Author/s:
Lamboley, CR;Xu, H;Dutka, TL;Hanson, ED;Hayes, A;Violet, JA;Murphy, RM;Lamb, GD

Title:
Effect of androgen deprivation therapy on the contractile properties of type I and type II skeletal muscle fibres in men with non-metastatic prostate cancer

Date:
2018-02-01

Citation:
Lamboley, C. R., Xu, H., Dutka, T. L., Hanson, E. D., Hayes, A., Violet, J. A., Murphy, R. M. & Lamb, G. D. (2018). Effect of androgen deprivation therapy on the contractile properties of type I and type II skeletal muscle fibres in men with non-metastatic prostate cancer. Clinical and Experimental Pharmacology and Physiology, 45 (2), pp.146-154. https://doi.org/10.1111/1440-1681.12873.

DR. CEDRIC ROGER LAMBOLEY (Orcid ID : 0000-0002-4008-4929)

Article type : Original Article - Cellular and Molecular Biology

Effect of androgen deprivation therapy on the contractile properties of type I and type II skeletal muscle fibres in men with non-metastatic prostate cancer

Lamboley C.R. [1,2], Xu H. [3], Dutka T.L. [2], Hanson E.D. [1,4,5], Hayes A. [1,4,5], Violet J.A. [6], Murphy R.M. [3], and Lamb G.D. [2]

[1] *Institute of Sport, Exercise and Active Living (ISEAL), College of Sport and Exercise Science, Victoria University, PO Box 14428, Melbourne, VIC, 8001, Australia*

[2] *School of Life Sciences, La Trobe University, Melbourne 3086, VIC, Australia*

[3] *Department of Biochemistry and Genetics, La Trobe Institute for Molecular Science, La Trobe University, Melbourne 3086, VIC, Australia.*

This is the author manuscript accepted for publication and has undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process, which may lead to differences between this version and the Version of Record. Please cite this article as doi: 10.1111/1440-1681.12873

[4] *Australian Institute for Musculoskeletal Science (AIMSS), Sunshine Hospital, Western Health, Melbourne 3021, VIC, Australia*

[5] *College of Health and Biomedicine, Victoria University, PO Box 14428, Melbourne, VIC, 8001, Australia*

[6] *Division of Radiation Oncology and Cancer Imaging, Peter MacCallum Cancer Centre, East Melbourne, VIC, Australia.*

Running Head: Testosterone depletion and muscle properties

Correspondence: Dr. Cedric Lamboley, ISEAL, Victoria University, Melbourne 8001, VIC, Australia (e-mail: cedric.lamboley@vu.edu.au; phone: +61399194287; fax: +61399194891)

Abstract

The contractile properties of *vastus lateralis* muscle fibres were examined in prostate cancer (PrCa) patients undergoing androgen deprivation therapy (ADT) and in age- and activity-matched healthy male subjects (Control). Mechanically-skinned muscle fibres were exposed to a sequence of heavily Ca^{2+}-buffered solutions at progressively higher free [Ca^{2+}] to determine their force-Ca^{2+} relationship. Ca^{2+}-sensitivity was decreased in both type I and type II muscle fibres of ADT subjects relative to Controls (by -0.05 and -0.04 pCa units, respectively, $P<0.02$), and specific force was ~13% lower in type I fibres of ADT subjects than in Controls ($P=0.02$), whereas there was no significant difference in type II fibres. Treatment with the reducing agent

dithiothreitol slightly increased specific force in type I and type II fibres of ADT subjects (by ~2 to 3%, $P<0.05$) but not in Controls. Pure type IIx fibres were found frequently in muscle from ADT subjects but not in Controls, and the overall percentage of myosin heavy chain IIx in muscle samples was 2.5 times higher in ADT subjects ($P<0.01$). The findings suggest that testosterone suppression can negatively impact the contractile properties by (i) reducing Ca^{2+}-sensitivity in both type I and type II fibres and (ii) reducing maximum specific force in type I fibres.

Keywords: calcium sensitivity, inactivity, muscle fibre, prostate cancer, testosterone deprivation.

Introduction

Increasing numbers of men with prostate cancer (PrCa) are receiving androgen deprivation therapy (ADT) (1). ADT leads to profound declines in serum testosterone levels and suppression of tumor growth. However, the benefits of ADT are partially offset by its adverse effects, which include musculoskeletal changes, with negative impacts on quality of life (2). In humans, a reduced level of total testosterone is linked to muscle atrophy and weakness (3, 4). Many studies have been conducted to examine the short- and long-term effects of ADT on muscle strength, physical function and body composition (for review see (5)). Cross-sectional and longitudinal studies (6-10) with PrCa patients treated with or without ADT, as well as with age-matched healthy men, have shown that the deleterious effects on muscle function, physical performance and body composition seen in PrCa patients are related to ADT and not to PrCa *per se*. These studies also found that the side effects of ADT occur shortly after the start of the treatment (~3 months). Men with non-metastatic well-controlled PrCa undertaking ADT offer a unique model of profound androgen deficiency and provide an important opportunity to investigate if muscle weakness occurring with the treatment is the results of atrophy alone or is due to some intrinsic factors within the individual muscle fibres.

The exact mechanisms by which testosterone alters muscle strength are not fully understood, but appears to be multi-factorial. Testosterone suppression has been showed to reduce resting

muscle protein synthesis in young men (4) and in prostate cancer patients as well (3). Over time, ADT significantly reduces lean mass (7, 11), maximal strength (12) and also muscle quality (13, 14) suggesting additional factors beyond muscle atrophy cause the decrease of muscle strength. The effects of ADT on muscle quality in human can only be indirectly inferred from whole muscle function, which is a current limitation. Interestingly, studies on castrated rats indicated that testosterone suppression has a negative effect on the intrinsic quality of the muscle (15, 16), although not all data support this (17). The effects of testosterone suppression on fibre type distribution and fibre contractile properties are also not well defined, particularly in humans (18). Some studies in men (18) and in rats (19, 20), with decreased endogenous testosterone level or supraphysiological anabolic steroids administration, found no effect of testosterone on the relative proportion of different muscle fibre types, whereas other studies in rats observed an increase in the proportion of type II fibres with testosterone (21-23). Clearly, studies at the single fibre level examining the effect of androgenic hormone suppression on muscle function are needed to unveil novel targets for therapy for the prevention and treatment of muscle dysfunction induced by ADT.

Additionally, several studies have suggested that blockade of androgen receptor (AR) signaling may induce oxidative stress in various systems (for a review see (24)). It has been shown that castration in rat induced oxidative stress by significantly upregulating reactive oxygen species (ROS)-generating NADPH oxidases and downregulating ROS-detoxifying enzymes (25). These findings indicate that ADT, which mimics castration or AR blockade, may also induce oxidative stress in human tissues, including skeletal muscle. We previously showed in human (26) that oxidative modification of some key excitation-contraction coupling proteins occurs with ageing and it probably deleteriously affects normal muscle function. For this reason it is important to investigate whether oxidative stress is a cause of the muscle function alteration usually observed in PrCa patients undergoing ADT.

The present study examined the contractile apparatus properties of mechanically-skinned fibres obtained from fresh biopsies of the *vastus lateralis* muscle in PrCa patients undergoing ADT and healthy age- and activity-matched males. We assessed (i) whether there were any group

differences for specific force and Ca^{2+}-sensitivity in type I and type II fibres, (ii) whether ROS can partially explain these differences, and (iii) whether the muscle phenotype in PrCa patients is altered by ADT.

Results

Hormonal parameters are presented in Table 1. Briefly, men on ADT had castrated levels of total testosterone (< 50 ng/dL) that were ~10 times lower than the Control group (34 ± 6 and 351 ± 37 ng/dL, respectively, P <0.001). There were no other physical characteristics differences between groups.

Specific force and contractile properties of fibres

We measured force responses in a total of 79 muscle fibres from 8 PrCa patients undergoing ADT. Subsequent dot blotting of myosin heavy chain (MHC), as presented in Fig. 1, showed that the sample of ADT fibres consisted of 44 type I, 20 type IIa, 1 type IIax, 12 type IIx and 2 'mixed' fibres (fibres containing both MHCI and MHCII, see Methods). The sample of 97 muscle fibres from 10 healthy older men consisted of 59 type I, 31 type IIa or IIax fibres and 7 mixed fibres. No pure IIx fibres were found in the randomly selected muscle fibres from the Control subjects. The high frequency of IIx fibres with ADT was in accord with the proportions of the various MHC isoforms found in the muscles of these subjects (see Percentage of MHC isoforms later).

Maximum force production and Ca^{2+} sensitivity in skinned fibres from both groups of subjects were assessed by activating the contractile apparatus in a series of solutions with the free [Ca^{2+}] heavily buffered at progressively higher levels, as in Fig. 2. Specific force in type I fibres was on average ~13% lower in fibres of ADT patients compared to Control participants (P=0.02), whereas specific force in type II fibres did not differ significantly between the groups (P=0.52) (Fig. 3). Type IIa and IIax fibres were found in each subject and had similar specific forces and shown pooled together in Figure 3, whereas type IIx fibres were found only in ADT patients and consequently are shown as a separate group. Specific force in the IIa/IIax fibres was not significantly different between Control and ADT subjects. It was apparent however that specific

force in the small number of pure type IIx fibres examined from ADT patients was significantly lower (P=0.03) than in the IIa/IIax fibres (129 ±1 and 176 ±9 $mN.mm^{-2}$, respectively).

Ca^{2+} sensitivity in both type I and type II fibres was significantly lower in the ADT subjects relative to Controls, with the pCa_{50} being ~ 0.05 and 0.04 pCa units lower in type I and type II fibres, respectively (Fig. 4 & Table 2). However, Ca^{2+}-sensitivity was not significantly different (P=0.68) between the pure IIx and other type II fibres from ADT subjects; pCa_{50} of 5.91 ±0.03 (n=4) and 5.92 ±0.01 (n=19), respectively. As such, data for all type II fibres were subsequently pooled together. The Hill coefficient (*h*) in type I fibres of ADT subjects was slightly steeper than in Controls (P<0.01), whereas it was not significantly different in type II fibres (P=0.19). As expected, type II fibres in both groups of participants showed a lower Ca^{2+} sensitivity (lower pCa_{50}) and steeper Hill coefficient than type I fibres in their matching group (P<0.01) (Table 2).

Effects of DTT and S-glutathionylation

To examine if the contractile properties were affected by some reversible oxidative modification, the properties were tested both before and after strong reducing treatment with 10 mM DTT (e.g. Fig. 2). DTT treatment increased the maximal force production by ~2 and 3 % respectively in the type I and type II fibres of the ADT subjects (P <0.05), whereas no significant effect of DTT was found in either fibre type in the Control group (Table 2). In type II fibres of the ADT patients, DTT treatment also produced a larger decrease in Ca^{2+} sensitivity (by -0.015 pCa units) than in the Control group (P=0.046), whereas it had no effect in either group in type I fibres (Table 2).

We have previously shown that treating type II fibres successively with the sulphydryl-specific oxidant DTDP (100 μM, 5 min) and then reduced glutathione (GSH) (5 mM, 2 min) (e.g. Fig. 2), results in S-glutathionylation of the troponin I fast isoform (TnIf), which induces a large increase in myofibrillar Ca^{2+} sensitivity (27, 28). In the present study, when we applied this DTDP-GSH treatment to type II fibres of ADT subjects, there was a large increase in Ca^{2+} sensitivity (+0.139 pCa units) which was indistinguishable from that found in the type II fibres of the age-matched Control group (Table 2).

Percentage of different MHC isoforms in muscle samples

The MHC isoform composition in the *vastus lateralis* muscle biopsy samples of all subjects was determined by electrophoresis (Fig. 5A). As presented in Fig. 5B, a significant group difference was found only for the percentage of MHCIIx, which was ~2.5 times higher in ADT subjects compared to Controls (15 ± 3 and 6 ± 1 %, respectively, $P < 0.01$). The proportions of MHCI or MHCIIa were not significantly different between the groups. Finally, no significant correlation was found between the percentage of MHCIIx in muscle of ADT patients and particular characteristics of that group, such as the time since PrCa diagnosis or the duration of ADT treatment.

Discussion

To our knowledge, this is the first study reporting the effects of testosterone suppression on human single muscle fibre contractility. The study identified appreciable deficiencies in the contractile properties of *vastus lateralis* muscle fibres in PrCa cancer patients undergoing ADT relative to similar fibres in age-matched Control subjects. Maximum specific force in type I fibres in ADT subjects was on average ~13% lower than in Controls (Fig. 3) and the Ca^{2+}-sensitivity of both type I and type II fibres was also decreased (Table 2). There was no significant difference in specific force on average in the type II fibres in ADT patients, but there was a large increase in the proportion of type IIx fibres, and although we only tested 4 of them collected from 3 patients these seemingly had comparatively reduced specific force (-26 %) (Fig. 3); their presence could be indicative of poorer contractile performance (see later). As illustrated in Fig. 4, both type I and type II fibres of the ADT patients produce less force at any given submaximal free Ca^{2+} level than do similar fibres from Control subjects. This decrease in the Ca^{2+} sensitivity of contractile apparatus, together with the decrease in specific force in type I fibres, would have appreciable deleterious effects on overall muscle performance in ADT patients. These deficits in the contractile properties in the individual muscle fibres must be expected to exacerbate the weakness that arises from any net loss of muscle mass occurring with the testosterone suppression. It can be expected that the decrease in maximum force and the reduced responsiveness of the contractile apparatus to myoplasmic [Ca^{2+}] are likely to be

significant factors in the decrease in muscle force production and fatigue resistance observed in patients undergoing ADT (2).

To examine whether the differences in specific force and Ca^{2+} sensitivity seen in the fibres of ADT patients were attributable to reversible oxidative modification, we exposed a subset of the skinned fibres to a strong reducing treatment (DTT). As presented in Table 2, this reducing treatment slightly increased maximum force in both fibre types in the ADT subjects (by ~2 to 3 %, $P < 0.05$), whereas there was no change in maximum force in either fibre type in the Control group. This indicates that there was a small reversible oxidation-linked depression of maximum Ca^{2+}-activated force in the fibres of the ADT subjects, though this by no means accounts for the ~13% decrease in specific force seen in type I fibres. The DTT reducing treatment also decreased type II fibre Ca^{2+} sensitivity to a slightly greater extent in ADT patients than in the Controls (Table 2). This suggests that there was a greater level of oxidation-induced sensitization in the type II fibres in the ADT subjects, presumably due to a greater resting level of S-glutathionylation of fast troponin I (see ( )). This and the small oxidation-linked depression of maximum force both indicate a slightly higher level of reversible oxidative modification at rest in the fibres of the ADT patients relative to Controls.

We also examined whether there was any reduction in the maximal Ca^{2+}-sensitivity increase occurring with full S-glutathionylation in the fibres of the ADT subjects. We have shown previously that the level of S-glutathionylation of troponin I increases with exercise in humans, and the resulting sensitization of the Ca^{2+}-sensitivity of the contractile apparatus would be expected to aid muscle performance by compensating to some extent for the actions of the many metabolic factors that decrease contractile Ca^{2+}-sensitivity with exercise (28). Furthermore, we have shown that strong oxidative treatment in-vitro irreversibly depresses the extent of the Ca^{2+}-sensitivity increase occurring with S-glutathionylation ( ), presumably owing to irreversible oxidative modification of the key cysteine residue on troponin I (28). Such irreversible oxidative modification may be the reason why S-glutathionylation in type II fibres of older subjects (~70 years) only elicits ~80% of the increase in Ca^{2+}-sensitivity seen in fibres from young subjects (~22 years) (~0.136 and 0.168 pCa unit increase, respectively) ( ). In the present study we

found that the Ca^{2+} sensitivity increase occurring with S-glutathionylation treatment in the type II fibres of ADT subjects was no different from that in similarly aged old Controls (+0.139 and +0.136 pCa units, respectively, Table 2). Thus, there was no indication that the profound lack of testosterone in the ADT patients exacerbated or otherwise altered the putative irreversible oxidative changes to troponin I occurring in muscle with normal aging ( ).

Importantly, it was also found that total MHCIIx was much higher in the muscle of the ADT patients (see Results and Fig. 5). This increase in total MHCIIx is in apparent accord with the finding of a population of pure type IIx fibres in muscles of ADT patients; such fibres were not found in Control subjects. To our knowledge, a shift of muscle fibre-type induced by testosterone suppression has not been reported before in humans. A previous study (18) in young men undergoing a 20 week treatment with gonadotropin-releasing hormone agonist combined with a low dose of exogenous testosterone did not observe any change in the relative amount of mRNA for any of the MHC isoforms following the treatment. However, it is important to note that the level of testosterone in the young male participants in that study were only reduced to levels similar to healthy aged individuals (234 ± 60 ng/dL) rather than to medical castration levels (<50 ng/dL) observed in the patients in the present study (34 ± 16 ng/dL).

Very few or no pure IIx muscle fibres are typically found in the *vastus lateralis* muscle in normally active individuals, either young or old (27, 30). Pure IIx fibres however are seen frequently in sedentary or immobilized individuals (31). Importantly, it was further found in all fibre types examined that the maximum specific force became progressively lower (by ~10 to 40%) across the subjects in the order: trained, normally active, sedentary and immobilized (31). Thus, it appears that the presence of pure IIx fibres and a relatively high overall percentage of MHCIIx in the muscle are usually indicative that i) the subject is relatively inactive or sedentary, and ii) of a decrease in the average specific force in the individual fibres.

Conclusion

This study shows for the first time the role of ADT on the skeletal muscle fibre contractile properties and muscle phenotype in men being treated for PrCa. The ablation of testosterone in these patients led to a decrease in Ca^{2+} sensitivity in both fibre-types and also a decrease in specific force in type I fibres. These modifications, which for the most part do not seem to be directly related to contractile protein oxidation, could be expected to have significant deleterious effects on the muscle strength and performance, and may be significant contributory factors to the muscle weakness usually reported in men undergoing ADT. Finally, our results indicate that testosterone suppression modifies muscle phenotype, specifically by increasing the proportion of MHCIIx.

Methods

Participants and recruitment

The PrCa patients undergoing ADT were recruited from local physicians in the greater Melbourne area. Patients were screened via medical records and those who met the inclusion criteria were contacted by study physicians regarding their interest in participating. All patients received medical clearance from their physician before enrolling in the study. Inclusion criteria included having being diagnosed prostate cancer by a physician and undergoing ADT (injection of LHRH agonist, Zoladex®) for at least 3 months prior to study. Exclusion criteria were any of the following: uncontrolled prostate cancer, symptomatic cardiovascular disease, inability to engage safely in exercise, and type 1 diabetes. Healthy age-matched control subjects were a subset of male participants involved in our previous studies described elsewhere (26, 27) as well as five new male participants. Further, Control subjects had no history of prostate cancer nor were undergoing any form of testosterone suppression.

This study was approved by the Human Research Ethics Committees of Victoria University and the Peter MacCallum Cancer Centre, and conformed to the Declaration of Helsinki. After reading all information, a total of fourteen control participants and eight ADT patients gave signed informed consent.

Prior to inclusion in the study, the current level of self-reported physical activity of participants was determined with a physical activity questionnaire about the preceding 7 days (Active Australia Survey, Canberra, Australia) (32, 33), and subjects in the Control and ADT groups were recruited with a similar number of self-reported hours of physical activity. The type of physical activity reported by the participants ranged from walking and gardening to swimming, running and cycling. The questionnaire included questions about all exercise, including occupational tasks, household duties and sports activities and the exercise was split into three categories of intensity: moderate, hard and very hard exercise. Overall, there was no significant difference between Control and ADT participants either in the number of hours of physical activity conducted in total (7.6 ± 4.1 and 6.6 ± 4.7 h, respectively) or in the three different exercise categories.

Testosterone level

The level of total testosterone in plasma was assessed using a commercially available kit (Abnova, Taipei City, Taiwan) according to the manufacturer's instructions in all ADT patients and in ten Control subjects. We were unable to include data on testosterone level from all Control subjects as these participants were drawn from two previous study (26, 27) in which some blood samples were no longer available. Baseline blood samples were drawn at ~07:30 am just before the muscle biopsy procedure and after an overnight fast of at least eight hours.

Muscle biopsy

Skinned muscle fibres were obtained from *vastus lateralis* resting muscle biopsies from all subjects described above. After injection of local anaesthetic (2% lidocaine) into the skin and fascia, an experienced medical practitioner made a small incision in the middle third of the *vastus lateralis* muscle of each subject and took a muscle sample using a Bergstrom biopsy needle (34). The excised muscle sample was rapidly blotted on filter paper to remove excess blood and placed in paraffin oil (Ajax Chemicals, Sydney, Australia) and then brought down to ~10°C for ~45 min before individual muscle fibres were dissected. The remaining muscle sample from the biopsies was frozen and stored in liquid nitrogen for later analyses.

Preparation and force recording

The muscle biopsy was pinned at resting length in a petri dish containing paraffin oil and kept cool (~10°C) on an icepack. Individual fibre segments were mechanically skinned as described previously (34, 35), and pinned out unstretched under oil and the diameter measured at three places along the fibre. As described previously (27), fibre cross-sectional area (CSA) was calculated in order to estimate the specific force. The skinned fibre segment was then mounted at 120 % of resting length on a force transducer (AME801, SensoNor, Horten, Norway), and then transferred in a Perspex bath containing 2 ml of a relaxing buffer solution that broadly mimicked the intracellular milieu (see solutions section below). Force responses were recorded using a Bioamp pod and Powerlab 4/20 series hardware (ADInstruments, Sydney, Australia). All experiments were performed at room temperature (~23 ± 2 °C).

Skinned fibre solution

All chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA) unless specified otherwise. As described previously (34), the properties of the contractile apparatus were examined using a mixture of two heavily Ca^{2+}-buffered solutions, namely the relaxing solution and the maximal Ca^{2+}-activating solution. The relaxing solution contained (in mM) 50 EGTA, 90 Hepes, 10.3 total Mg^{2+} (giving 1 mM free), 126 K^{+}, 36 Na^{+}, 8 total ATP and 10 creatine phosphate, pH 7.10, pCa (=-$\log_{10}[Ca^{2+}]$) ~9. Maximal Ca^{2+}-activating solution contained (in mM) 50 CaEGTA, 90 Hepes, 8.1 total Mg^{2+} (giving 1 mM free), 126 K^{+}, 36 Na^{+}, 8 total ATP and 10 creatine phosphate, pH 7.10 and pCa ~4.7. Appropriate ratio of the relaxing solution and maximal Ca^{2+}-activating solutions were mixed to produce heavily buffered solutions with free $[Ca^{2+}]$ in the required range (pCa 6.7 to 4.7). Where required, 10 mM dithiodithreitol (DTT) was added to relaxing solution from a 1 M stock prepared in distilled water. A 100 mM stock of reduced glutathione (GSH) was made in relaxing solution with pH re-adjusted to 7.10 with KOH, and then diluted 20 fold to give 5mM in the final relaxing solution. Finally, a 100 mM stock solution of 2,2'-dithiodipyridine (DTDP) was made in absolute ethanol and diluted 1000-fold in the final relaxing solution to 100 μM.

Force-Ca^{2+} relationship and analysis

The force–Ca^{2+} relationship in each individual muscle fibre was assessed by exposing the skinned fibre segment to a sequence of solutions with the free $[Ca^{2+}]$ strongly buffered at

progressively higher levels over the relevant range (pCa 6.7 to 4.7, the latter solution eliciting maximum force) and then the fibre was fully relaxed again in the relaxing solution. As described previously (27), this sequence was performed twice for each of the four different conditions: (a) Control in relaxing solution, (b) after 10 min exposure in 10 mM DTT, (c) after 5 min exposure in 0.1 mM DTDP followed by 2 min exposure in 5 mM GSH, and again (d) after 10 min exposure in DTT.

Isometric force responses produced at each [Ca^{2+}] within a given sequence were expressed as a percentage of the corresponding maximum force generated in that same sequence, and analyzed by fitting a Hill curve using GraphPad Prism 6 software, to ascertain values of pCa_{50} (pCa at half-maximum force) and the Hill coefficient (*h*) for each sequence. Finally, maximum force reached during each sequence (at pCa 4.7) was expressed relative to the control level before any treatment in the given fibre, after correcting for the small rundown occurring with each repetition of the sequence.

Muscle homogenates and myosin heavy chain composition assay

Snap frozen muscles were cross-sectioned by cryostat (Leica CM 1950, Germany) and the 10 µm sections were placed into relaxing buffer then followed by adding 3x SDS loading buffer consisted of 0.125 M Tris•HCl pH 6.8, 4% SDS, 10% glycerol, 4 M urea, 10% β-mercaptoethanol (β-ME) and 0.001% bromophenol blue as we described before (36). The denatured muscle samples were separated on SDS-PAGE gels. The separating gel consisted of 32% v/v glycerol, 8% w/v acrylamide, N'-ethylenebisacrylamide (Bis), with a 50:1 ratio of acrylamide to Bis, 0.2 M Tris–HCl (pH 8.8), 0.1 M glycine, 0.4% w/v SDS, 0.1% w/v ammonium persulfate, and 0.05% v/v N,N,N',N'-tetramethylethylenediamine (TEMED). The stacking gel consisted of 32% v/v glycerol, 4% w/v acrylamide, and Bis at the same ratio (50:1) to acrylamide, 70 mM Tris–HCl (pH 6.7), 4 mM EDTA, 0.4% w/v SDS, 0.1% w/v ammonium persulfate, and 0.05% v/v TEMED (37). For running the gel, two different buffers were used, the lower running buffer consisting of 0.05 M Tris (base), 75 mM glycine, and 0.05% w/v SDS, while the upper running buffer was at 6x the concentration of the lower running buffer and had β-ME added (final concentration: 0.12% v/v) (38). The gel was run at 4 ℃ for 24 hours, and immediately after running, Coomassie brilliant blue G250 was used to stain the gel and visualize

the MHC bands. The images were collected by G:BOX Chemi (Syngene, USA) and the densitometry analysis was conducted using Quantity One software (Bio-Rad, USA).

Fibre typing

Dot blotting was performed to determine the fibre type of each muscle fibre segment. Briefly, PVDF membrane was activated with 95% ethanol and equilibrated in transfer buffer, 1 µL of each sample was applied to the wet membrane and allowed to dry. The dry membrane was then reactivated with 95% ethanol, equilibrated in transfer buffer, washed in TBST for 5 min, and then placed in blocking buffer for 5 min. The presence of myosin heavy chain (MHC) types IIx, IIa and I were determined with sequential probing of the membrane with antibodies specific to MHC IIx (mouse monoclonal IgM, clone 6H1 Developmental Studies Hybridoma Bank [DSHB], 1 in 100 in 1% BSA/PBST), MHC IIa (mouse monoclonal IgG, clone A4.74, DSHB, 1 in 200 in 1% BSA/PBST) and MHC I (mouse monoclonal IgM, clone A4.840, DSHB, 1 in 200 in 1% BSA/PBST). The MHC IIx and IIa antibodies were removed from the membrane with stripping buffer (Pierce) prior to incubation in the subsequent antibody.

Statistics

Values are presented as mean ± SE, with n denoting the number of fibres examined and N the number of subjects from which the fibres were obtained. Parametric testing was used only after examining whether the data were normally distributed. Statistical significance ($P < 0.05$) was determined with two-tailed Student's t test.

Acknowledgements

The monoclonal antibodies used in the present study were obtained from the Development Studies Hybridoma Bank, under the auspices of the NICHD and maintained by the University of Iowa, Department of Biological Sciences, Iowa City, IA 52242, USA. The antibodies directed against adult human MHC I and IIa isoforms (A4.840 and A4.74) were developed by Dr H. Blau and that for MHC IIx (6H1) was developed by Dr C. Lucas.

This project was supported by the National Health and Medical Research Council of Australia (grant number 1051460).

Disclosures

No conflict of interests, financial or otherwise, are declared by the authors.

References

1. Cooperberg MR, Grossfeld GD, Lubeck DP, Carroll PR. National practice patterns and time trends in androgen ablation for localized prostate cancer. Journal of the National Cancer Institute. 2003;95(13):981-9.
2. Alibhai SM, Gogov S, Allibhai Z. Long-term side effects of androgen deprivation therapy in men with non-metastatic prostate cancer: a systematic literature review. Critical reviews in oncology/hematology. 2006;60(3):201-15.
3. Hanson ED, Nelson AR, West DW, Violet JA, O'Keefe L, Phillips SM, et al. Attenuation of Resting but Not Load-Mediated Protein Synthesis in Prostate Cancer Patients on Androgen Deprivation. The Journal of clinical endocrinology and metabolism. 2017;102(3):1076-83.
4. Mauras N, Hayes V, Welch S, Rini A, Helgeson K, Dokler M, et al. Testosterone deficiency in young men: marked alterations in whole body protein kinetics, strength, and adiposity. The Journal of clinical endocrinology and metabolism. 1998;83(6):1886-92.
5. Storer TW, Miciek R, Travison TG. Muscle function, physical performance and body composition changes in men with prostate cancer undergoing androgen deprivation therapy. Asian journal of andrology. 2012;14(2):204-21.
6. Basaria S, Lieb J, 2nd, Tang AM, DeWeese T, Carducci M, Eisenberger M, et al. Long-term effects of androgen deprivation therapy in prostate cancer patients. Clinical endocrinology. 2002;56(6):779-86.
7. Galvao DA, Spry NA, Taaffe DR, Newton RU, Stanley J, Shannon T, et al. Changes in muscle, fat and bone mass after 36 weeks of maximal androgen blockade for prostate cancer. BJU international. 2008;102(1):44-7.
8. Hanson ED, Hurley BF. Intervening on the side effects of hormone-dependent cancer treatment: the role of strength training. Journal of aging research. 2011;2011:903291.
9. Segal RJ, Reid RD, Courneya KS, Malone SC, Parliament MB, Scott CG, et al. Resistance exercise in men receiving androgen deprivation therapy for prostate cancer. Journal of clinical oncology : official journal of the American Society of Clinical Oncology. 2003;21(9):1653-9.

10. Stone P, Hardy J, Huddart R, A'Hern R, Richards M. Fatigue in patients with prostate cancer receiving hormone therapy. European journal of cancer. 2000;36(9):1134-41.

11. van Londen GJ, Levy ME, Perera S, Nelson JB, Greenspan SL. Body composition changes during androgen deprivation therapy for prostate cancer: a 2-year prospective study. Critical reviews in oncology/hematology. 2008;68(2):172-7.

12. Galvao DA, Taaffe DR, Spry N, Joseph D, Turner D, Newton RU. Reduced muscle strength and functional performance in men with prostate cancer undergoing androgen suppression: a comprehensive cross-sectional investigation. Prostate cancer and prostatic diseases. 2009;12(2):198-203.

13. Chang D, Joseph DJ, Ebert MA, Galvao DA, Taaffe DR, Denham JW, et al. Effect of androgen deprivation therapy on muscle attenuation in men with prostate cancer. Journal of medical imaging and radiation oncology. 2014;58(2):223-8.

14. Girard D, Marino FE, Cannon J. Evidence for reduced neuromuscular function in men with a history of androgen deprivation therapy for prostate cancer. Clinical physiology and functional imaging. 2014;34(3):209-17.

15. Brown M, Fisher JS, Hasser EM. Gonadectomy and reduced physical activity: effects on skeletal muscle. Archives of physical medicine and rehabilitation. 2001;82(1):93-7.

16. Prezant DJ, Karwa ML, Kim HH, Maggiore D, Chung V, Valentine DE. Short- and long-term effects of testosterone on diaphragm in castrated and normal male rats. Journal of applied physiology. 1997;82(1):134-43.

17. Storer TW, Magliano L, Woodhouse L, Lee ML, Dzekov C, Dzekov J, et al. Testosterone dose-dependently increases maximal voluntary strength and leg power, but does not affect fatigability or specific tension. The Journal of clinical endocrinology and metabolism. 2003;88(4):1478-85.

18. Sinha-Hikim I, Artaza J, Woodhouse L, Gonzalez-Cadavid N, Singh AB, Lee MI, et al. Testosterone-induced increase in muscle size in healthy young men is associated with muscle fiber hypertrophy. American journal of physiology Endocrinology and metabolism. 2002;283(1):E154-64.

19. Hourde C, Jagerschmidt C, Clement-Lacroix P, Vignaud A, Ammann P, Butler-Browne GS, et al. Androgen replacement therapy improves function in male rat muscles independently of hypertrophy and activation of the Akt/mTOR pathway. Acta physiologica. 2009;195(4):471-82.

20. Noirez P, Ferry A. Effect of anabolic/androgenic steroids on myosin heavy chain expression in hindlimb muscles of male rats. European journal of applied physiology. 2000;81(1-2):155-8.

21. Bisschop A, Gayan-Ramirez G, Rollier H, Dekhuijzen PN, Dom R, de Bock V, et al. Effects of nandrolone decanoate on respiratory and peripheral muscles in male and female rats. Journal of applied physiology. 1997;82(4):1112-8.

22. Holmang A, Svedberg J, Jennische E, Bjorntorp P. Effects of testosterone on muscle insulin sensitivity and morphology in female rats. The American journal of physiology. 1990;259(4 Pt 1):E555-60.

23. Ophoff J, Van Proeyen K, Callewaert F, De Gendt K, De Bock K, Vanden Bosch A, et al. Androgen signaling in myocytes contributes to the maintenance of muscle mass and fiber type regulation but not to muscle strength or fatigue. Endocrinology. 2009;150(8):3558-66.

24. Shiota M, Yokomizo A, Naito S. Oxidative stress and androgen receptor signaling in the development and progression of castration-resistant prostate cancer. Free radical biology & medicine. 2011;51(7):1320-8.

25. Tam NN, Gao Y, Leung YK, Ho SM. Androgenic regulation of oxidative stress in the rat prostate: involvement of NAD(P)H oxidases and antioxidant defense machinery during prostatic involution and regrowth. The American journal of pathology. 2003;163(6):2513-22.

26. Lamboley CR, Wyckelsma VL, McKenna MJ, Murphy RM, Lamb GD. Ca(2+) leakage out of the sarcoplasmic reticulum is increased in type I skeletal muscle fibres in aged humans. The Journal of physiology. 2016;594(2):469-81.

27. Lamboley CR, Wyckelsma VL, Dutka TL, McKenna MJ, Murphy RM, Lamb GD. Contractile properties and sarcoplasmic reticulum calcium content in type I and type II skeletal muscle fibres in active aged humans. The Journal of physiology. 2015;593(11):2499-514.

28. Mollica JP, Dutka TL, Merry TL, Lamboley CR, McConell GK, McKenna MJ, et al. S-glutathionylation of troponin I (fast) increases contractile apparatus Ca2+ sensitivity in fast-twitch muscle fibres of rats and humans. The Journal of physiology. 2012;590(Pt 6):1443-63.

29. Murphy RM, Dutka TL, Lamb GD. Hydroxyl radical and glutathione interactions alter calcium sensitivity and maximum force of the contractile apparatus in rat skeletal muscle fibres. The Journal of physiology. 2008;586(8):2203-16.

30. D'Antona G, Pellegrino MA, Adami R, Rossi R, Carlizzi CN, Canepari M, et al. The effect of ageing and immobilization on structure and function of human skeletal muscle fibres. The Journal of physiology. 2003;552(Pt 2):499-511.

31. D'Antona G, Pellegrino MA, Carlizzi CN, Bottinelli R. Deterioration of contractile properties of muscle fibres in elderly subjects is modulated by the level of physical activity. European journal of applied physiology. 2007;100(5):603-11.

32. Brown W BA, Timperio A, Salmon J, Trost S. Measurement of adult physical activity: reliability, comparison and validity of self-report surveys for population surveillance. Summary and recommendations. Unpublished report to the Department of Health and Ageing. 2002.

33. Australian Institute of Health and Welfare. The Active Australia Survey: a guide and manual for implementation, analysis and reporting. Canberra, Australia. 2003.

34. Lamboley CR, Murphy RM, McKenna MJ, Lamb GD. Endogenous and maximal sarcoplasmic reticulum calcium content and calsequestrin expression in type I and type II human skeletal muscle fibres. The Journal of physiology. 2013;591(Pt 23):6053-68.

35. Dutka TL, Lamboley CR, McKenna MJ, Murphy RM, Lamb GD. Effects of carnosine on contractile apparatus Ca(2)(+) sensitivity and sarcoplasmic reticulum Ca(2)(+) release in human skeletal muscle fibers. Journal of applied physiology. 2012;112(5):728-36.

36. Xu H, Stapleton D, Murphy RM. Rat skeletal muscle glycogen degradation pathways reveal differential association of glycogen-related proteins with glycogen granules. Journal of physiology and biochemistry. 2015;71(2):267-80.

37. Mizunoya W, Wakamatsu J, Tatsumi R, Ikeuchi Y. Protocol for high-resolution separation of rodent myosin heavy chain isoforms in a mini-gel electrophoresis system. Analytical biochemistry. 2008;377(1):111-3.

38. Kohn TA, Myburgh KH. Electrophoretic separation of human skeletal muscle myosin heavy chain isoforms: the importance of reducing agents. The journal of physiological sciences : JPS. 2006;56(5):355-60.

Table 1: Characteristics of prostate cancer patient undergoing ADT and healthy age-matched controls. Mean (±SE) of some of baseline characteristics of both groups of participants. N denotes the number of subjects. P value was determined with a Student's two-tailed *t* tests.

Table 2: Contractile properties and response to S-glutathionylation treatment in muscle fibres of ADT and Control subjects. Means ± SE of pCa_{50}, Hill coefficient (*h*), and change (Δ) in pCa_{50} and maximum force (Max) following DTT treatment in type I and type II fibres, as well as change following S-glutathionylation treatment (S-Glut) in type II fibres (as in Fig. 1). n denotes number of fibres and N the number of subjects. # Value in ADT group significantly different from matching value in Control group. (Student's two-tailed t test, $P<0.05$).

Fig. 1. Representative dot blot showing the detection of myosin heavy chain (MHC) IIx, IIa and I. Following physiological measurements, individual fibre segments were collected and analysed for fibre type according to the presence of myosin heavy chain (MHC) isoforms using dot blotting. The representative dot blot shows 9 fibres that were probed for, sequentially, MHC IIx (red circles), MHC IIa (blue circles) and MHC I (green circles).

Fig. 2. Effect of DTT and DTDP-GSH exposure on Ca^{2+}-sensitivity of contractile apparatus in type II fibre from patient undergoing ADT. Representative force responses upon directly activating the contractile apparatus with heavily Ca^{2+}-buffered solutions at progressively higher free [Ca^{2+}] (pCa of successive solutions: >9.00, 6.7, 6.4, 6.22, 6.02, 5.88, 5.75, 5.48, 4.7, then back to >9.00, marked by ticks under each force trace). Force-pCa staircases elicited successively twice for each of the four different conditions: (1) Control, (2) after 10 min exposure to 10 mM DTT, (3) after 5 min exposure to 0.1 mM DTDP followed by 2 min exposure to 5 mM GSH, and again (4) after 10 min exposure to DTT (only one force-pCa staircases shown). Horizontal arrows show force levels produced at pCa 5.88 in the different conditions. For conditions 1 to 4, the averaged Ca^{2+}-sensitivity of the contractile apparatus (pCa_{50}) values for this representative fibre were 5.865, 5.831, 5.974 and 5.789, respectively.

Fig. 3. Specific force in type I fibres is significantly lower in ADT patients. Mean (+SE) specific force in type I, type IIa/IIax and type IIx fibres of ADT patients and age-matched healthy Controls. 'n' denotes number of fibres and 'N' the number of subjects from which the fibres were obtained. No pure IIx fibres were found in any of the 10 Control subjects. '#'

indicates that value in ADT patients is significantly different from same fibre type in Control group; not tested for IIx fibres. '*' indicates value in ADT patients for type IIx fibres is significantly different from value for type IIa/IIax fibres (Student's two tailed t tests).

Fig. 4. Average force-Ca^{2+} relationship in type I and type II *vastus lateralis* fibres from ADT patients and Control subjects. Type I and type II fibres in ADT patients are less sensitive to Ca^{2+} than those in Control subjects. Dotted lines indicate that the [Ca^{2+}] producing 50% of maximum force in fibres from Control subjects elicits only ~37% of maximum force in the fibres from ADT patients. See Table 2 for mean values and error ranges of the Ca^{2+}-sensitivity parameters.

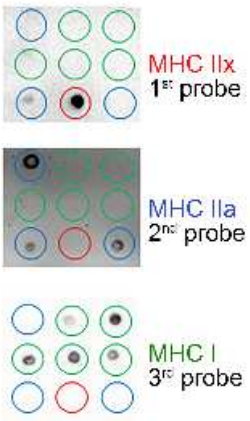
Fig. 5. MHC isoforms in *vastus lateralis* muscle of ADT patients and Controls. *A*: representative gel showing the three separated MHC isoforms in individual ADT and Control subjects. *B*: Mean (+SE) percentage of MHC isoforms in muscle of 8 ADT and 14 Control subjects. '#' indicates value is significantly different from Control group.

Table 1

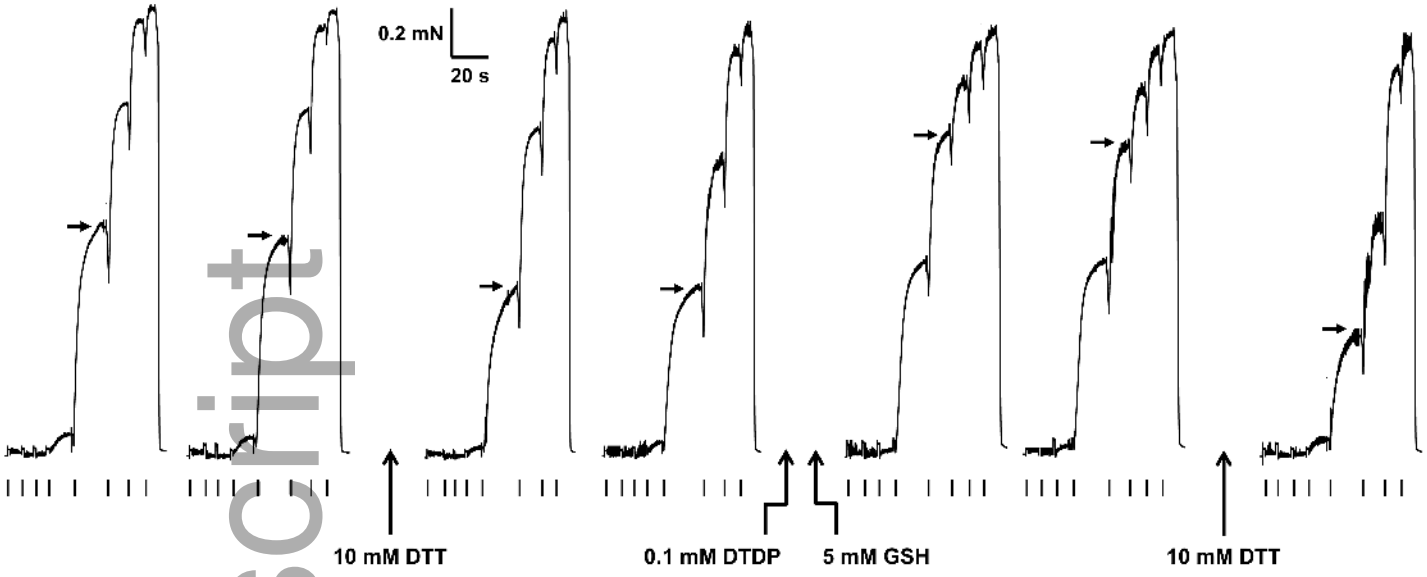
Variables	ADT (N = 8)	Controls (N = 14)	P value
Age (years)	68 ± 2	71 ± 1	0.222
Height (cm)	172.3 ± 2.6	175.5 ± 2.1	0.361
Weight (kg)	87.6 ± 3.4	81.5 ± 2.5	0.149
Physical activity (hours/week)	6.6 ± 1.7	7.6 ± 1.1	0.624
Time since diagnosis (days)	1571 ± 527	N/A	N/A
Gleason score	8 ± 0	N/A	N/A
Tumour stage	2 ± 0	N/A	N/A
Length of ADT (days)	485 ± 117	N/A	N/A
Total testosterone (ng/dL)	33.9 ± 5.5	351.3 ± 36.6	< 0.01

Table 2:

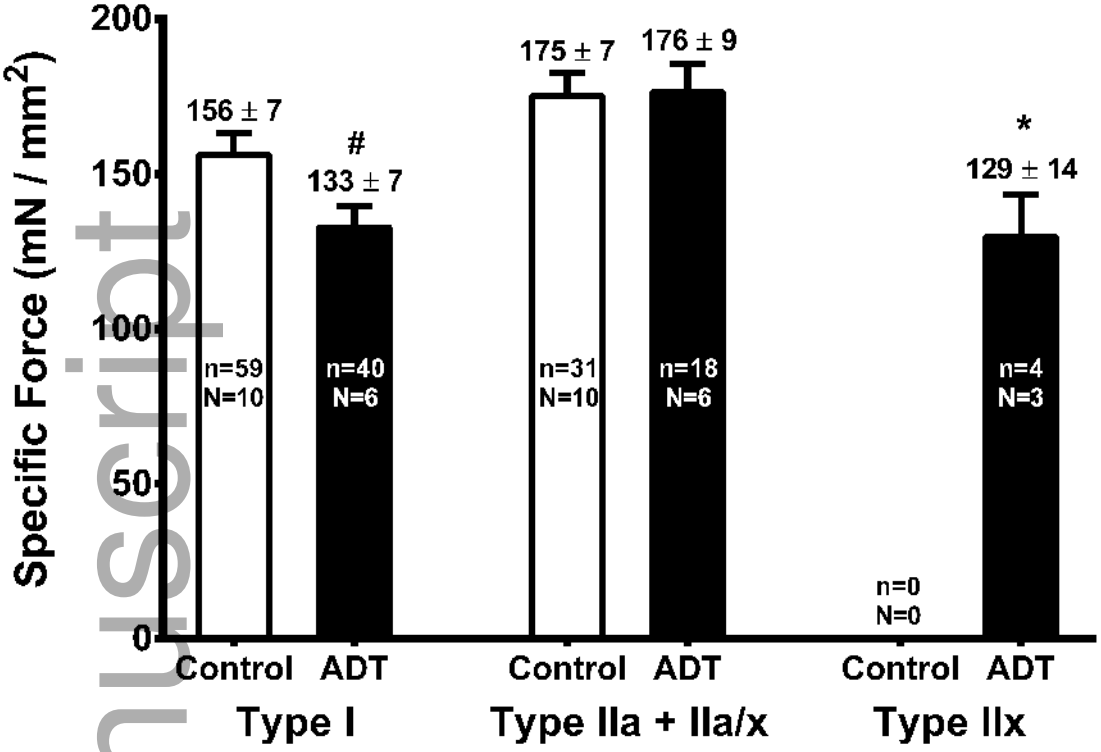
Variables	Type I fibre		Type II fibre	
	Control	ADT	Control	ADT
	(n = 27, N = 4)	(n = 41, N = 6)	(n = 16, N = 4)	(n = 23, N = 6)
pCa_{50}	5.99 ± 0.01	5.94 ± 0.01 # (P=0.013)	5.95 ± 0.01	5.91 ± 0.01 # (P=0.007)
h	3.98 ± 0.12	4.80 ± 0.17 # (P<0.001)	5.32 ± 0.18	5.90 ± 0.33 (P=0.187)
	Control	ADT	Control	ADT
	(n = 21, N = 3)	(n = 34, N = 8)	(n = 14, N = 3)	(n = 30, N = 8)
ΔpCa_{50} DTT	-0.002 ± 0.002	-0.002 ± 0.002 (P=0.818)	-0.016 ± 0.003	-0.031 ± 0.005 # (P=0.046)
ΔMax DTT (%)	0.1 ± 0.5	1.6 ± 0.5 # (P=0.032)	0.4 ± 0.5	3.4 ± 1.0 # (P=0.037)
			Control	ADT
			(n = 14, N = 3)	(n = 15, N = 4)
ΔpCa_{50} S-Glut			0.136 ± 0.004	0.139 ± 0.004 (P=0.651)



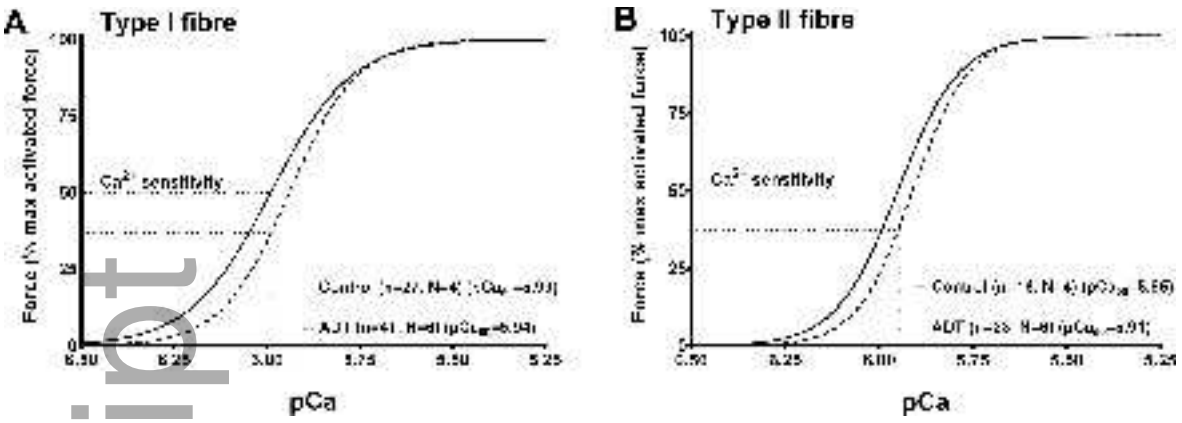
cep_12873_f1.tif



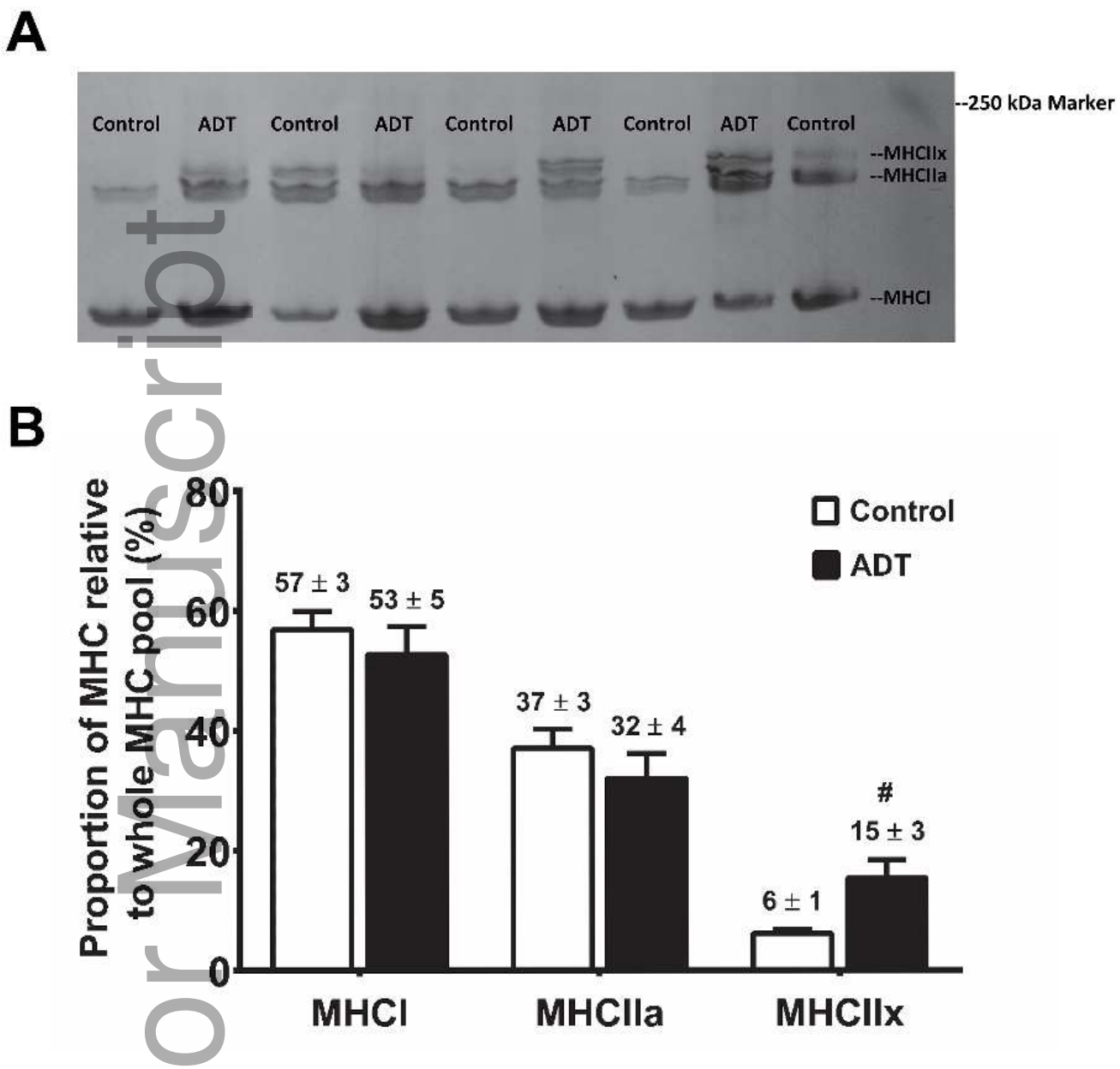
cep_12873_f2.tif



cep_12873_f3.tif



cep_12873_f4.tif



cep_12873_f5.tif