

Perturbations in skeletal muscle sarcomere structure in patients with heart failure and Type 2 diabetes: restorative effects of (–)-epicatechin-rich cocoa

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Abstract

HF (heart failure) and T2D (Type 2 diabetes) associate with detrimental alterations in SkM (skeletal muscle) structure/function. We have demonstrated recently that (–)-ERC (epicatechin-rich cocoa) improves SkM mitochondrial structure [Taub, Ramirez-Sanchez, Ciaraldi, Perkins, Murphy, Naviaux, Hogan, Ceballos, Maisel, Henry et al. (2012) Clin. Trans. Sci. 5, 43–47]. We hypothesized that an improved mitochondrial structure may facilitate the reversal of detrimental alterations in sarcomeric microstructure. In a pilot study, five patients with HF and T2D consumed ERC for 3 months; treadmill testing [$\dot{V}O_{2\max}$ (maximum oxygen consumption)] and SkM biopsies were performed. Western blot analysis, immunohistochemistry and electron microscopy were used. We report severe perturbations in components of the DAPC (dystrophin-associated protein complex) as well as sarcomeric microstructure at baseline. ERC induced recovery/enhancement of DAPC protein levels, sarcomeric microstructure and, in a co-ordinated fashion, alterations in markers of SkM growth/differentiation consistent with myofibre regeneration. $\dot{V}O_{2\max}$ increased (~24%) but did not reach statistical significance. These initial results warrant further rigorous investigation, since the use of ERC (or pure epicatechin) may represent a safe and novel means of improving muscle function.

Key words: atrophy, dystrophin, mitochondrion, myofibre, skeletal muscle, Type 2 diabetes

INTRODUCTION

Fatigability, a common symptom in HF (heart failure) patients, is associated with SkM (skeletal muscle) atrophy, including reductions in mitochondrial volume and cristae abundance and myosin fibre content, leading to loss of function [1–3]. In patients with T2D (Type 2 diabetes), perturbations in SkM are also observed such as decreased intermyofibrillar mitochondrial content and abnormal lipid deposition [4,5]. HF and T2D frequently co-exist and may synergize to cause profound alterations in SkM.

The integrity of the DAPC (dystrophin-associated protein complex) is essential for maintaining SkM structure/function [6].

Members include dystrophin, β -DG (dystroglycan), utrophin, α -SG (sarcoglycan), β -SG, γ -SG and δ -SG, among others [7]. In HF patients, myocardial dystrophin levels are reduced [8]. However, no such information is available for any SkM DAPC members. It is reasonable to surmise that perturbations in members of the SkM DAPC in the setting of HF and/or T2D may translate into alterations in sarcomere microstructure, leading to an impaired function.

Currently, only exercise is known to improve HF- or T2D-induced changes in SkM structure/function. However, HF patients tend to be older and their mobility can be restricted by a variety of conditions and diseases, such as arthritis, thus there

Abbreviations: BNP brain natriuretic peptide; DAPC, dystrophin-associated protein complex; DG, dystroglycan; EM, electron microscopy; ERC, epicatechin-rich cocoa; HbA_{1c}, glycated haemoglobin; HDL, high-density lipoprotein; HF, heart failure; LDL, low-density lipoprotein; MEF2, myocyte enhancer factor 2; Myf5, myogenic regulatory factor 5; MyoD, myogenic differentiation; S6RP S6 ribosomal protein; SG, sarcoglycan; SkM, skeletal muscle; T2D, Type 2 diabetes; $\dot{V}O_{2\max}$, maximum oxygen consumption.

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Table 1 Characteristics of patients with HF and T2D before and after ERC treatment

All patients were undergoing continuous treatment with a diuretic, β -blocker, statins and either an angiotensin-converting enzyme inhibitor or angiotensin II type 1 receptor blocker. Deviations from such treatment for each patient are denoted in the table. EF, ejection fraction.

(a) General characteristics

| Characteristic | Patient 1 | Patient 2 | Patient 3 | Patient 4 | Patient 5 | Mean \pm S.D. |
|----------------|------------------------------------|---|------------------------------------|---|---------------------------------------|-----------------|
| Age (years) | 70 | 71 | 54 | 62 | 47 | 60.8 \pm 10.3 |
| HF stage | II | III | II | III | III | – |
| EF% | 47 | 41 | 55 | 22 | 26 | 38.2 \pm 13.9 |
| Medication | Amioradone, glipizide and warfarin | Digoxin, nitrates, warfarin, allopurinol and colchicine | Metformin, warfarin and oxybutynin | Spironolactone, metformin and glipizide | Digoxin, spironolactone and metformin | – |

(b) Effect of ERC treatment

| Parameter | Patient 1 | | Patient 2 | | Patient 3 | | Patient 4 | | Patient 5 | | Mean \pm S.D. | |
|-------------------------|-----------|-------|-----------|-------|-----------|-------|-----------|-------|-----------|-------|-------------------|------------------|
| | Before | After | Before | After |
| Cholesterol (mg/dl) | 176 | 188 | 136 | 205 | 202 | 160 | 159 | 137 | 165 | 158 | 167.6 \pm 24.2 | 169.6 \pm 26.8 |
| HDL (mg/dl) | 40 | 51 | 39 | 45 | 36 | 43 | 28 | 32 | 48 | 49 | 38.2 \pm 7.2 | 44 \pm 7.4 |
| LDL (mg/dl) | 87 | 102 | 66 | 102 | 131 | 95 | 64 | 30 | 95 | 83 | 88.6 \pm 27.2 | 82.4 \pm 30.3 |
| Triacylglycerol (mg/dl) | 243 | 176 | 156 | 292 | 176 | 109 | 364 | 374 | 108 | 131 | 209.4 \pm 99.1 | 216.4 \pm 113 |
| HbA _{1c} | 6 | 6.2 | 6 | 6.3 | 8.9 | 7.2 | 7 | 7.6 | 6.8 | 8.3 | 6.9 \pm 1.2 | 7.1 \pm 0.9 |
| BNP (pg/ml) | 78.2 | 74.5 | 316.2 | 264.1 | 57.4 | 25.4 | 537.8 | 122.9 | 104.8 | 50 | 218.9 \pm 206.2 | 107.4 \pm 94.7 |

is a need to identify alternative safe and effective therapeutic interventions [9,10]. We have demonstrated previously that the administration of the flavanol (–)-epicatechin to mice led to notable improvements in exercise capacity that correlated with increased mitochondrial volume, cristae abundance and capillarity in SkM and myocardium [11]. In an attempt to translate these observations, we pursued a pilot study in five HF/T2D patients using ERC (epicatechin-rich cocoa) for 3 months [12]. Administration of ERC led to a notable recovery of SkM markers of mitochondrial biogenesis and of cristae abundance, indicating improved microstructure and, possibly, bioenergetics. Interestingly, perturbations in mitochondrial biogenesis impair the ability of muscle to regenerate and repair [13]. Thus we hypothesized that improvements in mitochondrial microstructure may allow myofibres to ‘regenerate’ and thus display more normal architecture.

Modulators of SkM growth and repair include myostatin, follistatin and dysferlin. Myostatin and follistatin negatively and positively modulate SkM growth respectively [14], whereas dysferlin facilitates cell membrane (patch-like) repair [15]. The transcriptional factors MEF2 (myocyte enhancer factor 2), myogenin, Myf5 (myogenic regulatory factor 5) and MyoD (myogenic differentiation) participate in SkM cell differentiation [16]. Thus evidence favouring new myofibre production is likely to include the up-regulation of these factors.

On the basis of our previous results demonstrating the beneficial effects of ERC on mitochondria structure [12], we performed a subanalysis of samples to ascertain changes in sarcomere microstructure including members of the DAPC as well as regulators of SkM growth and regeneration, and compare before and after ERC treatment with those of healthy control muscle.

MATERIALS AND METHODS**Clinical information**

Five male patients with NYHA (New York Heart Association) stage II/III HF and non-insulin dependent T2D (47–71 years old) were recruited from the San Diego Veterans Administration Medical Center. General patient characteristics are described in Table 1. All patients had standard therapy for HF and T2D and were on stable medical management for ≥ 6 months. No changes in medication were made during the course of the study. This open label protocol involved patients consuming Hershey’s Extra Dark 60% Cacao chocolate and cocoa beverages containing 18 g of natural cocoa powder for 3 months with a total of 100 mg of (–)-epicatechin content/day with 390 calories and 18 g of fat. During the duration of the treatment period, patients were instructed to refrain from consuming other forms of chocolate and were monitored for treatment compliance every 2 weeks by telephone. Patients underwent muscle biopsies from quadriceps femoris before and after ERC consumption. For comparison purposes, three SkM biopsy samples from healthy subjects (male, aged 50–53 years) were obtained from the Medical Center using the same procedures. UCSD’s Institutional Review Board approved protocols and all subjects gave informed consent.

Exercise treadmill testing

We employed the standard Bruce protocol for treadmill testing. $\dot{V}O_{2\max}$ (maximal oxygen consumption) was estimated using the formula = 1.444T + 14.99, where T is the total time of the test expressed in min and fractions of a min [17].

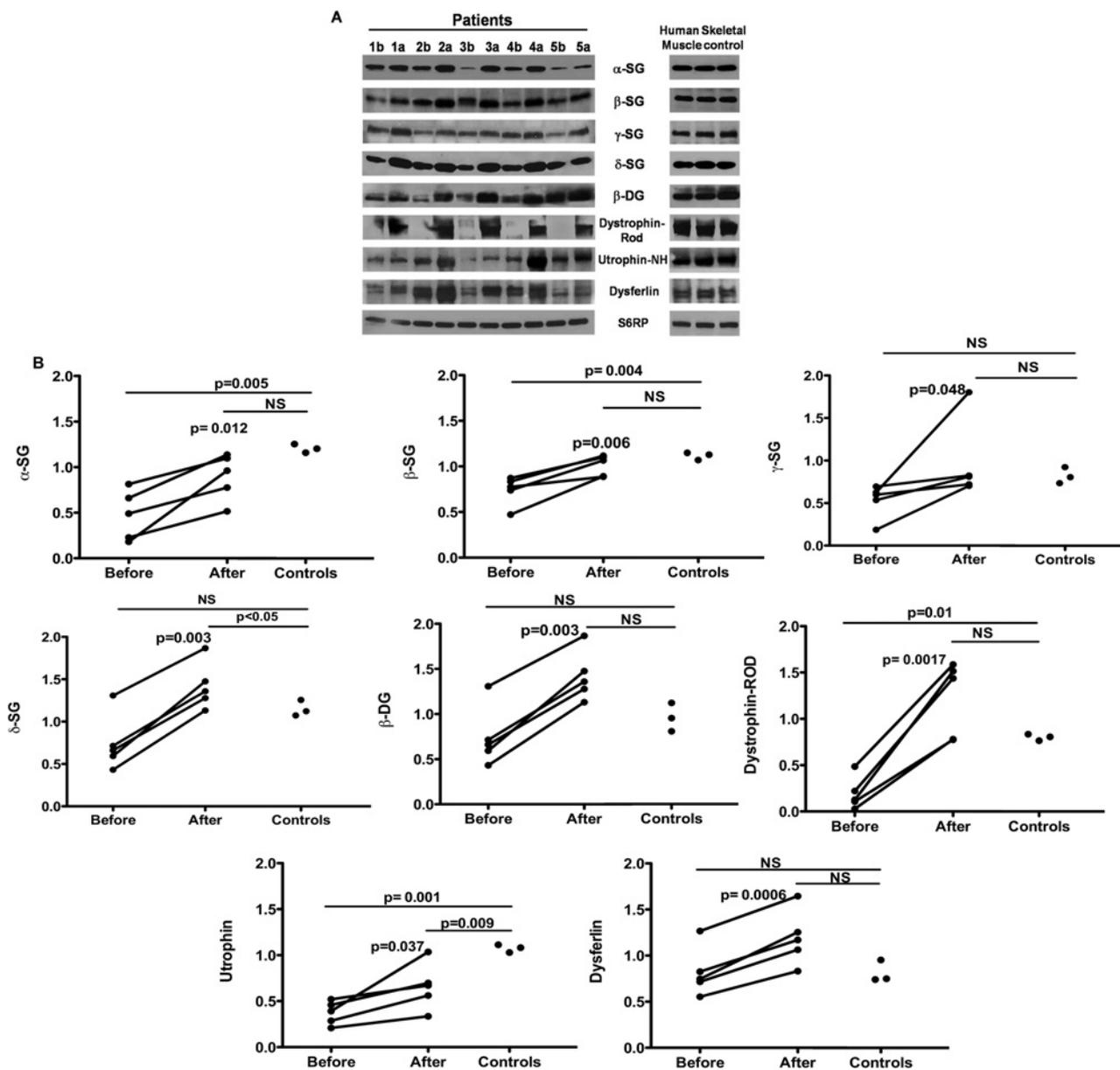


Figure 1 Comparison of changes observed in protein levels before and after treatment with ERC

(A) Changes observed in α -SG, β -SG, γ -SG, δ -SG, β -DG, dystrophin (rod), utrophin and, dysferlin. S6RP protein levels were used for normalization purposes and a representative image is included. Protein levels observed in control (healthy) muscles are shown for comparison. Grouped results and their statistical comparison are shown in (B). NS, not significant.

Biochemical and histological analysis

SkM samples were frozen or fixed for EM (electron microscopy). Western blots of SkM homogenates or plasma samples and immunohistochemistry were performed using standard methods [11,12]. Blot images were obtained within the linear range of X-ray film to ensure accuracy. Values were normalized for loading differences using S6RP (S6 ribosomal protein). Antibodies against dystrophin (rod, C- and N- terminus) β -DG (Vector Labs), utrophin (San Cruz Biotechnology), α -SC, β -SG, γ -SG, δ -SG (Novocastra), dysferlin, myostatin, myogenin, MEF2, MyoD, Myf5 (Abcam), follistatin (Santa Cruz Biotechnology) and S6RP (Cell Signaling Technology) were used. EM images of

SkM with an area of $134 \mu\text{m}^2$ each were scored by two independent, blinded, trained graders on a 1–4 scale, with 4 being highest quality sarcomere presentation. A total of 51 before-treatment and 54 after-treatment images were used.

Statistical analysis

Values are expressed as means \pm S.D. For data from Western blots, a paired Student's *t* test was used to compare differences observed before and after treatment (an Kolmogorov–Smirnov test was performed to assess for Gaussian distribution). ANOVA (and a post-hoc Tukey's analysis), and was used to compare differences observed before and after compared with control SkM.

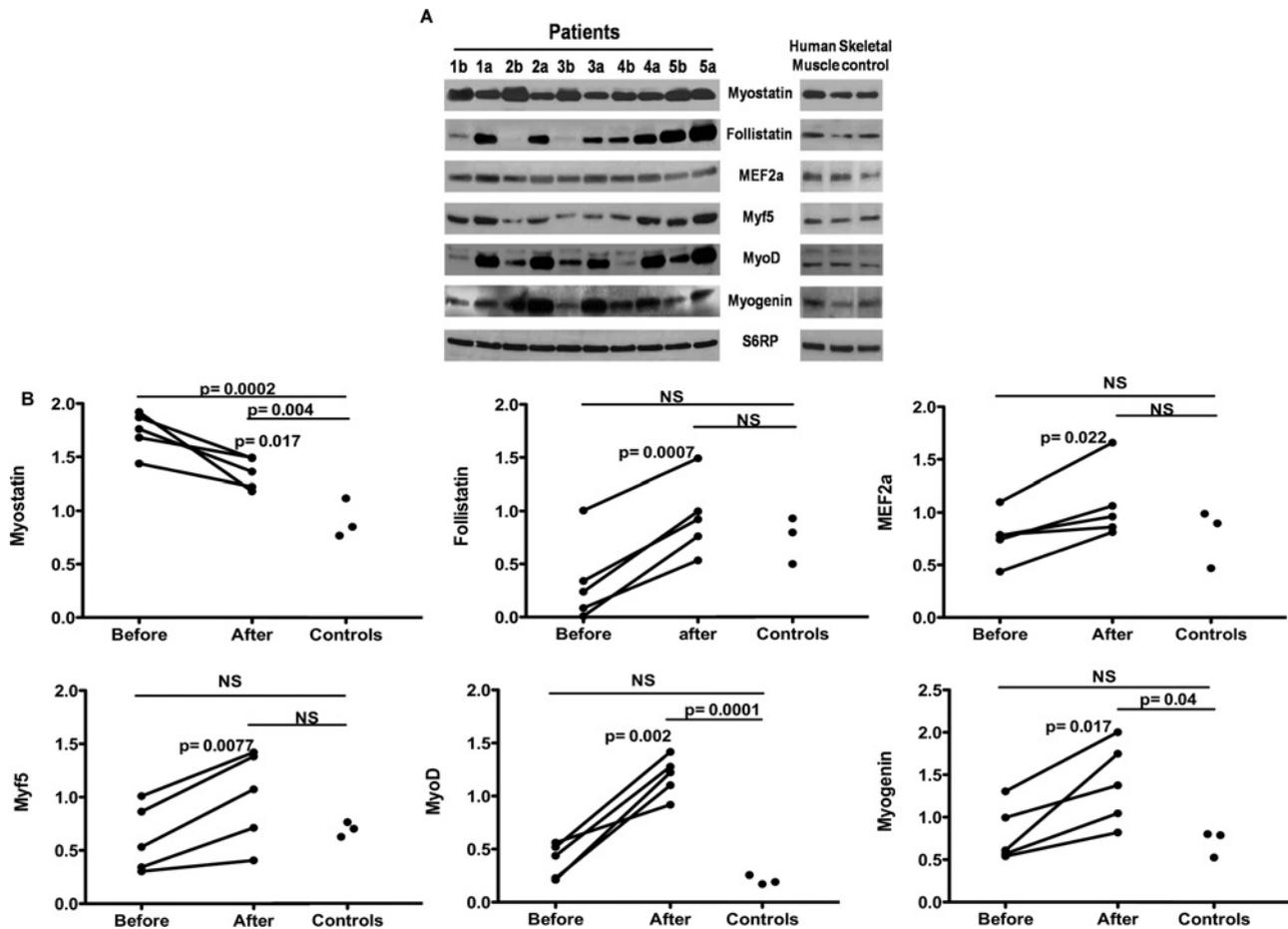


Figure 2 Comparison of changes observed in protein levels before and after treatment with ERC (A) Changes observed in myostatin, follistatin, MEF2, Myf5, MyoD and, myogenin. S6RP protein levels were used for normalization purposes and a representative image is included. Protein levels observed in control (healthy) muscles are shown for comparison. Grouped results and their statistical comparison are shown in (B). NS, not significant.

For the comparison of before compared with after EM images, a Student's *t* test was used. Statistical significance was defined when $P < 0.05$.

RESULTS

Clinical characteristics of the patients are summarized in Table 1. All patients reported no adverse effects during treatment. Significant, albeit modest, improvements were observed in HDL (high-density lipoprotein) levels and a trend in BNP (brain natriuretic peptide) levels ($P = 0.06$), whereas no major changes were detected in cholesterol, LDL (low-density lipoprotein), triacylglycerols and HbA_{1c} (glycated haemoglobin). There were no statistically significant changes in blood pressure, body weight and C-reactive protein (results not shown). The average increase in plasma (–)-epicatechin concentration 3 months after ERC administration was $1.06 \pm 0.3 \mu\text{M}$. $\dot{V}\text{O}_{2\text{max}}$ treadmill testing increased by 23.6% (18.6 ± 5.1 to 23 ± 2.2 ml/kg of body weight per min) was not significant ($P = 0.11$). The level of self-reported spontaneous physical activity was unaltered.

Figure 1(A) illustrates changes in α -SG, β -SG, γ -SG, δ -SG, β -DG, dystrophin (rod), utrophin, dysferlin before and after treatment compared with healthy (control) muscle, and Figure 1(B) plots the differences observed. At baseline, significant decreases compared with healthy controls were observed in α -SG, β -SG, δ -SG, dystrophin (rod) and utrophin expression, which were restored by ERC. γ -SG, β -DG and dysferlin levels, although not significantly decreased at baseline, increased with ERC.

Figure 2(A) shows changes in myostatin, follistatin, MEF2, Myf5, MyoD and myogenin, and Figure 2(B) plots the results. Myostatin levels were high at baseline and were significantly reduced by ERC (although remaining higher compared with controls). Conversely, follistatin levels were reduced at baseline and increased above controls with treatment. MEF2, Myf5, MyoD and myogenin levels were significantly induced with treatment. The plasma follistatin/myostatin ratio increased significantly from 0.65 ± 0.35 to 0.96 ± 0.35 ($P = 0.01$) with treatment.

Figure 3(A) shows representative normal (control), and before and after-treatment SkM staining for dystrophin (using C- and N-terminus-directed antibodies), suggesting the recovery of dystrophin levels with ERC. Microstructural changes observed

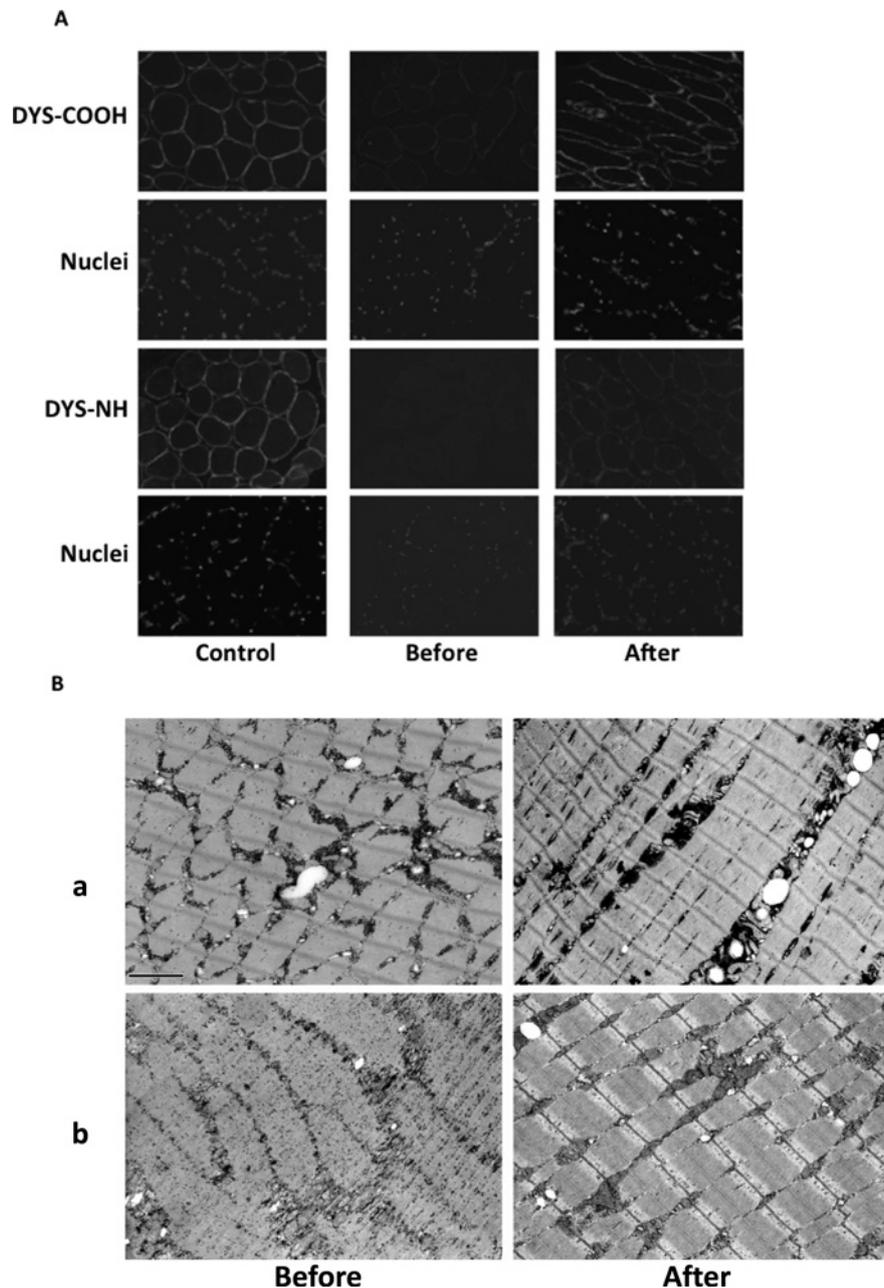


Figure 3 ERC-induced changes in dystrophin and sarcomeric structure

(A) Comparison of changes observed before and after ERC treatment dystrophin (DYS) levels in one (representative) patient by immunohistochemistry and nuclei disposition by 4',6-diamidino-2-phenylindole (DAPI) staining (including a control sample). C- and N-terminus-directed antibodies against dystrophin (-COOH and -NH respectively) were used ($\times 20$ magnification, $25 \mu\text{m}$ scale bar included). (B) Representative changes observed in sarcomeric microstructure by EM ($2 \mu\text{m}$ scale bar included) in two patients before and after treatment with ERC.

in sarcomeric organization from two representative patients are shown in Figure 3(B). At baseline, there was a severe perturbation of sarcomere microstructure which was restored with treatment. EM analysis of images obtained from all five patients yielded the following: the mean score from the first grader was 1.7 ± 0.1 (S.E.M.) before and 3 ± 0.1 ($P < 0.001$) after treatment; the mean score from the second grader was 1.8 ± 0.1 before and 3.1 ± 0.1 ($P < 0.001$) after treatment.

DISCUSSION

Initial findings indicate that, in HF/T2D patients, severe perturbations in SkM sarcomeric microstructure are evident. Alterations appear more severe than reported previously in HF alone such as those of myofibre atrophy and loss of mitochondrial volume [1–3], suggesting that the combination of HF and T2D (potentially compounded by aging) results in profoundly

greater derangements. A striking recovery (dystrophin) or induction of DAPC protein levels and sarcomeric microstructure occurred with a 3-month treatment with ERC. Accompanying these changes were 'co-ordinated' shifts in modulators of SkM growth/differentiation suggesting regeneration.

The DAPC plays an important role in muscle function, maintaining membrane stability during contraction and participating in signal transduction events [6]. Reductions observed in SkM DAPC members (in particular, dystrophin) are likely to have major implications for function, as can be inferred by the perturbations observed in sarcomeric microstructure. Given the magnitude of dystrophin loss and sarcomere perturbations, the SkM of these patients could be described as in a state of 'acquired muscular dystrophy'. Only one other study has reported changes in SkM members of the DAPC (specifically dystrophin) in humans with non-dystrophic diseases. Acharyya et al. [18] reported a substantial loss of dystrophin in terminal cancer cachectic patients. They postulated that the loss of dystrophin could account for the muscle-wasting phenomena observed. In support of this hypothesis, they documented that SkM overexpression of a dystrophin minigene was able to prevent cancer-induced muscle wasting in mice. Although no studies have examined the effects that T2D has on the DAPC complex in humans, severe reductions (>80%) occur in SkM dystrophin levels in a rat model of T2D [19]. A recent report using 20-month-old mice (as a model of aging-induced sarcopenia) also documented perturbations in SkM β -DG [20].

A number of studies substantiate the beneficial effects of cocoa consumption. A meta-analysis reported a 37% reduction in cardiovascular risk in subjects routinely consuming modest amounts of dark chocolate [21]. Dark chocolate contains high amounts of the flavanol (–)-epicatechin, a compound linked to cocoa's healthy effects [22]. We performed studies in animals that have shown the beneficial effects of (–)-epicatechin in models of cardiac ischaemic injury [23], including stimulatory effects on exercise capacity, cardiac and SkM mitochondrial volume and cristae abundance [11]. As pure (–)-epicatechin is unavailable for human consumption, we performed a study assessing effects on mitochondria volume, cristae abundance and biogenesis-related pathways using ERC for 3 months [12]. Positive effects were observed in modulators of biogenesis and cristae abundance. Improvements in mitochondria structure suggest a restoration of ATP production. ERC was capable of stimulating the recovery of SGs, utrophin and, most dramatically, dystrophin. β -DG was stimulated beyond normal levels. Improved EM scoring substantiated the favourable nature of these changes. The recovery of SkM DAPC probably represents the assembly of new complexes, which can improve contractile function and associated signalling [6].

As the results suggested a structural recovery, we speculated on the ability of muscle to undergo repair/regeneration. A co-ordinated shift in myostatin and follistatin levels was observed with ERC treatment. Myostatin levels, although remaining high, decreased with ERC, whereas those of follistatin recovered to control levels. Additional validation was obtained by significant increases observed in the plasma follistatin/myostatin ratio with ERC. Our results agree with those of Lenk et al. [24], where SkM

myostatin levels were high in HF patients and were reduced by ~25% with exercise. Of interest is that the inhibition of myostatin by follistatin binding has been associated with SkM growth and reduced fibrosis [14]. Signatures for muscle differentiation were also observed as shown by increases in MEF2, myogenin, Myf5 and, MyoD levels. Furthermore, dysferlin levels also increased, suggesting the patching of damaged cell membranes. Taken together, a coherent signature of molecular events has been reported, supporting ERC-induced microstructure improvements. As to the underlying mechanisms explaining the recovery of muscle microstructure, it is possible that at baseline a state of catabolism predominated and that following ERC treatment as mitochondria partially recovered, a more 'normal' metabolic state ensued allowing muscle regeneration to take place.

CLINICAL PERSPECTIVES

- HF and T2D associate with detrimental alterations in SkM structure/function and thus effective therapeutic interventions to prevent these effects are being investigated.
- In the present study, we found that levels of members of the DAPC and regulators of growth were modified in HF/T2D patients, which were accompanied by major perturbations in SkM structure. These effects were reversed by treatment with a cocoa formulation rich the flavanol epicatechin. This recovery observed in SkM structure should translate into improved exercise capacity; however, the 24% apparent improvement in $\dot{V}O_{2\max}$ observed in these patients did not reach statistical significance.
- These results are provocative and future clinical trials need to be powered to validate improved functional effects, be blinded and placebo-controlled. The use of ERC or pure (–)-epicatechin may hold promise as an effective treatment for muscle-wasting conditions. However, it will not replace exercise as a proven beneficial intervention, and their combined effects should be explored in the future.

AUTHOR CONTRIBUTION

Pam Taub was the lead clinical investigator for the study, and designed and managed the study with input from the group. Israel Ramirez-Sanchez was the lead biochemist, and designed and managed tissue analysis work with input from the group. Theodore Ciaraldi, Silvia Gonzalez-Basurto, Ramon Coral-Vazquez, Michael Hogan, Alan Maisel, Robert Henry, Guillermo Ceballos and Francisco Villarreal participated in study design, literature searches, Figure generation, data analysis and interpretation, and writing of the paper. Guy Perkins designed, implemented, performed analysis and generated Figures from microscopy studies and also participated in all other manuscript duties. Francisco Villarreal drafted the first and subsequent versions of the paper with input and key revisions by all authors, who reviewed and approved the final submitted paper.

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