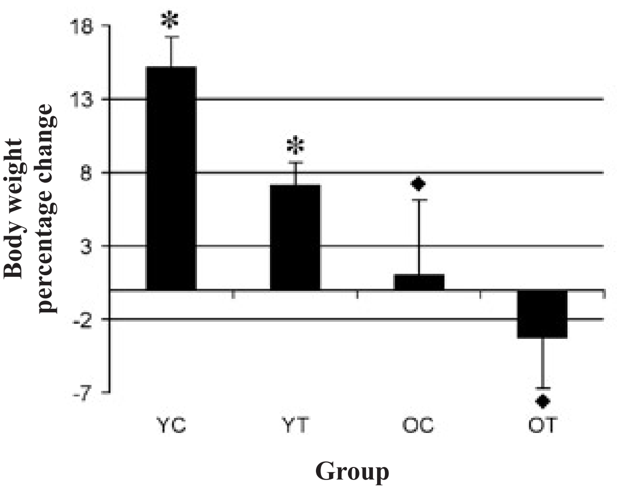

Figure 2. Body weight changes in the YC, YT, OC and OT groups. Values are reported as mean ± SD. * p<0.05 for young groups. ◆ p<0.05 for old groups.

As expected, all fiber types could be found in the EDL muscle, and their representative percentages are shown in figure 3B. There was no statistically significant shift in muscle fiber types between control and testosterone treated animals at any age group (Fig. 3B).

When the control groups (YC and OC) were compared, there was no significant difference in the fiber type composition profile in the SOL muscle, suggesting lack of an age-dependent effect. However, in the EDL muscle, the OC group presented a higher (p=0.022) percentage of type IIA fibers compared to YC group (Fig. 3B).

Morphometry of muscle fibers

In the SOL muscle, the cross-sectional area of type I fibers was larger in YT group (p=0.00001) than in the YC group (Table 2). This increase was not observed between OT and OC groups (Table 2).

In the EDL muscle, the area of type I and IIAD fibers in YT group was larger compared to YC group, p=0.023 and p=0.00008, respectively (Table 3). Type I fibers in the EDL muscle of OT group showed a smaller cross-sectional area (p=0.002) compared to its control (Table 3).

DISCUSSION

Animal weight

In contrast to what is observed in young/adult females [5,13,15,22,28,39], some authors have reported inhibition of growth in young male rats treated with an anabolic steroid [5,19,24,39]. This growth inhibition occurs when male animals are treated with high doses of the anabolic agent, which does not occur at low doses [5]. The reduction in body weight values may be accompanied by a reduction in adipose tissue volume, a fact more commonly observed in old than in young rats, because the latter display smaller amounts of fat [9]. The present data demonstrate that high doses of testosterone not only reduce the growth of young rats, but also reduce the weight of old rats. Reduced food ingestion due to decreased appetite [24] and increased fat metabolism [4,43] induced by testosterone are possible mechanisms related to the inhibition of growth and to the reduction in body weight.

Muscle weight

Testosterone effects on muscle weight are not clearly understood. In female rats testosterone treat-

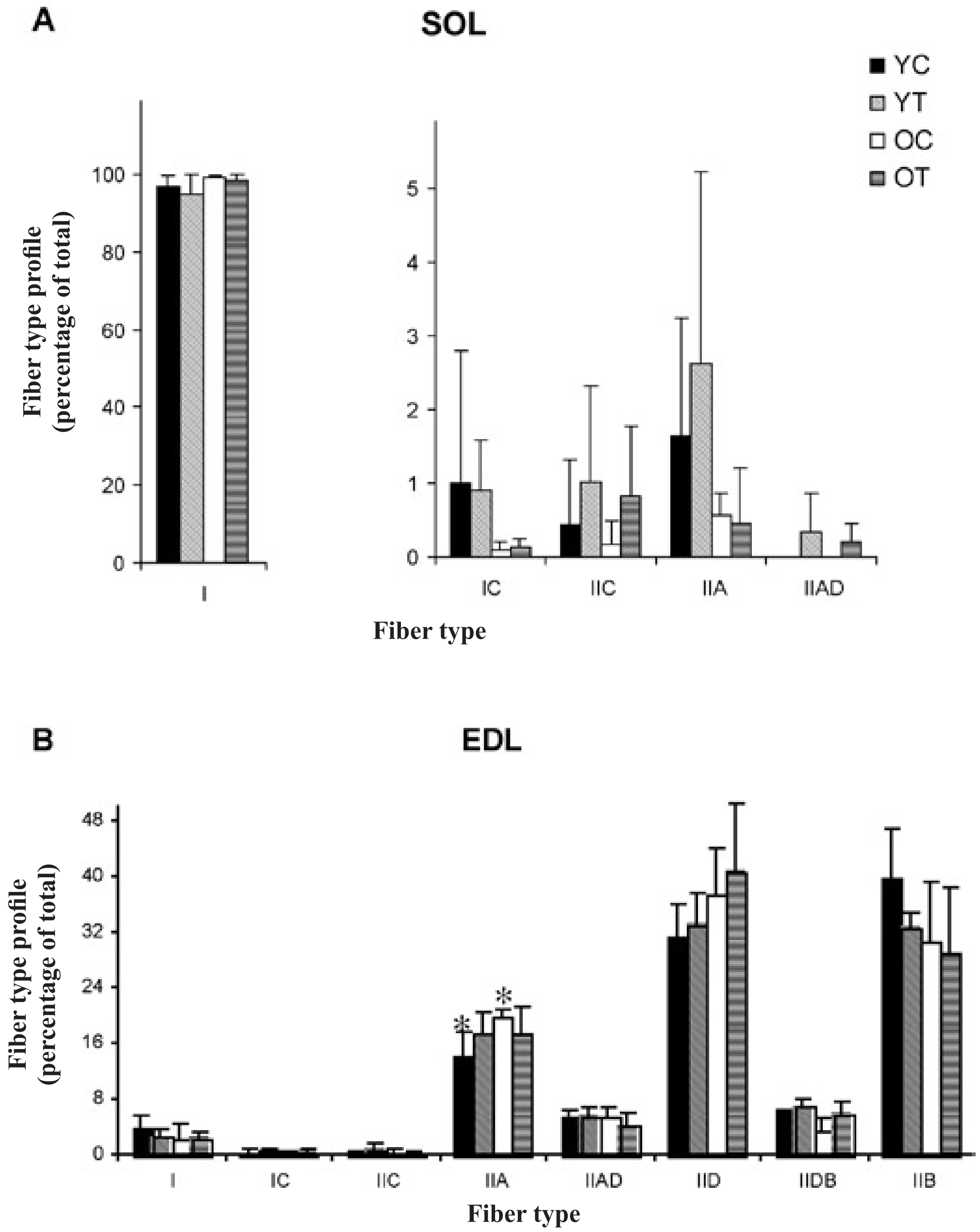

Figure 3. Fiber type (I, IC, IIC, IIA, IIAD, IID, IIDB and IIB) composition profile in the SOL (A) and EDL (B) muscles after treatment of the YC, YT, OC and OT groups. Values are reported as mean ± SD. * $p<0.05$ between YC and OC groups.

ment increases the weight of the skeletal muscle, an effect possibly related to the greater concentration of androgen receptors [12,22,40], which have been reported as potential modulators of muscle mass [9].

To our knowledge, there are no studies in which the weight of the SOL and EDL muscles was analyzed in young and old rats treated with testosterone. The present results demonstrate a reduction in body weight values of YT and OT groups comparing to their controls (YC and OC), with no change in relative weight of the SOL and EDL muscles. In other study, rats aged 5 to 25 months when treated with nandrolone, showed a fall in body weight without changes in the relative weight of the SOL and plantaris muscles [9]. However, it should be kept in mind that the animals used in both studies were sedentary and confined to their

Table 1. Relative weight (mg/g) in the SOL and EDL muscles of the YC, YT, OC and OT groups.

	YC	YT	OC	OT
SOL	0.394 ± 0.01	0.396 ± 0.04	0.35 ± 0.03	0.395 ± 0.02
EDL	0.438 ± 0.03	0.415 ± 0.02	0.461 ± 0.06	0.435 ± 0.02

Table 2. Cross-sectional area (μm²) of the type (I, IC, IIC, IIA, IIAD, IID, IIDB and IIB) fibers in the SOL muscle of the YC, YT, OC and OT groups. Values are reported as mean ± SD. + p<0.001 between young groups.

	I	IC	IIC	IIA	IIAD	IID	IIDB	IIB
YC	2863 ± 760 +	2067 ± 256	1701 ± 160	2980 ± 615	-	-	-	-
YT	3363 ± 1078 +	3900 ± 3628	1951 ± 921	2600 ± 763	2824 ± 81	-	-	-
OC	3357 ± 1129	2139 ± 807	1350 ± 0	1896 ± 950	-	-	-	-
OT	3451 ± 945	2015 ± 224	3230 ± 1405	2451 ± 1245	3629 ± 433	-	-	-

Table 3. Cross-sectional area (μm²) of the type (I, IC, IIC, IIA, IIAD, IID, IIDB and IIB) fibers in the EDL muscle of the YC, YT, OC and OT groups. Values are reported as mean ± SD . * p<0.05 and + p<0.001 between young groups. ⊡ p<0.05 between old groups.

	I	IC	IIC	IIA	IIAD	IID	IIDB	IIB
YC	1008 ± 218 *	1013 ± 134	1064 ± 146	1209 ± 218	1131 ± 217 +	1637 ± 415	2442 ± 739	3273 ± 704
YT	1205 ± 350 *	1234 ± 338	1124 ± 207	1254 ± 260	1441 ± 225 +	1808 ± 430	2337 ± 631	3158 ± 681
OC	1593 ± 266⊡	1205 ± 327	968 ± 302	1346 ± 316	1736 ± 316	2370 ± 661	3074 ± 823	3770 ± 916
OT	1313 ± 236⊡	1045 ± 412	1161 ± 167	1406 ± 269	1816 ± 236	2540 ± 758	3301 ± 765	3911 ± 966

cages. A different scenario perhaps would be seen if they were raised in a different environment, with physical activity [6,35].

Muscle fiber types

Muscle fiber phenotype is subjected to hormonal interference. Sexually dimorphic muscles show a transition from slow-twitch to fast-twitch fibers in testosterone-treated animals [16,29]. However, the effects of testosterone on fiber distribution in non-sexually dimorphic muscles are not completely understood. The present results demonstrate that testosterone does not cause significant shift in muscle fiber types in the SOL and EDL muscles, as also reported in studies involving the use of an anabolic agent [5,23,34].

Although controversial, aging is another factor that may alter muscle fiber composition. Some authors observed an increase in type I fibers [2,14,25,26,46], a decrease in type IIB fibers, and an increase in type IID fibers [26,27,46] with age. Others have not reported changes in the pattern of muscle fiber distribution with advancing age [47]. In the present study, comparison of the control groups (OC and YC) showed age-related changes. In the OC group, the percentage of type I fibers increased and types IIC and IIA fibers decreased in the SOL muscle; whereas in the EDL muscle type IID fibers increased and type IIB fibers decreased, when compared to YC group. These differences however, did not reach statistical significance. Even in older animals statistical difference is not reported by others [47], although, controversial data remain [2,26,27,46].

The percentage of type IIA fibers in the EDL muscle increased significantly with age. This data differs from Sugiura *et al.* [46] where no significant differences were observed in the older animals. We believe that this difference could arise from the technique used in their study, where hybrid fiber types would also be included.

When aging and testosterone effects were analyzed as a whole, testosterone treatment did not seem to reverse the age-related changes. This lack of response raises doubts about whether testosterone treatment may reverse or block changes in the distribution of muscle fiber types acquired during the aging process.

Morphometry of muscle fibers

To better investigate a possible hypertrophic effect of testosterone on muscle, we measured the cross-sectional area of the different types of muscle fibers. Some studies support the hypothesis that treatment with an anabolic agent hypertrophies type I fibers more than type II fibers in mammals [5,15,49], whereas others have reported a greater hypertrophic effect on type II fibers [28]. The present results demonstrate significant changes in the area of type I fibers in the SOL muscle of YT group and in the EDL muscles of YT and OT groups compared to their controls. There was also an increase in the area of IIAD type fibers in the EDL muscle of YT group. We believe that changes in the cross-sectional area in testosterone-treated animals may be fiber type- , muscle- and age-specific, which partially, could explain the differences obtained in the literature.

It is well known that anabolic effects of the testosterone are augmented in stimulated/exercised muscles [6,33,35,36,42,44,48] in mammals. However, testosterone addiction in non-exercised (sedentary) muscles of males is not as frequently reported as those of females [5,13,15,22,28,39]. Therefore, additional studies are needed for a complete understanding of testosterone's effect on skeletal muscle in males and its potential therapeutic benefits during the ageing process.

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