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Combined xIL-6R and urocortin-2 treatment restores *mdx* diaphragm muscle force.

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Abstract

Combined xIL-6R and urocortin-2 treatment restores *mdx* diaphragm muscle force.

Introduction

Duchenne muscular dystrophy (DMD) is characterized by progressive muscle degeneration leading to immobility, respiratory failure and premature death. As chronic inflammation and stress are implicated in DMD pathology, the efficacy of an anti-inflammatory and anti-stress intervention strategy in ameliorating diaphragm dysfunction was investigated.

Methods

Diaphragm muscle contractile function was compared in wild-type and dystrophin-deficient *mdx* mice treated with saline, anti-IL-6R antibodies (xIL-6R), the corticotrophin-releasing factor receptor 2 (CRFR2) agonist, urocortin 2 or both xIL-6R and urocortin 2.

Results

Combined treatment with xIL-6R and urocortin 2 rescued impaired force in *mdx* diaphragms. Mechanical work production and muscle shortening was also improved by combined drug treatment.

Discussion

Treatment which neutralizes peripheral IL-6 signaling and stimulates CRFR2 recovers force-generating capacity and the ability to perform mechanical work in *mdx* diaphragm muscle. These findings may be important in the search for therapeutic targets in DMD.

Keywords: Interleukin-6, corticotrophin-releasing factor, urocortin 2, *mdx,* diaphragm, monoclonal.

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Introduction

Patients with Duchenne muscular dystrophy (DMD) are deficient in the functional protein dystrophin, which protects muscle fibers from mechanical stresses induced by cellular contraction[1,2]. The most obvious and debilitating characteristic feature of DMD is the progressive degeneration and weakening of striated muscles resulting in severe disability and premature death[3]. Cardiopulmonary failure dominates disease morbidity in the later stages and pulmonary insufficiency is the leading cause of premature death[4]. As repeated contraction and load bearing on striated muscle determines the severity of the pathological signature in DMD muscle, continuous diaphragmatic contractions result in severe degeneration of this organ. Patients suffer reduced vital capacity, aberrant blood gas regulation and are prone to sleep-disordered breathing[5]. Indeed, artificial ventilation has proved effective in extending lifespan in DMD patients[6,7].

Dystrophin-deficient skeletal muscles exhibit altered calcium handling and abnormal regulation of reactive oxygen species and nitric oxide[8]. Additionally, chronic inflammation as evidenced by elevated levels of cytokines, leukocyte adhesion and complement system activation[9], is likely to contribute to muscle dysfunction. Pro-inflammatory cytokines such as tumor necrosis factor (TNF)[10] and interleukins (IL)-1 and -6[9] are early indicators of the disease, and the inflammatory response worsens with disease progression. The pro-inflammatory cytokine IL-6, is secreted by skeletal muscle following exercise and due to local inflammation[11,12] but is also released by immune cells, neurons[13] and epithelial cells[14]. Circulating IL-6 levels are elevated in DMD patients[15,16] and in the diaphragm of dystrophin-deficient *mdx* mice[17]. Although *mdx* mice generally exhibit a milder phenotype than the human disease, pathological changes in the diaphragm are faithfully recapitulated[18-20],

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making the *mdx* mouse appropriate for studying interventions seeking to ameliorate diaphragm dysfunction. The diaphragm muscle undergoes inflammation and becomes fibrotic, with collagen replacing functional fibers[22], resulting in a substantial loss of force production[1,18,21]. Interestingly, Kostek *et al.* demonstrated that blocking IL-6 signaling with an IL-6 receptor antibody, raised levels of inflammatory markers in gastrocnemius muscle in *mdx* mice, with an 11% increase in hind limb strength, which failed to reach statistical significance. There was no effect on forelimb strength[23]. Conversely, in *mdx* diaphragm, this treatment strategy favored an anti-inflammatory response and improved muscle repair[24]. However, the functional effects of this strategy have not been assessed.

DMD patients frequently exhibit co-morbid anxiety and depression[25] and depression- and anxiety-like behaviors are also evident in *mdx* mice[26]. Indeed, amitriptyline, an anti-depressant with anti-inflammatory effects caused alterations in circulating IL-6 levels associated with decreased inflammation in skeletal limb muscles[26]. Although *mdx* mice are stress-sensitive[26], the hypothalamic-pituitary-adrenal (HPA) stress axis, which is activated by the binding of corticotrophin-releasing factor (CRF) to either CRF1 or CRF2 receptors (CRFR1 or CRFR2), appears to be intact[27]. Nonetheless, treatment with the CRFR2 agonist, urocortin2 (Uro2), reduced inflammation in skeletal muscle with an associated improvement in diaphragm function in *mdx* mice[28], an effect attributed to increased muscle mass, modulated proteolysis and activation of anabolic signaling pathways[29]. Given that the current treatment for DMD, corticosteroids, are associated with unwanted side-effects, we sought to investigate whether more targeted treatment strategies using monoclonal IL-6 receptor (xIL-6R) antibodies and/or Uro2, which have both been shown to be beneficial with regard to

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dystrophinopathies, may be beneficial in terms of ameliorating diaphragm muscle dysfunction in the *mdx* mouse.

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Material and Methods

Ethical approval

All experiments involving animals were conducted under national license in full accordance with European Union directives and following institutional animal ethics committee approval.

Animals and Experimental Design

Breeding pairs of dystrophin-deficient C57BL/10ScSn-Dmd^{Mdx}/J (*mdx*, dystrophic) and C57BL/10ScSn wild type (WT) mice were purchased from Jackson Laboratories (Bar Harbor, Maine, U.S.A). Colonies were established and maintained in our institutional animal facility. Weaned mice were housed in groups of up to 4 per cage, and kept in a 12h light/12h dark cycle (06:00-18:00 light hours), with free access to drinking water and standard chow. Male mice were used for studies. Power calculations with an effect size of 2, power of 0.9 and false positive rate (α) of 0.05, were used to determine group sizes of at least n=6 per group. Diaphragm tissue was harvested from animals used in a previously published study[30].

Diaphragm contractile function was assessed in untreated dystrophin-deficient *mdx* mice compared with WT mice (10 weeks old) confirming a significant respiratory muscle phenotype in *mdx* at this age (figure 1A). In the intervention study, a treatment regimen amended from previous studies[23,28] was used. *Mdx* mice were randomly assigned to one of four groups, administered either saline (0.9% NaCl, 6 subcutaneous injections on alternate days over two weeks); anti-IL-6 receptor antibodies (xIL-6R, 0.2mg/kg body weight, 6 subcutaneous injections on alternate days over two weeks (MR16-1, Chugai Pharmaceutical Co., Ltd, Tokyo, Japan); the CRFR2 agonist, urocortin 2 (Uro2, 30µg/kg body weight, 6

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subcutaneous injections on alternate days over two weeks, Sigma Aldrich, St Louis, MO, USA) or a combination of both xIL-6R and Uro2 (6 subcutaneous injections on alternate days over two weeks). The experimental protocol is illustrated in Figure 1B. Animals were handled prior to the intervention to habituate animals to the associated stress and all injections were carried out by the same researcher. The investigator was not blinded to the intervention groups during data collection but subsequent analysis of the data was blinded.

Tissue collection

Mice were euthanized by decapitation and exsanguination. The diaphragm was excised and maintained in ice-cold 95% O_2 / 5% CO_2-bubbled Krebs solution consisting of (in mmol/L): NaCl, 117; KCl, 4.8; $CaCl_2$, 2.5; $MgCl_2$, 1.2; $NaHCO_3$, 25; NaH_2PO_4, 1.2; and D-glucose 11.

***Ex vivo* diaphragm muscle function**

A 3mm longitudinal strip of costal diaphragm was dissected with central tendon attached, extending to a rib. Cotton thread was tied to the central tendon and the preparation was arranged vertically in a tissue bath with 95% O_2 / 5% CO_2-bubbled Krebs solution maintained at 37°C, with the rib tied to a hook at the base of the tissue holder and the tendon attached to a dual-mode force transducer (Aurora Scientific Inc., ON, Canada), which can isolate force and length independently allowing assessment of isometric and isotonic muscle performance. Electrical field stimulation of the diaphragm strips was evoked using two silver electrodes running vertically on either side of the diaphragm strip. The optimum length (L_O) of each muscle preparation (i.e. the length which produces the maximal isometric twitch force in response to supra-maximal stimulation) was determined by incrementally adjusting a

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micropositioner which altered muscle length between intermittent stimulations (1ms, 80V). Once determined, the muscle was held at L_O for the duration of the experiment.

A single twitch was elicited (supra-maximal voltage) and the twitch force, contraction time (time to peak force) and half-relaxation time (time for peak force to decay by 50%) were determined. Tetanic force in response to supra-maximal stimulation (100Hz, 300ms) was determined. Muscle peak shortening and maximum shortening velocity were determined under zero load conditions, the latter from the first detectable length change during the first 30ms of each contraction[31–33] .Mechanical work was calculated as the product of force *x* shortening (normalized to L_O). Mechanical power was calculated as the product of force *x* velocity (L_O/s) of muscle shortening. Peak work and peak power were determined from contractions performed under varying load conditions[31–33].

Statistical Analysis

The data are represented as mean values ± the standard error of the mean (SEM). Student t-tests or one-way ANOVA (with Newman-Keuls *post hoc* tests) were used to compare parameters between groups; $p<0.05$ was considered statistically significant.

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Results

Combination treatment with xIL-6R and Uro2 recovers *mdx* diaphragm force production

Mdx diaphragms have significantly reduced peak specific twitch force in response to a single supra-maximal stimulus compared with WT mice (Figure 2A). However, time to peak in *mdx* (30±2 ms) and WT (20±3 ms, $p>0.05$) and the time taken for the peak force to decay by 50% in *mdx* (20±5 ms) and WT (20±2 ms, $p>0.05$) mice were not different. In a separate cohort of *mdx* mice, assessment of diaphragm peak specific twitch force production determined that intervention with xIL-6R did not improve muscle function compared with saline-treated *mdx* mice ($p>0.05$). Diaphragms from *mdx* animals treated with Uro2 or the combined treatment of xIL-6R and Uro2 showed trends towards higher peak specific twitch force compared with saline-treated *mdx* mice (Figure 2A). In comparison to WT forces, combined treatment with xIL-6R and Uro2 in *mdx* mice, reflects a recovery to 97% of the control values.

In response to tetanic stimulation (100Hz, 300ms), peak specific tetanic forces evoked in *mdx* diaphragms were significantly reduced compared with WT mice (Figure 2B). The weak peak specific tetanic force in saline-treated *mdx* diaphragm muscles was not significantly improved by treatment with either xIL-6R ($p>0.05$) or Uro2 alone ($p>0.05$). However, peak specific tetanic force was significantly increased following combined treatment with xIL-6R and Uro2 (Figure 2B) compared with saline-treated *mdx*, restoring values to 93% of the WT diaphragm.

Work and power production in *mdx* diaphragm are improved by treatments

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Further analysis of diaphragm muscle function in the *mdx* cohort demonstrated that peak specific shortening (L/*Lo*) was increased in xIL-6R-treated *mdx* diaphragms and combined xIL-6R- and Uro2-treated *mdx* diaphragms, but not Uro2-treated diaphragms compared with saline-treated *mdx* controls (Figure 3A). Peak diaphragm specific work, the product of contractile force and length of shortening, was significantly higher in *mdx* diaphragms from animals receiving the combined treatment of xIL-6R and Uro2 compared with saline-treated *mdx*, but not for either treatment alone (Figure 3B). Contraction kinetics were significantly improved in xIL-6R-treated group, but not different between saline treated and Uro2 or combination-treated *mdx* mice (Figure 3C). Peak specific power production of *mdx* diaphragm muscle strips was increased by all treatments compared with saline-treated *mdx* diaphragms ($p<0.05$ ANOVA), but this failed to achieve statistical significance for any of the three independent treatment groups (Figure 3D).

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Discussion

Diaphragm degeneration, fibrosis and dysfunction are evident in the *mdx* mouse, faithfully recapitulating human DMD[18,35,36]. Consistent with reports of reduced muscle strength, elasticity, twitch speed and fiber length[18], we have similarly demonstrated that contraction force is attenuated in *mdx* diaphragm muscle. Our data demonstrate that an intervention treatment which inhibits peripheral IL-6 signaling and activates CRFR2 signaling has beneficial outcomes for diaphragm function in *mdx* mice, improving force-generating capacity and work production.

Dystrophin anchors the extracellular matrix to cytoskeletal F-actin and is associated with intermediate muscle fiber filaments in the extracellular matrix protein complex. This complex spans the sarcolemma and provides skeletal muscle with protection from contraction-induced damage[1]; thus dystrophin deficiency is likely to alter the threshold for work-induced injury in muscle fibers. Isotonic muscle contractions that induce changes in muscle length can cause micro lesions in muscle fibers, which initiate a cascade of events that are detrimental to fiber health and function[37,38]. Thus, the isotonic properties of *mdx* muscle fibers are particularly relevant to understanding dystrophin-deficient muscle fiber pathology. Similar to other reports[18,19,39–41], our study in 10-week old *mdx* mice established that diaphragm weakness is a hallmark signature of dystrophin loss. In response to single and tetanic stimuli *mdx* diaphragm muscle strips generated considerably lower contractile forces. As force generation is relative to the number of functional cross bridge connections made during skeletal muscle contraction[42,43], muscle weakness is likely to be due to the loss of functional muscle fibers and an increase in connective tissue[22,44]. Changes in isometric contractile kinetics can indicate changes in muscle fiber type or changes in calcium handling in myocytes. However,

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despite evidence that aged *mdx* mice exhibit slowing of actomyosin interactions[45,46], we found no significant differences between WT and *mdx* mice in contractile kinetics, although the data should be viewed cautiously as the study may have been underpowered for this analysis. Peak specific tetanic force was the primary outcome measure in our study. Impaired mechanical work production has been reported previously in *mdx* diaphragm[47].

In addition to loss of the regenerative capacity of muscle fibers[48,49] and weaker branched muscle[50], loss of dystrophin from striated muscles is associated with inflammatory upregulation[9,51,52]. Indeed, the extent of the chronic inflammatory response is thought to predict the severity of the pathological changes associated with dystrophin-deficiency[9]. The potent anti-inflammatory effects of glucocorticoids, which are secreted following activation of the HPA axis by CRF, are the most effective therapy to date to slow down progression of DMD but are associated with unwanted side-effects. More specific targeting of immune and/or stress factors may provide novel therapeutic strategies with fewer side-effects. Indeed, inhibitors of NFκB have shown promise in improving *mdx* muscle pathology[53] such as a recent study which revealed improvements in *mdx* diaphragm force following NFκB inhibition with ursodeoxycholic acid[54]. The pro-inflammatory cytokine IL-6, involved in skeletal muscle metabolism and maintenance and remodeling of myocytes, as well as regeneration after exercise and damage[55,56], is reported to be elevated in *mdx* tissue[23,26,57].

The efficacy of blocking IL-6 signaling with xIL-6R in other inflammatory diseases such as rheumatoid arthritis and Castleman disease has been demonstrated[56,59]. Blocking IL-6 signaling can result in muscle regeneration via immune modulation[60] and *mdx* mice perform better on a treadmill test following 2 weeks of treatment with xIL-6R with associated

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downregulation of diaphragm pro-inflammatory markers[61]. We have previously determined that xIL-6R administration prevents gastrointestinal dysfunction in the same animals as those used in the present study[30]. However, another report unexpectedly detected increased inflammation in limb muscles and found no changes in diaphragm muscle regeneration[23]. In our study, *in vivo* administration of xIL-6R alone over two weeks had only modest protective effects on *mdx* diaphragm function; *mdx* muscle force was not significantly changed by xIL-6R treatment. The degree of muscle shortening and the velocity of this contraction was increased compared with saline-treated *mdx* tissue, but *mdx* diaphragm muscle peak work and peak power were only partially recovered and did not reach significance. As implicated by some studies using this treatment strategy, global reduction in inflammation is a possible mechanism underlying improved muscle function in *mdx* mice. However, Kostek and colleagues reported increased inflammation in *mdx* limb muscles following administration of xIL-6R after 5 weeks of treatment[23]. Evidence accruing is starting to suggest that the timing of the xIL-6R treatment may be crucial in terms of improved muscle function and altered inflammatory profiles[24].

We also investigated the potential therapeutic benefits of the CRFR2 agonist, Uro2. Previous studies demonstrated that activation of CRFR2 reduces skeletal muscle atrophy in limb muscles and increases muscle mass[29,62,63]. Uro2 has also recently been shown to improve skeletal muscle structure and function in *mdx* mice when administered in early life[28]. In our studies, 2 weeks of treatment with Uro2 in *mdx* mice increased diaphragm muscle force, although the effects failed to achieve statistical significance. Uro2 did not alter the contractile kinetics of *mdx* diaphragm contractions. These results differ to the observation of increased contractile kinetics following urocortin treatment reported by others[28]. However, as the Uro2

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treatment group in our studies only had an n of 5, we acknowledge that this group was likely underpowered and this is a limitation of our study. CRFR2 agonists stimulate anabolic signaling pathways, promoting hypertrophy and reducing muscle necrosis, and have been shown to have beneficial effects in the limb muscles of *mdx* mice[29,40]. Uro2 probably promotes muscle hypertrophy and delays apoptosis of macrophages, as well as slowing muscle atrophy. However, it is worth noting that activation of CRFR2 may promote a pro-inflammatory environment through NFκB dependent induction of pro-inflammatory IL-8[64], which could limit its potential use in the treatment of DMD.

Since evidence exists for crosstalk between the stress factor, CRF and the immune mediator, IL-6 in neural[65], smooth[66] and cardiac tissues[67], we assessed the potential benefits of a combination therapy of both xIL-6R and Uro2 to improve the inflammatory environment of *mdx* diaphragm muscle using different but complementary mechanisms. Our study revealed that combined drug therapy was most effective in restoring diaphragm force-generating capacity. Indeed, peak specific force and peak specific mechanical work production were equivalent in *mdx* mice receiving the combination treatment compared with WT diaphragm. Peak shortening, which occurs under zero load conditions was improved by xIL-6R treatment, which may relate to decreased muscle fibrosis and improved muscle mechanics. There was no significant effect of Uro-2 alone on peak shortening. As such the combination therapy was equivalent to xIL-6R alone. Improvement in peak shortening does not translate to improved peak work, as peak work occurs at 30-40% load (% of max force) in mouse diaphragm[30]. xIL-6R alone did not affect peak work, whereas the combination therapy significantly increased peak work, illustrating that the beneficial effect of the combined

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therapy on mechanical work related predominantly to the positive inotropic effect of the drug interventions on muscle force.

In conclusion, our findings show that xIL-6R and Uro2 treatments alone have modest beneficial effects on *mdx* diaphragm muscle function, but when administered together the inotropic effects are such that diaphragm peak force-generating capacity is impressively restored to WT values. Diaphragm muscle force is regarded as a clinically relevant parameter given that diaphragm weakness has prognostic value for patient outcome in the critical care setting[68]. Our study implicates IL-6R- and CRFR-mediated signaling in dystrophin-deficient respiratory muscle pathophysiology and has identified a combinational therapy which restores aspects of diaphragm function in the dystrophic *mdx* mouse, representing a strategy worthy of further consideration in the search for therapies for the treatment of DMD.

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Abbreviations: Ca^{2+}, calcium; CRF, corticotropin releasing factor; CRFR, CRF receptor; HPA, hypothalamic-pituitary-adrenal; IL, interleukin; IL-6R, interleukin-6 receptor; xIL-6R, anti-IL-6R.

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Figure Legends

Figure 1: Experimental protocol

A: The flow chart shows the experimental protocol to establish the baseline characteristics of muscle function in wildtype (WT) and *mdx* diaphragms. **B:** The flow chart illustrates the experimental protocol in the interventions study which compares *mdx* mice treated with saline, anti-interleukin-6 receptor antibodies (xIL-6R), urocortin 2 (Uro2) or a combination treatment with both xIL-6R and Uro2. Behavioral assessments on day 13 have been previously published (Manning *et al*, 2016).

Figure 2: Contractile forces are improved by intervention treatments in *mdx* diaphragm

A: Bar charts and representative traces show weaker twitch force in *mdx* (n=6, gray line) compared with WT (n=6, black line) diaphragms and the effect of treatment with saline, (n=8, black line), monoclonal interleukin-6 receptor antibodies (xIL-6R) (n=6, gray line), urocortin 2 (Uro2, n=5, light gray line) and combined xIL-6R and Uro2 (n=7, dashed black) in *mdx* diaphragm tissue. **B:** Bar charts and representative traces show weaker tetanic force in *mdx* compared with WT diaphragms and the effect of treatment with saline, xIL-6R, Uro2, and combined xIL-6R and Uro2 in *mdx* diaphragm tissue. * and *** indicate $p<0.05$ and $p<0.001$, respectively.

Figure 3: Diaphragm function is improved by combined xIL-6R and Uro2 treatment.

Bar charts show **A:** peak specific shortening, **B:** peak specific mechanical work, **C**: peak specific velocity of contraction and **D:** peak specific mechanical power in saline-treated *mdx* mice compared with *mdx* mice treated with xIL-6R, Uro2 and combined xIL-6R and Uro2 treatment. * indicates $p<0.05$.

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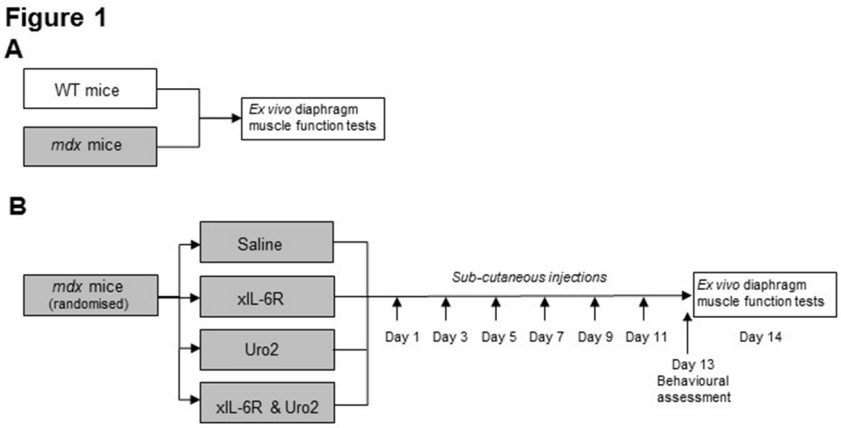


Figure 1

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Figure 2

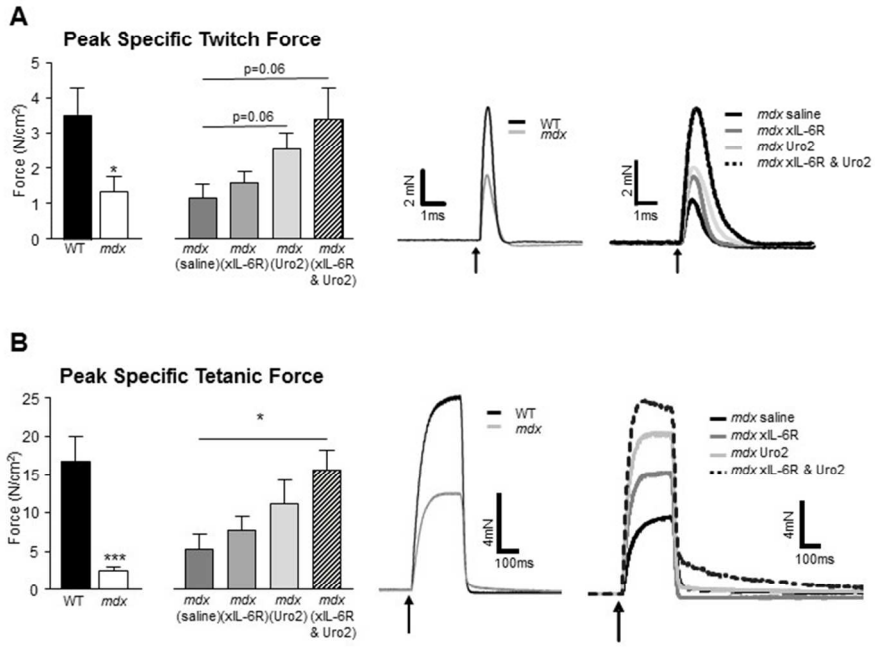


Figure 2

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Figure 3

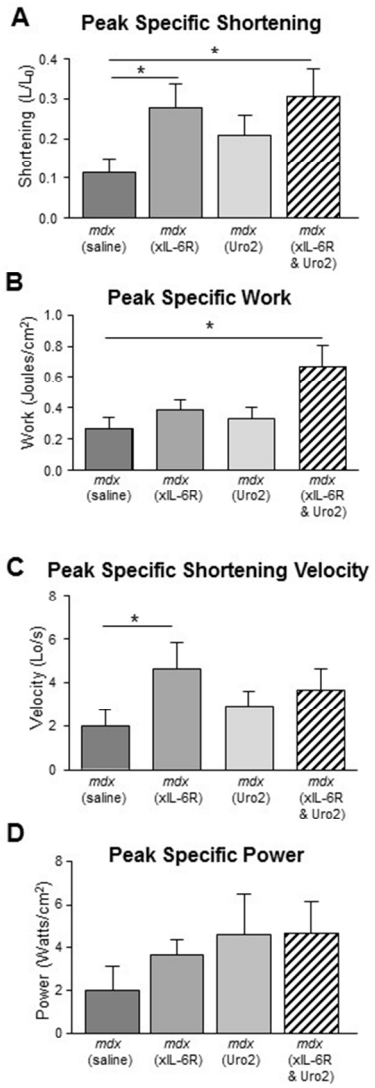


Figure 3

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