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Low-dose tamoxifen and raloxifene enhance therapeutic effect of ribitol in mouse model of FKRP mutation-related muscular

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ABSTRACT

Tamoxifen and raloxifene are selective estrogen receptor modulators (SERM) used to treat or reduce the risk of breast cancer as well as for osteoporosis. These drugs act to block estrogen in various tissues while having a pro-estrogen activity in others. Furthermore, they have been shown to have anti-fibrotic and anti-inflammatory properties making them a potential therapeutic option for muscular dystrophy. Previous studies by our lab and others have examined tamoxifen and raloxifene treatments in muscular dystrophy mouse models with success. These include numerous studies in the mdx mouse resulting in improved muscle function and pathology. Studies in our lab demonstrated long-term, dose dependent improvements in muscle function and pathology with daily treatment of up to 50 mg/kg tamoxifen and up to 100 mg/kg raloxifene. Additionally, ribitol has been shown to be effective in mitigating the dystrophic pathology in the FKRP-P448Lneo- mutant mouse, thus providing a potential supplement option for improving the effectiveness of current drugs. Here we examined the effectiveness of low-dose tamoxifen and raloxifene with and without ribitol supplement. FKRP-P448Lneo-mutant mice were treated with 1 mg/kg tamoxifen or 10 mg/kg raloxifene with and without 2 g/kg ribitol daily for 12 months. Muscle function was examined using grip force, treadmill, whole body plethysmography and echocardiogram at 6 and 12 months. Muscle histology and pathology was examined at 12 months. Muscle function improvements, specifically in treadmill distance and grip force, were seen after 6 months of treatment with combined treatment showing further improvement over each drug alone. Improvements were also noted in respiratory and cardiac parameters. Although there was a reduction in improvements after 12 months of treatment the treated animals retained higher function over untreated controls. Furthermore, an increase in alpha-dystroglycan glycosylation was noted in the combination treated groups as well as improvements in fibrosis and central nucleation in all treated groups but more prominent with the combined treatment. Taken together these data suggest the combinatorial treatment is effective over drug alone. These results demonstrate the potential benefit of low dose tamoxifen and raloxifene for the treatment of FKRP-related muscular dystrophies and other muscular dystrophies.

Introduction

O-manno glycosylation of alpha-dystroglycan (α-DG) is crucial for binding of the membrane to extracellular matrix proteins, maintaining muscle membrane integrity and protecting from contractile induced damage[1]. Dystroglycanopathies are a group of diseases caused by the reduced or lack of α-DG glycosylation (hypoglycosylation) which leads to a loss of muscle cell membrane integrity and repeated cycles of degeneration/regeneration. This process is accompanied by the accumulation of fibrotic and fat tissues in the muscle, leading to loss of function in both cardiac and skeletal muscles. Mutations in the fukutin-related protein (*FKRP*) gene are one of the major causes of dystroglycanopathies with clinical manifestation ranging from mild LGMD2I to severe congenital muscular dystrophy. Currently, there is no approved treatment for FKRP-related diseases. However, several experimental therapies have been identified by pre-clinical studies in animal models and two of them are under clinical trials. One promising treatment is ribitol administration which has been demonstrated with safety and efficacy in both animal models and in phase I/II clinical trials[2-10].

Ribitol is a natural metabolite widely present in organisms including mammals although its metabolic pathway has not been clearly understood. Recent studies demonstrate that ribitol can be converted to ribitol-5 phosphate (Rbo5P) by the kinase FGGY and subsequently converted to CDP-ribitol by CDP-Rbo pyrophosphorylase A/isoprenoid synthase domain-containing (CRPAA/ISPD)[11,12]. FKRP has been identified as a Rbo5P transferase, utilizing CDP-ribitol for the addition of Rbo5P to the core glycan chain of α-DG, providing the base necessary for the addition of a repeating disaccharide of xylose (Xyl) and glucuronic acid (GlcA) (matriglycan) required for the binding of the extracellular matrix proteins[1]. Increase in the substrate CDP-ribitol enhances the efficiency of mutant FKRP function and, consequently, the levels of matriglycan. It has now been demonstrated that ribitol treatment indeed can increase levels of Rbo5P and CDP-ribitol in muscle tissues and is associated with improved muscle pathology and function in disease relevant mouse models with FKRP mutation. Studies by our lab using the FKRP-P448Lneo-(P448L) mutant mouse model have demonstrated recovery of matriglycan synthesis following ribitol treatment[10]. Ribitol treatment dose dependently increases matriglycan and improves muscle pathology and function[6]. A clinical trial is also underway to test ribitol in LGMD2I patients (NCT05775848) with positive Phase 2 results reported including improved matriglycan expression in muscles, improved serum CK and sustained 10 meter walk test (MLBio/BridgeBio results). No severe side effects have been identified. The safety and effectiveness of ribitol as a therapeutic for FKRP-related muscular dystrophy provide a potential means of supplementing other therapeutics such as gene or drug therapies to increase their effectiveness and possibly lower the required dose[13].

One of the pathogenic processes within dystrophic muscle is the persistent inflammation initiated by muscle degeneration. An inflammatory response is essential for normal skeletal muscle repair, but the chronic inflammatory environment impairs muscle regeneration, exacerbates the loss of muscle mass and accelerates accumulation of fibrotic and fat tissues within diseased muscles[14]. The process of fibrosis associated with inflammation involves many factors including transforming growth factor (TGF-β), which is activated chronically in dystrophic muscles, and induces the deposition of fibrotic proteins such as collagen replacing degenerated muscle fibers. Therefore, suppressing the inflammatory response and fibrosis has been the primary attempt as a potential therapy for muscular dystrophies in general, and remains one of the most practiced treatments despite well-recorded side effects and limited efficacy (reviewed in[15]). This line of drugs includes glucocorticoids, such as deflazacort, prednisone, and Vamorolone (VBP15).

Selective estrogen receptor modulators (SERMs) act on estrogen receptors (ERs) either as ER agonists or antagonists and induce a wide range of effects in a tissue and cell type specific manner. This is because estrogen receptors, as either a or b form, are widely present in nearly all tissues, but at different levels and in variable proportions. The use of SERMs therefore results in a broad spectrum of effects on functions of nearly all systems by differentially altering ERα and ERβ actions. SERM drugs have long been explored for treating diseases and its therapeutic effect have been well established for the prevention of breast cancer, bone loss and against inflammation and fibrosis. Other benefits include improvement to muscle repair and enhancements of muscle function likely

through stabilizing biological membranes and inhibition of apoptosis. These effects are considered desirable for treating muscular dystrophies with several reports of animal model trials of tamoxifen[16,17].

Our laboratory earlier examined the use of tamoxifen and raloxifene for their therapeutic potential in the P448L mutant mouse. The mice were treated with tamoxifen and raloxifene at doses ranging from either 2, 10 and 50 mg/kg/day or 50 and 100 mg/kg/day respectively for 12 months[7]. Treatments of each drug resulted in improved grip strength as well as running time and distance via treadmill exercise. Furthermore, enhanced cardiac and respiratory function were noted as well as a reduction in bone loss. These positive effects are apparently dose-dependent. No significant adverse effects were noted in the animals treated with 50 mg/kg raloxifene. However, tamoxifen treatment causes severe adverse effects on male reproductive organs and hernia with varying degree in severity, dose-dependently. Specifically, tamoxifen at the dose of 50 mg/kg greatly reduced the masses of vas deferens and testicles with seminal vesicles hardly visible. Reduction in size of these organs becomes less severe, but remained detectable even at the dose of 2 mg/kg. Therefore, while our results and those reported in animal models of other muscular dystrophies clearly suggest therapeutic potentials of tamoxifen for muscular dystrophy, the risks with high dose of the drug could well hinder its clinical application, especially for muscular dystrophies which requires life-long treatment.

Complementary mechanisms of action offered by SERMs and ribitol prompted us to examine the therapeutic values of a combined treatment of the SERMs with ribitol. In this study, we tested the long-term efficacy of tamoxifen and raloxifene at doses lower than the human equivalent in clinics in P448L mutant mice.

Methods

ANIMALS

The use of animals in this study was approved by the Atrium Health/Wake Forest Institutional Animal Care and Use Committee, Atrium Health Wake Forest School of Medicine (Charlotte, NC). All mice were housed in the vivarium at the Carolinas Medical Center following animal care and guidelines of the institute.

ANIMALS, DRUGS, AND IN VIVO DELIVERY METHODS

The P448L mutant mouse was used with C57Bl/6 mice as controls. The P448L mutant mouse contains a c.1343C>T point mutation resulting in an amino acid change from proline to leucine at the 448 position[33]. In each treatment group, 10 P448L or C57BL/6 mice (five female and five male mice), aged 8 weeks, were used. The mice were randomly assigned to control and different treatment groups, with littermates split between control and treatment groups.

Treated P448L mice were given either 1 mg/kg tamoxifen (Letco Medical, Decatur, AL) or 10 mg/kg raloxifene (Teva Pharmaceuticals, North Wales, PA) daily, 6 days per week, for 12 months via oral gavage with or without 2 g/kg ribitol (Biosynth, Gardner, MA). Control P448L mice were gavaged with the same amount of saline. Mice were sacrificed at scheduled time points, and muscles and organs snap frozen in cold isopentane (−80°C) and stored at −80°C. All attempts were made to analyze experiments blind to the treatment groups.

HISTOLOGY

As described previously, tissue sections (6 µm thick) were stained with monoclonal antibody IIH6C4 (Millipore, Temecula, CA) and Alexa Fluor 594–labelled goat–anti-mouse IgM (Invitrogen, Eugene, OR) for detection of functionally glycosylated α-DG. Sections were stained with hematoxylin and eosin or Masson's trichrome for histologic assessment of fiber size and centranucleation using the MetaMorph Basic Offline software version 7.7.0.0 (Molecular Devices LLC, Sunnyvale, CA). The percentages of fibrosis in diaphragm, heart, and biceps muscles were measured from Masson's trichrome staining (ImageJ software version 1.42; NIH, Bethesda, MD; http://imagej.nih.gov/ij). The level of serum components was determined by Charles Riverside Laboratories International (Wilmington, MA).

PROTEIN EXTRACTION AND WESTERN BLOT ANALYSIS

Total proteins were extracted from each muscle shaving using TX-100 buffer [1% Triton X-100, 50

mmol/L Tris (pH 8.0), 150 mmol/L NaCl, and 0.1% SDS] supplemented with protease inhibitor cocktail (Roche, Mannheim, Germany). Samples were homogenized in TX-100 buffer, and the supernatants were collected by centrifugation at 16,000 × g for 10 minutes. Protein concentration was determined by Bradford assay (Bio-Rad DC protein assay). The lysates were then loaded onto 4% to 20% Tris-glycine gel (Invitrogen, Carlsbad, CA). The proteins were transferred to polyvinylidene difluoride membranes with constant ampere at 200 mA for 2 hours in cold room (4°C). Polyvinylidene difluoride membranes were incubated with protein-free T20 blocking buffer (Pierce, Rockford, IL). The antibodies against α-DG (IIH6C4) and α-actin (Sigma, St. Louis, MO) were incubated in 20 mmol/L Tris (pH 7.4), 150 mmol/L NaCl, and 0.1% Tween 20 at 1:2000 dilutions. α-DG and α-actin antibodies were detected by horseradish peroxidase–goat anti-mouse IgM (Invitrogen, Carlsbad, CA) and goat anti-rabbit IgG–horseradish peroxidase conjugate (Bio-Rad, Hercules, CA), respectively. Blots were developed with electrochemiluminescence (PerkinElmer, Waltham, MA), and the images were exposed and processed by an LAS-4000 imaging system (Fujifilm, Valhalla, NY).

GRIP STRENGTH AND TREADMILL TEST

Grip strength was measured using a horizontal forelimb mesh and hindlimb angled mesh grip strength meter (Columbus Instruments, Columbus, OH). Five successful forelimb and hind limb strength measurements were recorded within 2 minutes with the highest and lowest measurements removed. Data was averaged and normalized to body weight (BW).

Treadmill test was performed on LE8700 treadmill (Panlab/Harvard Apparatus, Barcelona, Spain). Mice were acclimated with a 0-degree plane for 5 minutes at a speed of 7 cm/second. After the acclimation the speed was increased by 1cm/second every 30 seconds until a maximum speed of 25 cm/second was reached. A 0.2-mA shock grid was used throughout the procedure for motivation. Mice were considered exhausted if they remained on the shock grid for 10 consecutive seconds without getting off or 50% on/off within 1-minute period. Mice were also monitored for any signs of distress or injury and removed promptly if any signs appeared.

PLETHYSMOGRAPHY AND ECHOCARDIOGRAPHY

Whole body plethysmography was performed using the Emka Technologies Whole Body Plethysmograph with IOX Respiratory function analysis software (Emka Technologies, Falls Church, VA). Mice were acclimated to individual chambers for 5 minutes prior to recording respiratory data. Immediatelys following the acclimation period the data was collected for 15 minutes with continuous exchange of air.

Echocardiography was performed as previously described by Blaeser et al (Blaeser 2016) using the BioScan SonixTablet High Frequency Ultrasound (Analogic Ultrasound, Peabody, MA).

STATISTICAL ANALYSIS

All of the results were expressed as means ± SEM. The data were analyzed using two-tailed t-test to compare tamoxifen- or raloxifene-treated groups with untreated controls. $P \leq 0.05$ is considered to be significant.

Results

1. Recovery of glycosylated α-DG after 12 months of treatment with tamoxifen and raloxifene.

Hypoglycosylation of α-DG is the key characteristic of FKRP-related dystroglycanopathies and a primary therapeutic target. The glycosylation is detected using the IIH6 antibody with affected muscle of P448L mutant mice showing little to no IIH6 staining, except for those regeneration-related revertant fibers[18]. We examined glycosylation in the TA, heart and diaphragm of control and treated mice to determine if treatment with low doses of tamoxifen (1 mg/kg) or raloxifene (10 mg/kg) could enhance ribitol-induced matriglycan synthesis. We've previously demonstrated restoration of α-DG glycosylation in FKRP mutant mice within a range of doses of ribitol[6,10]. P448L mice treated with 2 g/kg ribitol showed an increase in glycosylation, especially in the diaphragm and heart tissues, consistent with our previous results[6]. Treatments with 1 mg/kg tamoxifen and 10 mg/kg raloxifene alone did not show significant restoration of α-DG glycosylation. Combined treatments of tamoxifen or raloxifene with ribitol showed a substantial increase in IIH6 staining in TA, diaphragm and heart (Fig 1A). This is confirmed by Western blot (Fig 1B). However, signal intensity in matriglycan is not clearly different between the combined treatment compared to ribitol alone.

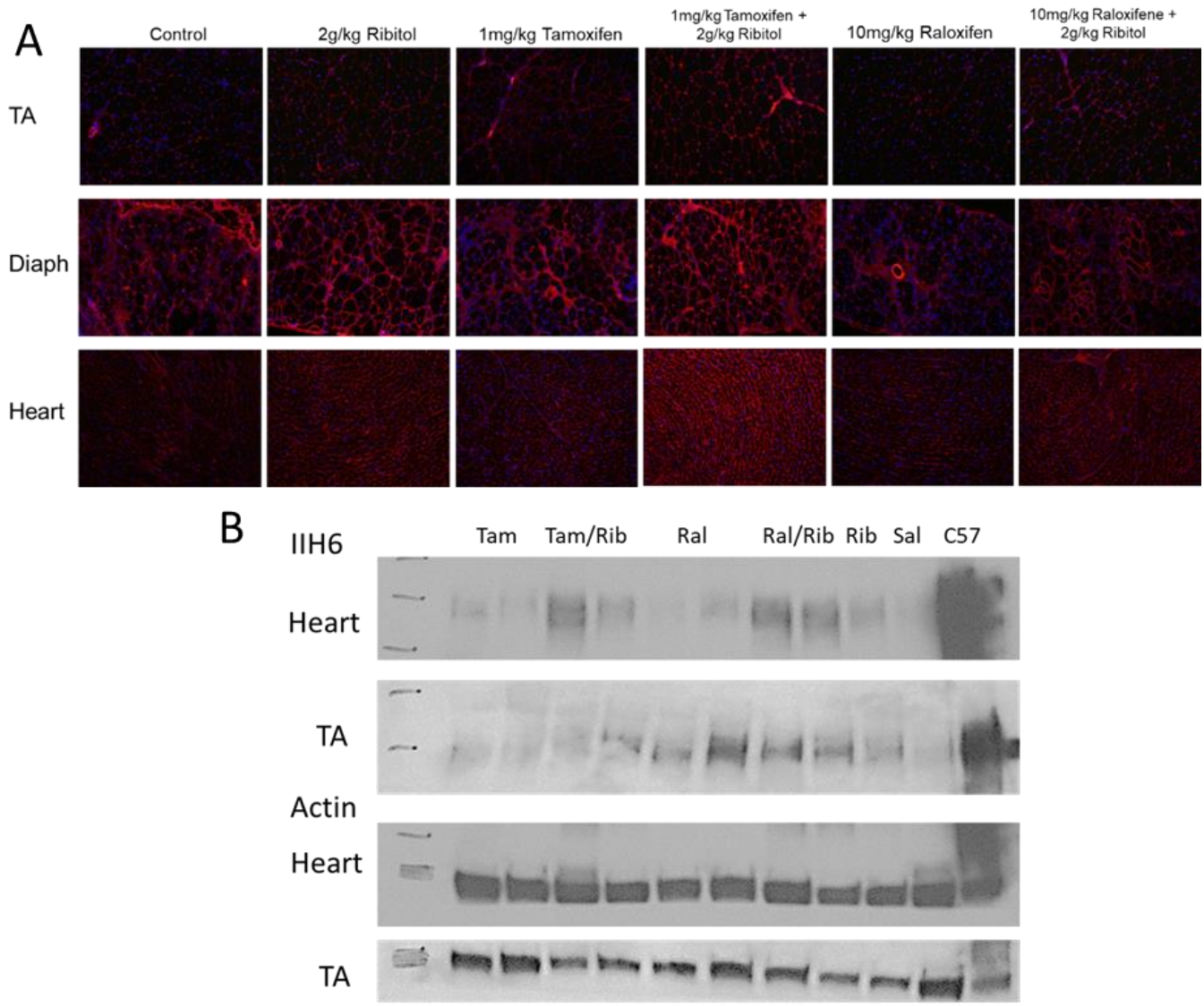

Figure 1. Matriglycan expression detected by IIH6 staining in tibialis anterior (TA), diaphragm (Diaph) and heart of FKRP-P448Lmutant mice. A) IIH6 staining of tissue sections. Treatment with 2 g/kg ribitol shows increased expression (membrane localized staining) in all three tissues. Treatment with 1 mg/kg tamoxifen and 10 mg/kg raloxifene shows no clear improvement. Combined tamoxifen and raloxifene treatment with ribitol shows similar increase in IIH6 signal intensity in TA, diaphragm and heart to ribitol alone. B) Western blot.

2. Histological analysis of P448L mutant mice following tamoxifen and raloxifene treatment.

A hallmark of pathology in the P448L mutant mouse is an increase in centrally nucleated fibers (CNF) due to repeated degeneration and regeneration. These CNFs are of various sizes and tend to be in clusters signifying regeneration. Control P448L mice had an average CNF of ~71% in the TA. Our earlier study showed that treatments with 2 mg/kg Tamoxifen and 50 mg/kg Raloxifene demonstrate a small, but statistically significant decrease in the number of CNF in the TA[7]. Treatment with 1mg/kg tamoxifen or 10 mg/kg raloxifene, with and without ribitol, for 1 year showed a similar reduction in CNF in the TA. A small reduction in the number of CNF was seen in the ribitol treated mice (68%) with a further reduction in raloxifene (68%) with statistically significant reductions in tamoxifen (64%) and raloxifene/ribitol combination (63%) therapies. The largest, statistically significant, reduction was seen in the tamoxifen/ribitol group with a reduction in CNF to less than 61% (Fig 2). On the other hand, only a small reduction in centrally nucleated fibers was seen in the diaphragm of raloxifene treated animals with 30% CNF compared to control mice with 31%. Ribitol treatment resulted in similar CNF levels with approximately 31%. However, an increase in CNF was seen in the raloxifene/ribitol treatment with 34% CNF and a further increase in tamoxifen and tamoxifen/ribitol both resulting in CNF of 35%. It should be noted that none of the changes in CNF percentage in the diaphragm are statistically significant.

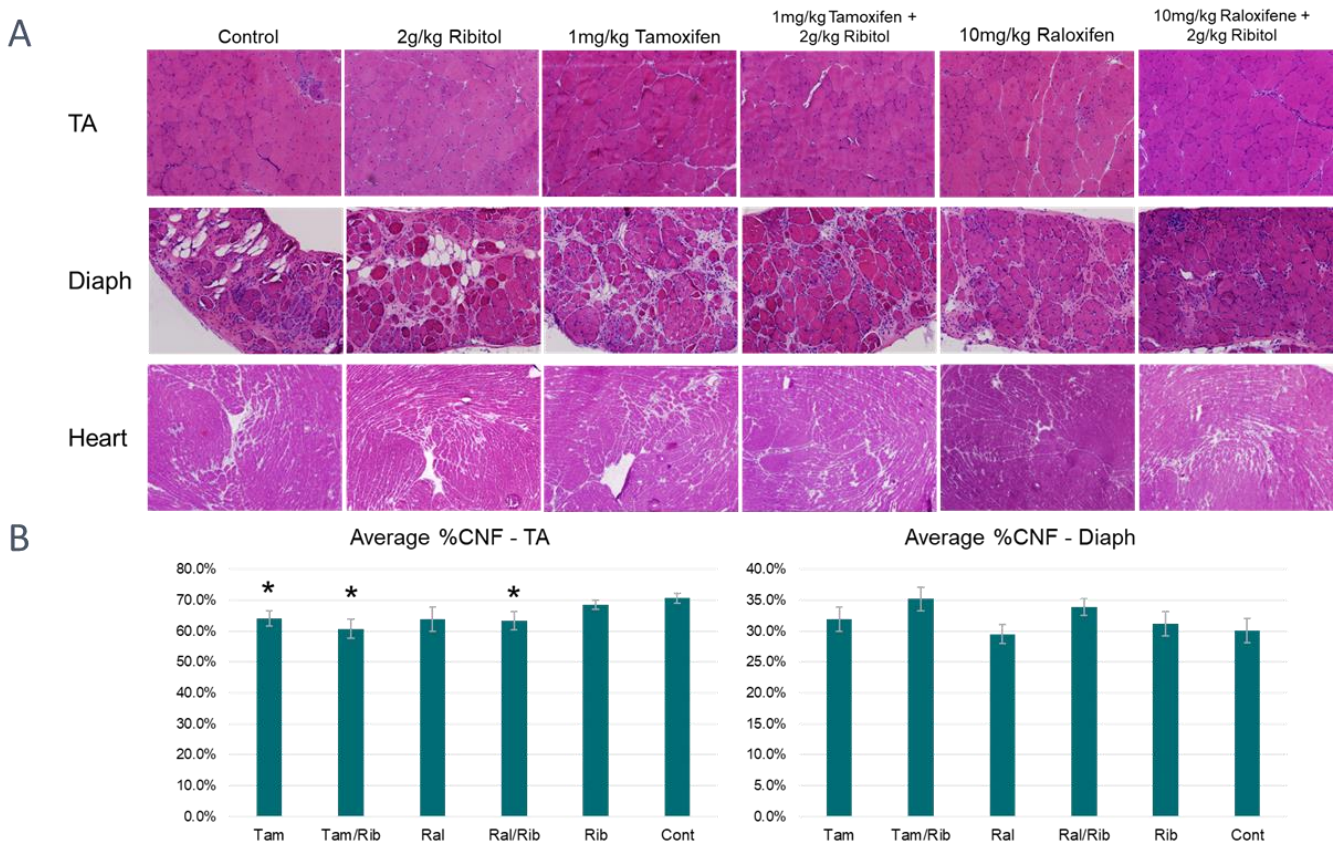

Figure 2. Histology of 12 month treated tissues from P448L mutant mice. H&E staining. A) Mice treated with tamoxifen with and without ribitol show improved numbers of muscle fibers and reduced fibrosis and streaks of mononuclear cells. Raloxifene with and without ribitol show the greatest improvement in diaphragm histology with least streaks of fibrosis and mononuclear cells. B) A reduction of CNF in the TA in all treatment groups with combination treatments showing fewer CNFs than single treatment. The % of CNF increases slightly in the diaphragm with combination s

The amount of fibrotic area was reduced with the treatment of SERMs and ribitol. (Fig 3). Fibrotic area consisted of approximately 4% of the tissue in the control TA muscles and was reduced by all the treatments especially the groups treated with tamoxifen/ribitol, raloxifene/ribitol and ribitol alone, down to 2.4, 2.4 and 2.0% of the tissue area respectively, However, statistical significance is only observed between the ribitol treated and the control group. The heart of control animals has limited fibrotic tissue, approximately 4.5% area. A reduction is detected in the treated groups with 4.0% in the tamoxifen alone and 3.6% in the tamoxifen combination groups. A further reduction is noted in the other three treatment groups with just above 3% fibrosis in both raloxifene treatment groups and ribitol alone, though not statistically significant. The progressive fibrosis is most evident in the diaphragm of the aged control P448L mouse accounting for about 60% total area of the tissue. Treatment with tamoxifen and raloxifene for 12 months with and without ribitol show a significant reduction in fibrosis. down to less than 50%. Interestingly, the combination treatments showed the most improvement with a reduction in fibrosis down to 33% and 28% in the tamoxifen/ribitol and raloxifene/ribitol treatment groups, respectively.

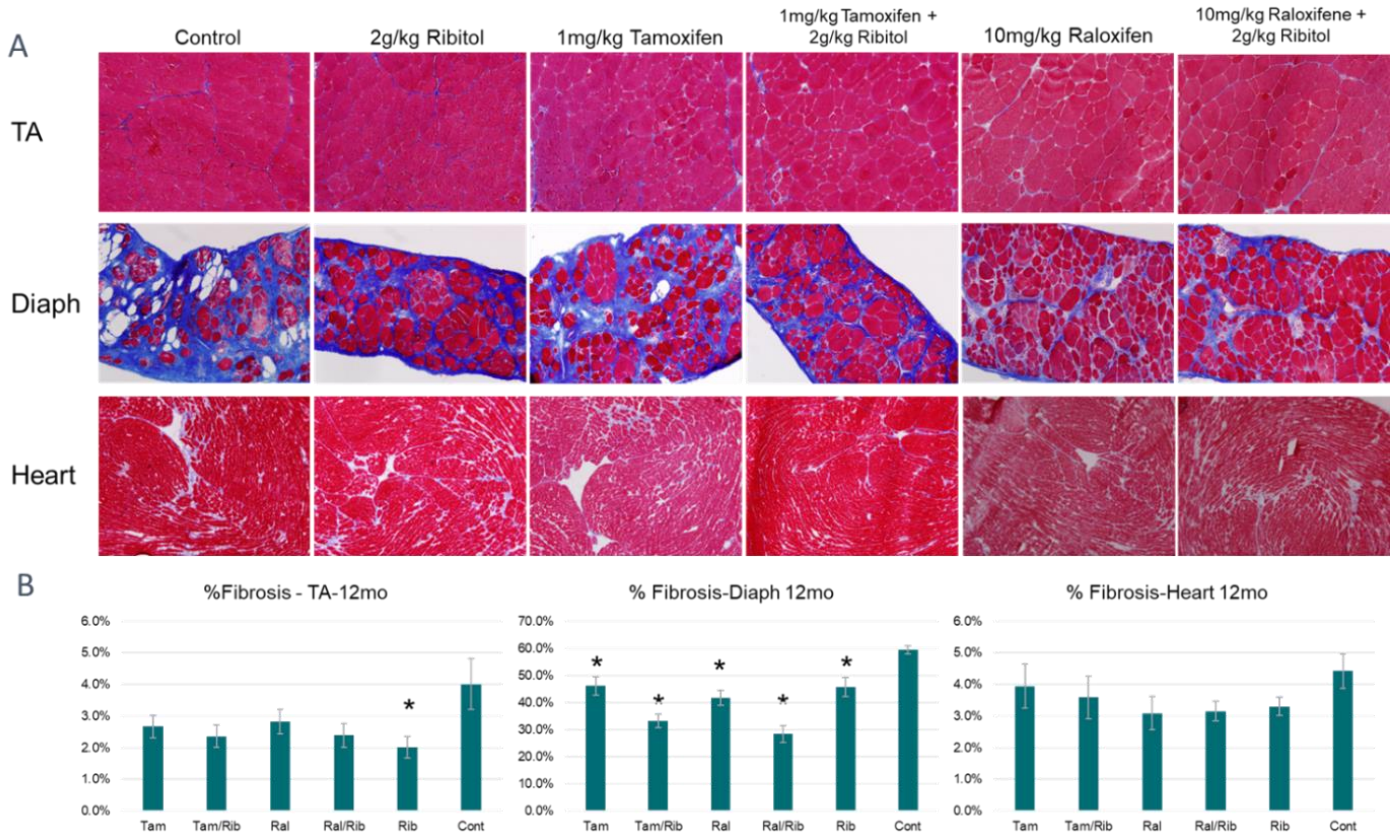

Figure 3. Histology of 12 month treated samples from P448L mutant mice. Mason's Trichrome staining. A) Fibrotic area shown as streaks (stained in blue) in tissue samples from all groups. B) Quantification of fibrosis represented as a percentage of the blue area to total tissue. TA, Tibialis anterior; Diaph, Diaphragm. * = $P<0.05$ compared to the control.

3. Evaluation of muscle function by treadmill and grip force

Muscle function was assessed twice, at 6- and 12-months post-treatment. Treadmill exercise showed that all the treatments improve performance when compared to the control P448L mice. Improved performance in both the mice treated with ribitol alone and tamoxifen alone was statistically significant in running distance at 12-month over the control. Furthermore, significant improvement was detected in both running distance and time at both time points in the mice treated with tamoxifen in combination with ribitol. Interestingly, treatment with raloxifene alone also significantly improved both running distance and time at both time points. Moreover, combined treatment of raloxifene and ribitol presented the most significant functional improvement, more than that achieved in the raloxifene alone at the 12 month time point, and with running distance and time almost doubled that achieved by the control mice (Figure 4). It should also be noted that the overall weight of the animals was only slightly less in the tamoxifen groups compared to control animals. However, animals of both raloxifene groups are significantly lighter at both 6-month and 12-month time-points. The association of significant improvement in muscle function with lower body weight is consistent with our earlier study of higher dose SERM treatment in the same mouse model[7]. These results together suggest that improvement in muscle functions may be linked to maintenance of proper body weight, or body weight itself can be an indicator of general health of the mice.

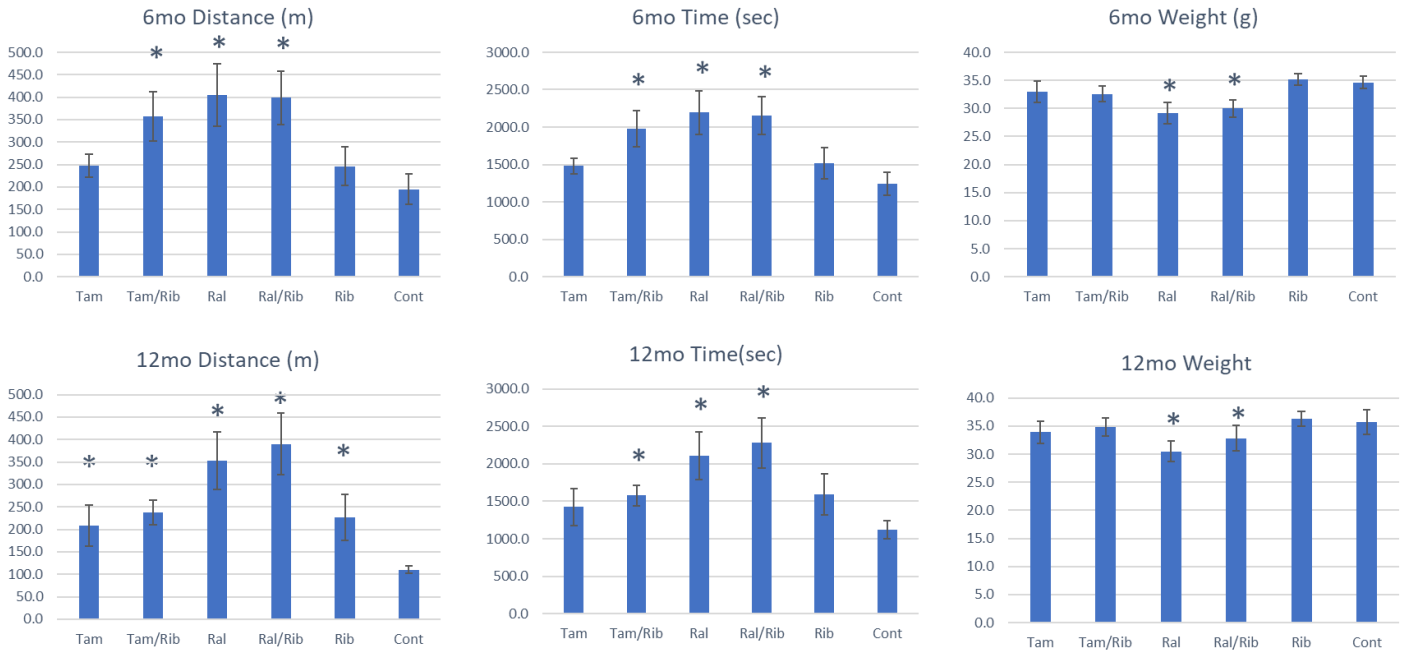

Figure 4. Treadmill and body weight 6- and 12-months post treatment in P448L mutant mice. Treadmill distance and time as well as weight was recorded for all groups at both 6 (top row) and 12 months (bottom row) post-treatment. Treadmill distance is shown in meters (m) with treadmill time as seconds (sec). Mouse weights are shown in grams (g) Tam, tamoxifen; Ral, raloxifene; Rib, ribtol; Cont, Control. * = $P<0.05$ compared to untreated control.

Measurement of muscle functions at 2 time points provides data for direct comparison of the same individual and groups of animals in treadmill performance over time. While there is a considerable variation between animals, a significant (~43%) reduction in performance at 12-month point was recorded when compared to that at 6-month point in the control mice. A much smaller reduction in performance was seen between the two time points in the tamoxifen treated groups, ~33% and 16% with and without ribitol, respectively. A minimal reduction was observed in the group with ribitol alone (~7%) especially in the raloxifene-treated groups, ~13% and only ~2% without and with ribitol respectively (Fig 5D). Also interesting is the change in body weight (BW) between control and treated groups. Control mice showed an increase of approximately 6.5% from 6 to 12 months post-treatment, while ribitol treated mice showed a smaller increase of ~3.4%. Tamoxifen treated mice also increased weight by ~5% and ~3% with and without ribitol, respectively. Interestingly, the mice treated with raloxifene alone and in combination gained just under 5% weight between 6-month and 12-month time points. (Fig 5B). However, BW increase was much slower during the first 6 months of raloxifene treatment. As a results, the raloxifene groups maintained an overall lower weight compared to all the other groups. It should also be noted that the raloxifene groups did not appear to be underweight as the average weight at 6 months post-treatment was ~30g with raloxifene alone and ~33g with combined treatment with ribitol after 12 months of treatment (Fig 4A). This is supported by the better maintenance of muscle function of the raloxifene treated mice than all other groups.

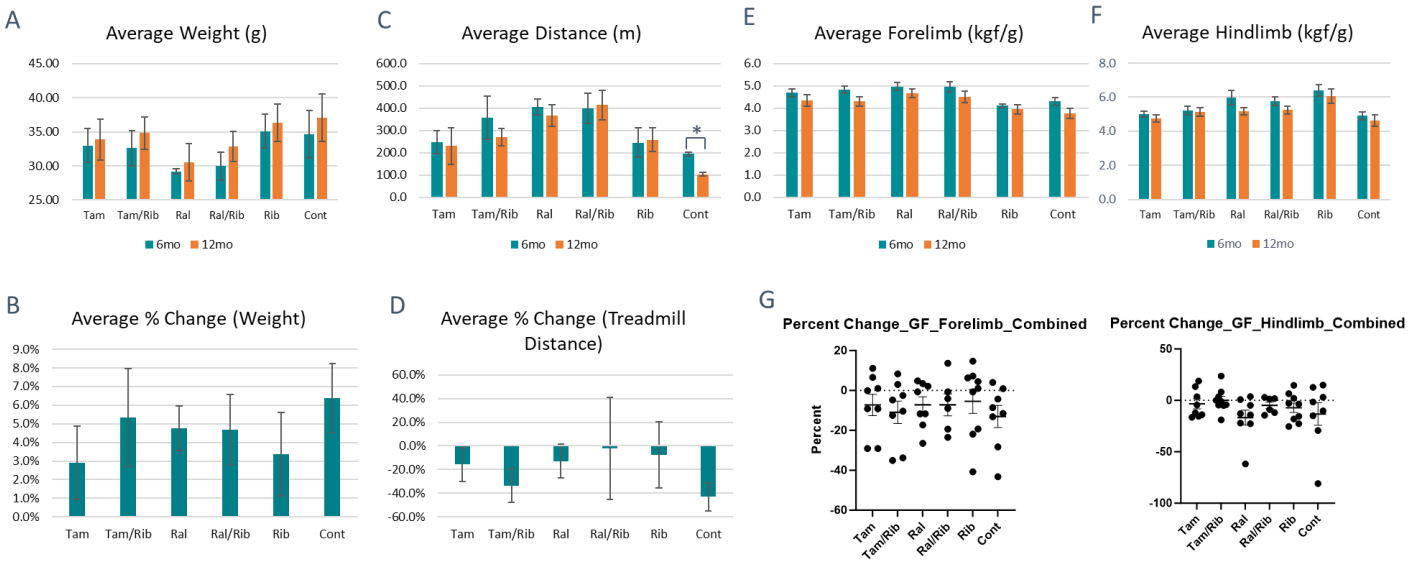

Figure 5. Change in body weight, treadmill distance and grip force from 6 to 12 months post treatment. A) Average body weight with and without treatment at 6 and 12 month treatment point. B) Percent change in weight from 6 to 12 months. C) Average treadmill distance with and without treatment at 6 and 12 month post-treatment start. D) Percent change in treadmill distance run. Average forelimb (E) and hindlimb (F) grip strength at 6 and 12 month post-treatment start. G) Percent change in forelimb and hindlimb grip strength with and without treatment. Tam, tamoxifen; Ral, raloxifene; Rib, ribtol; Cont, Control. * = P<0.05 compared to untreated control.

The improvement of the treatments on muscle functions was also demonstrated by grip strength measurement. Forelimb and/or hindlimb grip strength were improved at 6-month time point in all treatment groups, and significance was observed for forelimb strength for the groups with combination treatment and raloxifene alone (Fig 6). Hindlimb strength was significantly improved only in the groups treated with raloxifene and ribitol alone. These results are similar to the treadmill tests with both raloxifene treatment groups showing significant improvements over control. Significant improvement is maintained in hindlimb strength with ribitol treatment alone and in forelimb strength with raloxifene treated groups at 12-month compared to the control mice. However, improvements are not statistically significant in all other treatment groups. (Fig 6).

We also compared percent change in grip force between 6 and 12 month treatment points. Control mice showed a drop in strength of ~6.5% in the hindlimb and ~13% in the forelimb. A similar reduction in forelimb grip strength was seen with Tamoxifen with and without ribitol and with raloxifene alone. A drop of 12% in hindlimb force was also observed with raloxifene alone. However, reduction in hindlimb was much less in the tamoxifen groups (1% and 5% with and without ribitol respectively) than that of the control. Ribitol alone showed a reduction of only 4% and 5% in the forelimb and hindlimb, respectively. Interestingly, there is only a loss of ~4% forelimb strength and ~5% hindlimb strength in the raloxifene combination treatment allowing for the treated mice to maintain a grip strength above controls (Fig 5E-G). Overall, the functional data demonstrate efficacy with all the treatments and raloxifene groups present the most consistent improvements by both functional tests.

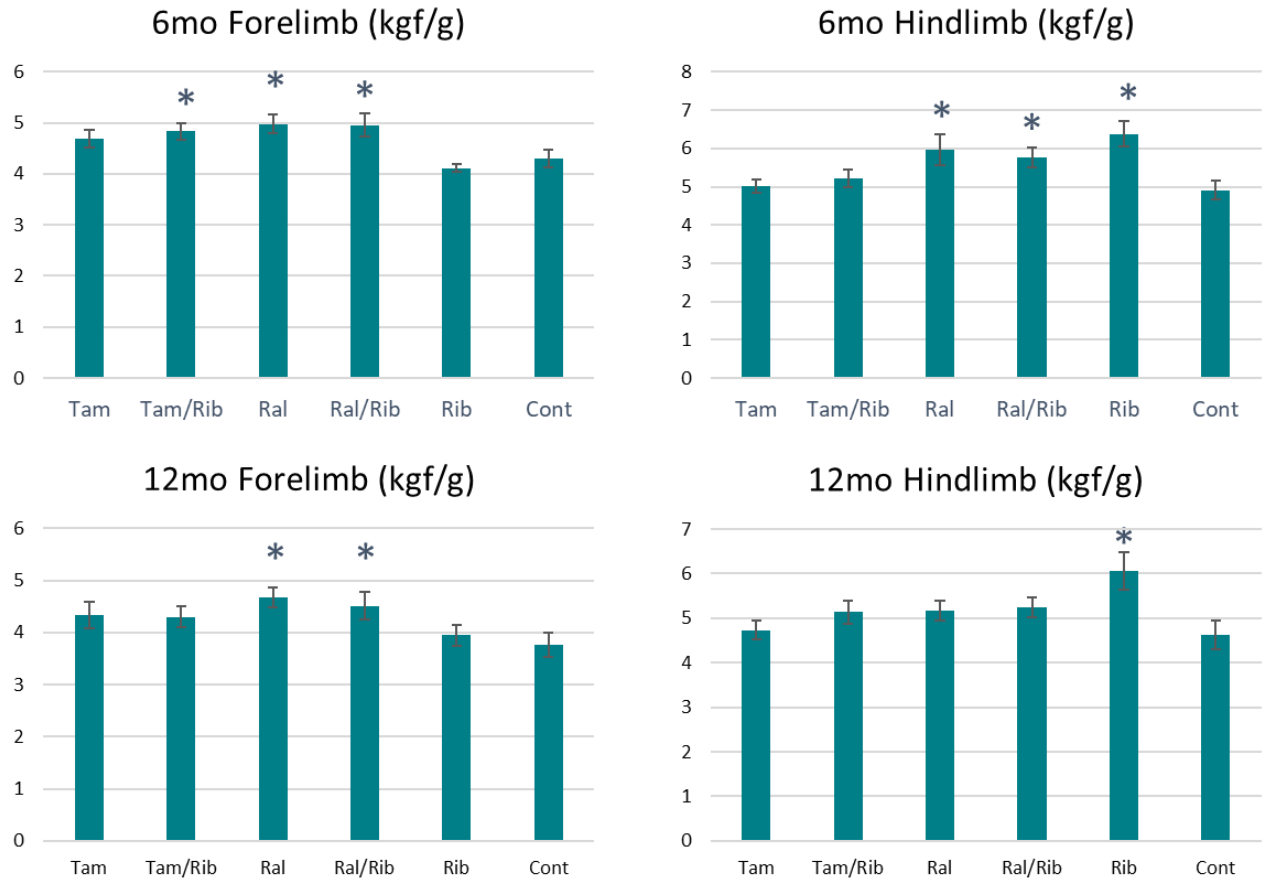

Figure 6. Grip force measurements at 6-month (top row) and 12-month (bottom row) treatment points. Forelimb and hindlimb grip strength measured at 6 and 12-months post treatment. Measurements represent as kilograms of force per gram (kgf/g). Tam, tamoxifen; Ral, raloxifene; Rib, ribtol; Cont, Control. * = $P<0.05$ compared to untreated control.

4. Plethysmography and ultrasound of P448L mice 12 months post treatment.

The progressive replacement of muscle with fibrotic tissue in the diaphragm results in significant dysfunction in respiratory parameters of the untreated P448L mouse. Using whole-body plethysmography, a number of respiratory parameters were measured to assess changes in respiration after treatment. Inspiratory (Ti) and expiratory (Te) times are increased and decreased, respectively, in the P448L mouse compared to C57 controls. While there is no change in Ti in the tamoxifen and ribitol treated groups a significant reduction was seen in all other treatment groups, though not reaching C57 levels. No apparent change was noted in the Te of tamoxifen and ribitol groups compared to controls, a significant increase however was seen in the tamoxifen/ribitol combination and raloxifene groups, reaching a similar level to C57 in the raloxifene/ribitol group (Fig 7A and B). Other respiratory parameters significantly affected in the P448L mouse is a reduction in the frequency of breath (F) and increase in peak inspiratory (PIF) which were improved with the two combination groups towards C57 control. The end inspiratory pause (EIP) is consistently longer in control P448L mice compared to C57 (Fig 7E). All treatments groups show significant reduction in EIP with levels at or below C57, and the largest decrease in the raloxifene groups compared to the controls. Interestingly, positive changes in end expiratory pause (EEP) is only detected with each of the SERM treatment, but not with combined groups (Fig 7C and D). Changes in PEF and MV was most obvious in the groups of raloxifene treatment, but significance is only detected with the raloxifene alone. This could be related to the body weight which is lower in the raloxifene alone group than in the combination group. Respiratory frequency (F) is normalized towards that in C57 mice in all group except for ribitol treatment alone with significance only in the two combination therapies when compared to the control P448L mice (Fig 7G). Interestingly, enhanced pause (Penh) is significantly higher in the P448L mutant mice when compared to the C57. All the SERM treated groups showed significant decrease whereas ribitol alone treatment increases the Penh although without significance.

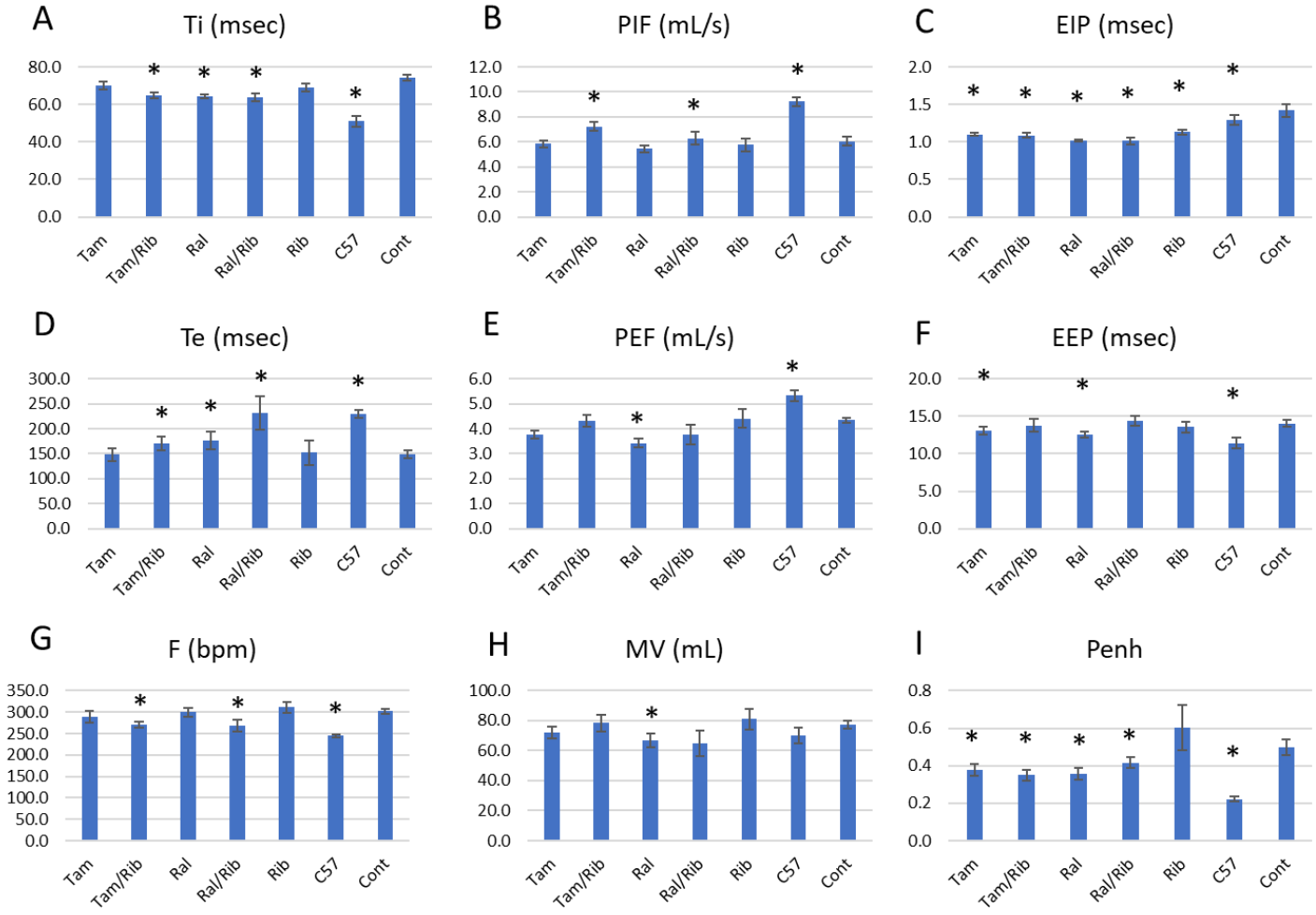

Figure 7. Whole-body plethysmography of the P448L- mutant mice. A and D) Ti and Te, Inspiratory time and Expiratory time measured in milliseconds (msec). B and E) PIF and PEF, Peak inspiratory flow and Peak expiratory flow measured in milliliters per second (mL/s). C and F) EEP and EIP, End expiratory pause and End inspiratory pause measured in milliseconds (msec). G) F, Respiratory rate measured in beats per minute (bpm). H) MV, Minute volume measured in milliliters (mL). I). Penh; Enhanced pause presented as index number. Tam, tamoxifen; Ral, raloxifene; Rib, ribtol; Cont, Control. * = $P<0.05$ compared to untreated control.

Cardiac function and morphology were also examined using high-frequency ultrasound. The P448L mutant mouse shows a reduced ejection fraction (EF) and stroke volume (SV) as well as an increase in myocardial thickness compared to C57 controls. This leads to a generally reduced overall cardiac output (CO)[19]. Treatment with tamoxifen and raloxifene, with and without ribitol, results in an increased EF of >50% compared to untreated controls (just over 48%) with statistical significance for the raloxifene alone group (Fig 8B). Furthermore, an increased SV is noted across all treatment groups, though statistical significance was not reached (Fig 8C). However, myocardial thickness is significantly reduced across all treatment groups with the biggest reduction noted in the raloxifene alone group (Fig 8D). These changes result in an improved CO in all treatment groups with combined treatments showing CO levels above those of drug alone (Fig 8A).

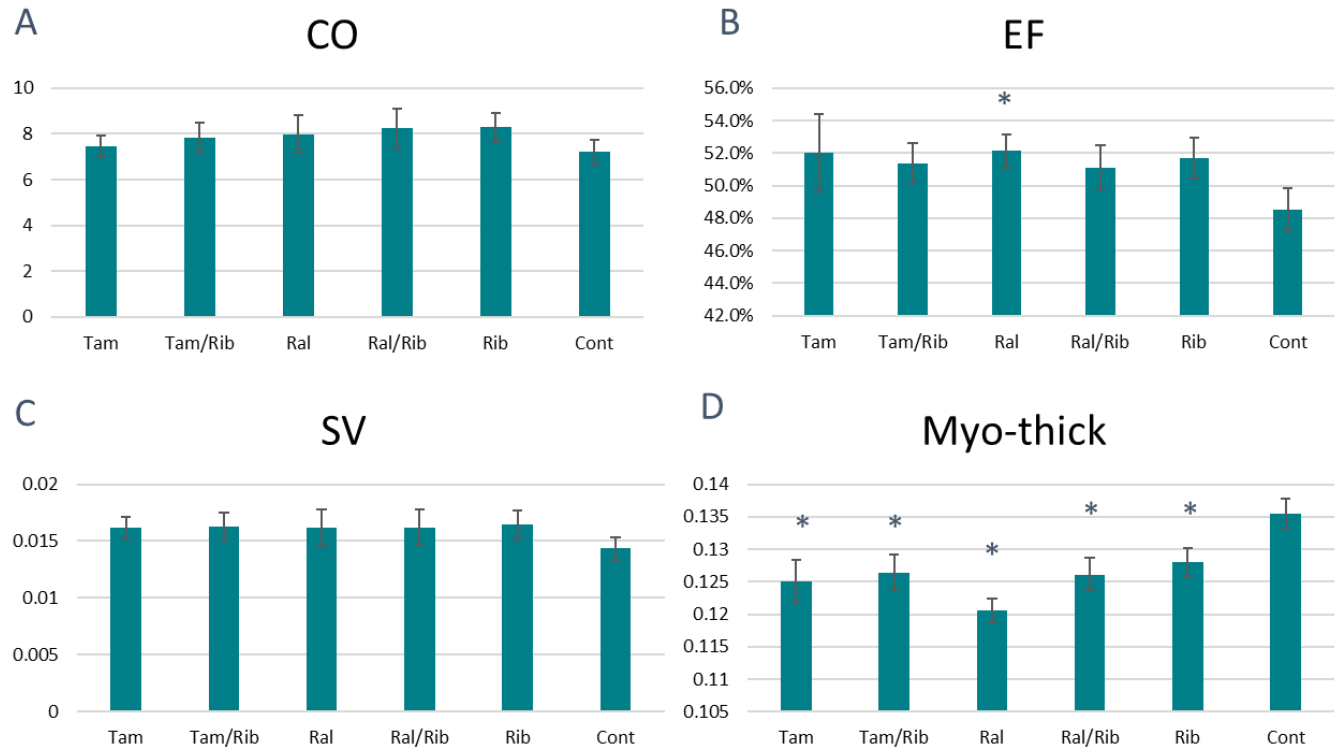

Figure 8. Echocardiogram of the P448L- mutant mice. A) Cardiac output (CO), the product of heart rate and stroke volume. B) Ejection fraction (EF), measurement of percent of blood leaving left ventricle during each contraction. C) Stroke volume (SV). D) Myocardial thickness (Myo-thick). Tam, tamoxifen; Ral, raloxifene; Rib, ribtol; Cont, Control. * = $P<0.05$ compared to untreated control.

Discussion

Clinical trials are currently underway for the use of tamoxifen for ALS (NCT01257581) and X-linked myotubular myopathy (NCT04915846), among other disorders. The use of tamoxifen for DMD was first examined by Dorchies et al in mdx mice. A moderate dose of tamoxifen, 10 mg/kg/day, was reported to improve muscle pathology and function with reductions in fibrosis[16,20]. Results from a 3-year clinical trial (NCT02835079) reported maintenance of muscle functions. A phase III clinical trial examined the effect of tamoxifen in two groups of DMD patients, aged 6.5 to 12 years with the ability to walk and under stable treatment with glucocorticoids and aged 10 to 16 years but unable to walk and not under glucocorticoid treatment (NCT03354039). Initial report published recently indicates that Tamoxifen at 20 milligrams (mg) once daily for 48 weeks is safe and well tolerated. The treatment also slows disease progression when compared to a placebo controls. However, the differences between the two groups are not clinically or statistically significant[21]. Another recent study in vitro reported that tamoxifen treatment ameliorates contractile dysfunction of stem cell-derived cardiomyocytes on bioengineered substrates[22]. Our earlier study in the P448L mutant mice shows a dose dependent effect of the treatment in both the efficacy and side effects. The lowest dose of 2 mg/kg daily produces improvements in histology and function, but still incurs detectable side effect. Daily dose of 20 mg for a life-long treatment may still risk significant side effects in male reproductive organs although this potential risk is not apparent after the 48 weeks of treatment from the abovementioned clinical trial. Furthermore, it is well acknowledged that long-term use of tamoxifen is associated with increased risk of endometrial cancer[23,24]. These risks seriously deter the willingness of patients and doctors to use tamoxifen for female muscular dystrophy patients. One possible solution to overcome this barrier is to establish a minimum but effective dose which avoids risk to both male and female patients. In this study we confirmed that a dose of only 1 mg/kg of tamoxifen still shows efficacy in mouse. Applying Food and Drug Administration recommended body surface area-based dosing conversion from mouse to human, the human equivalent dose would be less than 0.1 mg/kg body weight and equal to about 5 mg/kg for a 50 kg patient. This dose could be used as guidance for further clinical trials for efficacy and side effects in clinics.

One alternative means to avoid the side effects of tamoxifen is to explore the use of other SERMs with better safety record, specifically raloxifene. Raloxifene was first developed by Eli Lilly and approved by the FDA to treat postmenopausal osteoporosis more than twenty years ago. Raloxifene as an estrogen agonist acts on bone and lipid metabolism and as an estrogen antagonist for reproductive tissues[23,25]. Benefits of long-term use of raloxifene include enhancement to bone mineral density, decrease of serum concentrations of total and low-density lipoprotein cholesterol, and anti-inflammatory effect. In contrast to significant negative effect of tamoxifen on endometrium and male reproductive organs, raloxifene does not stimulate the endometrium proliferation[26]. Raloxifene does not cause significant changes in sperm production and quality, or male reproductive performance at doses as high as 100 mg/kg/daily[27]. Therefore, raloxifene has been used as medication for many diseases with well-established safety record. Daily doses of 60-120 mg have been widely used for postmenopausal osteoporosis, lowering the risk of invasive breast cancer in high risk population and as an adjuvant for antiviral treatment including hepatitis C and SARS-CoV-2 infection[28-31]. The reported effect on cell signaling and antioxidant free-radical scavenging leads to many reported applications to cardiovascular and neurodegenerative diseases such as dementia and schizophrenia[32]. Earlier we examined the raloxifene effect in the P448L mutant mice and reported efficacy with the low dose of 50 mg /kg body weight. Applying Food and Drug Administration recommended body surface area-based dosing conversion from mouse to human, with a factor of 12, the human equivalent dose would be 250 mg/day for a 60-kg individual which is clearly higher than the standard dose currently in use for many applications, including cancer and osteoporosis.

The results from this study now demonstrate that doses as low as 10 mg/kg body weight in mouse remain effective for improving muscle pathology and function. This dose will be the human equivalent dose of 50 mg daily for a 60-kg individual, well within the doses currently in clinics for long-term treatment of diseases such as osteoporosis. While treatment with a low dose of tamoxifen and raloxifene alone did not appear to restore glycosylation of α-DG, consistent with the earlier report using a high dose of tamoxifen and raloxifene[7], improved glycosylation was seen with combination treatment. However, this improvement is not above ribitol treatment alone suggesting a limited contribution from tamoxifen and raloxifene to matriglycan levels in the combination treatment. Additionally, a reduction in the number of centrally nucleated fibers is generally considered a sign of therapeutic efficacy. While reduced central nucleation is shown in the TA of all treatment groups an increase in centrally nucleated fibers is noted in most of the treated diaphragms, particularly the combination groups. Since CNF mainly represents regeneration, increase in CNF in all the treated groups except for the raloxifene treatment suggest a stronger regeneration capacity in the diaphragm compared to the aged control mice which may well have diminished capacity in regeneration at more than 13 months of age. This would be consistent with the lower density of fiber number in the diaphragm of the control mice (averaging ~150 fibers per 20X image area) when compared to that in the diaphragm of the treated mice (averaging >200 fibers per 20X image area). Clearly, difference in degree of degeneration, capacity in regeneration at different ages and amount of fibrosis determine the quantity of CNF in different muscle tissues. Improvements in pathology were also demonstrated with reduced fibrosis in heart, diaphragm and TA with combination treatment showing lower fibrosis than drug alone further supporting the beneficial effects of combination treatment. The histological improvements for both drug alone and combination therapies are further validated by improvements in functional results, particularly forelimb grip strength and treadmill performance as well as various plethysmography and echocardiography measures.

Conclusion

It needs to be reminded that efficacy achieved with the use of SERMs alone has been limited for treating muscular dystrophy in both human and in animal models. While the treatments alone can improve disease pathology and muscle function, averting disease progression is unlikely achievable. Combinatorial treatment is therefore clearly desirable. Here we show that combined treatment of ribitol and SERMs produces better improvement to the diseased muscles. Importantly, raloxifene and ribitol combination results in superior efficacy when compared to that achieved by individual

drugs used alone. This enhanced effect is observed in both skeletal and cardiac muscles. Furthermore, better respiratory function is also detected. The two drugs have different mechanisms of action and can be considered complementary to each other. Ribitol is a metabolite with its main effect on enhancing the glycosylation of α-DG and thus unlikely to cause any negative reactions between them. While ribitol is only applicable to the muscular dystrophy caused by FKRP mutations, we reason that the multifaceted benefits of raloxifene with limited side effect demonstrated in clinics allows the potential of its use alone and in combination with many experimental therapies for muscular dystrophies including gene therapy and oligonucleotide therapy for DMD and BMD. Furthermore, our results demonstrate, encouragingly, that the SERMs, especially raloxifene at clinically applicable dosage alone and in combination with ribitol, constitutes a highly promising treatment regime for FKRP-related muscular dystrophy. Low dose raloxifene alone could also benefit other muscular dystrophies.

Conflicts of Interest Statement

The authors have no conflicts of interest to declare.

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