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Choline administration attenuates aspects of the dystrophic pathology in *mdx* mice

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SUMMARY

Background & aims: Dystrophic muscle fibres are fragile and prone to breakage, leading to impaired Ca^{2+} homeostasis and excessive inflammation, resulting in muscle wasting and weakness. Choline, an essential water-soluble nutrient, is involved in multiple biological processes, including modulation of inflammation and oxidative stress. We tested the hypothesis that choline supplementation would ameliorate the dystrophic pathology in *mdx* mice.
Methods: Three-week old male *mdx* mice (n = 40) were fed control purified laboratory chow (CON; n = 20) or a choline-enriched diet (5 g/kg choline; CHL, n = 20) for four weeks. Rotarod performance, grip strength and running (wheel) distance were assessed during treatment. Markers of Ca^{2+}-handling, inflammation, oxidative stress and fibrosis were measured in the diaphragm, quadriceps muscle and the liver.
Results: Choline-treated *mdx* mice displayed less macrophage (CD68 -33%, $P < 0.05$) and collagen infiltration (−34%, $P < 0.05$) and reduced *Tgfβ3* mRNA expression ($P < 0.05$) in the diaphragm compared to untreated *mdx* mice. Choline supplementation increased maximal SERCA activity (38%, $P < 0.05$) and reduced markers of inflammatory (*Tnfα, F4/80* and *Cd206* mRNA, $P < 0.05$) processes compared with untreated *mdx* mice. In the liver there

Abbreviations: DMD, Duchenne muscular dystrophy; TMAO, Trimethylamine oxide; AST, Aspartate aminotransferase; ALT, Alanine aminotransferase.

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was a reduction in *Acta2* mRNA ($P < 0.05$) with choline treatment, as well as an improvement in serum ALT levels ($P < 0.01$). There were no differences between the groups for the whole-body functional analyses.

Conclusions: Choline supplementation attenuated the progression of the dystrophic pathology. Although choline did not alter functional performance, the reduction in fibrosis is clinically relevant for increasing the efficacy of therapies for DMD.

1. Introduction

Duchenne muscular dystrophy (DMD) is an X-linked neuromuscular disease affecting approximately 1:3600–6000 live born males [4] and is characterised by progressive muscle wasting and weakness. Patients experience reduced quality of life and die prematurely. DMD is caused by mutations in the *dystrophin* gene resulting in low expression or complete absence of dystrophin protein, rendering muscle fibres susceptible to contraction-mediated damage [4]. The loss of membrane integrity increases Ca^{2+} influx and impaired sarcoplasmic/endoplasmic reticulum Ca^{2+}-ATPase (SERCA) function [3], leads to increased production of reactive oxygen species (ROS), inflammation, impaired regeneration and eventually cell death [21]. In addition, the dystrophic pathology is associated with an inflammatory profile that alters metabolism in other tissues including liver [17]. Despite the current understanding of the underlying genetic cause and efforts to alleviate the pathology, there is still no effective treatment or cure for DMD.

One way to attenuate the disease progression is by modulating some of the underlying drivers of the pathology, including Ca^{2+} homeostasis, ROS and inflammation. Choline, an essential water-soluble nutrient, has multiple biological roles and is involved in inflammatory pathways, oxidative stress, neurotransmission, membrane composition and lipogenesis [23]. Interestingly, choline levels are reduced by almost 50% in skeletal muscles of *mdx* mice [22]. Although clinical studies have investigated the effects of choline supplementation on neuronal function [11], to our knowledge, no studies have assessed effects on skeletal muscle directly. We therefore tested the hypothesis that choline supplementation would ameliorate the dystrophic pathology by enhancing SERCA activity and improving Ca^{2+}-associated inflammation.

2. Materials and methods

2.1. Animals

Male *mdx* mice (3 weeks old; Animal Resource Centre, Canning Vale, WA, Australia) received a choline bitartrate enriched feed containing 5 g/kg choline (Sigma–Aldrich Co., Castle Hill, NSW; Australia) (CHL), or a control diet (modified AIN93G to contain 1.3 g/kg choline; Specialty Feeds, WA; Australia) (CON) for 4 weeks. Food intake was measured weekly. Experiments were approved by the Animal Ethics Committee of The University of Melbourne (1714140.2) and conducted in accordance with the Australian code of practice for the care and use of animals for scientific purposes as stipulated by the National Health and Medical Research Council (NHMRC, Australia).

2.2. Study design

Three-week-old mice received a choline bitartrate enriched feed for 4 weeks to ensure that we treated *mdx* mice during the critical period during which there is a peak muscle fibre degeneration,

regeneration and deposition of fibrosis. Food intake was measured over a 3-day period each weekend of the 4 weeks. Whole body functional assessments (rotarod, grip strength and running wheel) were conducted in week two and three of treatment as described previously [10]. Briefly, mice were given free access to a running wheel (~150 mm diameter) (Activity wheel, model 80820, Lafayette Instrument, Indiana, USA) for 24 h once per week (weeks 2 and 3). Due to the need to start treatment as young as possible, there was no time for a pre-treatment assessment. The running wheel of each cage was connected to the AWM Activity monitoring system, (Lafayette Instrument, Indiana, USA), where voluntary running measurements were automatically recorded. Data for analysis (week three) were collected from 7 pm to 7 am. Forelimb strength was assessed using a grip strength meter (Columbus Instruments, Columbus, OH) once per week (weeks 2 and 3). The maximum force achieved was normalised to their body mass at the time of assessment. The motor co-ordination and balance of mice was assessed twice (weeks two and three) on a Rotarod (Rotamex-5, Columbus Instruments). The test phase consisted of 3 trials separated by 15 min intervals. For each of these gross functional measurements, the week two assessment was used for familiarisation and week three for data analysis. At the end of the treatment period, blood was drawn from the tail vein of mice using a 27-gauge (G) needle following a 2-h fast, prior to anaesthesia. Blood glucose concentration was determined using a glucometer (Accu-Chek® Performa, Roche Diagnostics, VIC, Australia) and expressed in mmol/L. Mice were deeply anaesthetised deeply via intraperitoneal injection of sodium pentobarbitone (Nembutal; 60 mg/kg; Sigma–Aldrich). Serum was collected and tissues (including liver, quadriceps and diaphragm muscle) excised and stored at −80 °C.

2.3. Biochemical analysis

Serum choline and trimethylamine oxide (TMAO) were measured using an Agilent 1290 LC coupled to an Agilent 6550 QToFMS using targeted MS/MS methodology [8]. Aspartate aminotransferase (AST) and alanine aminotransferase (ALT) activity in serum were determined by enzymatic colorimetric assays [1,2]. Lipids were extracted from the liver as described previously [20] and triacylglycerol (TAG) content was determined using a colorimetric assay (Triglycerides GPO-PAP; Roche Diagnostics, Indianapolis, IN, USA).

2.4. Immunoblotting

Quadriceps (mixed) muscle (10–30 mg) was homogenised and immunoblotting was performed as described previously [6]. Antibodies were diluted in blocking buffer (5% BSA/TBST); SERCA1 (ab2818, Abcam, VIC, Australia; 1:1000) and SERCA2 (ma3-919, Thermo Fisher Scientific Inc., VIC, Australia; 1:1000) and membranes were incubated overnight at 4 °C. The density of bands was quantified using Image Lab software (Bio-Rad Laboratories) and normalised to total protein and expressed relative to the CON group.

2.5. q*PCR*

RNA extraction and cDNA synthesis from liver, diaphragm and quadriceps muscle (5–10 mg) was prepared as described previously [6,13]. qPCR was performed in triplicate using the CFX384 Real-Time PCR Detection System (Bio-Rad Laboratories) and data analysed and normalised to a reference gene (Rplpl1 [muscle] and *Hprt* [liver]) as described [6]. Primers were designed using the NCBI Primer BLAST tool from gene sequences obtained from GenBank. Primer pairs are listed in Table 1 or have been described elsewhere [5,10,19].

2.6. SERCA activity

Quadriceps (mixed) muscle (~80 mg) was minced with scissors in 200 μl of ice-cold homogenisation buffer (250 mM Sucrose, 5 mM HEPES, 0.2% sodium azide, pH 7.0) before homogenising in a glass homogeniser. Homogenates were subsequently sonicated (Microson Ultrasonic cell disruptor, ULAB

Table 1
Details of primer pairs used for qPCR.

Gene	Accession number	Forward primer (5′-3′)	Reverse primer (5′-3′)	Amplicon length
Acta2	NM_007392.3	CATCTTTCATTGGGATGGAG	TTAGCATAGAGATCCTTCCTG	97
Cd206	NM_008625	GTGGAGTGATGGAACCCCAG	CTGTCCGCCCAGTATCCATC	120
F4/80	NM_010130.4	CATCAGCCATGTGGGTACAG	CATCACTGCCTCCACTAGCA	256
Hprt	NM_013556.2	AGGGATTTGAATCACGTTTG	TTTACTGGCAACATCAACAG	116
Mmp2	NM_008610.3	ACAAGTGGTCCGCGTAAAGT	AAACAAGGCTTCATGGGGGC	187
Rplp1	NM_018853	GGCAGTCTACAGCATGGCTT	GAAAGGTTCGACGCTGACAC	139
Tgfβ3	NM_009368.3	GATGAGCACATAGCCAAGCA	GTGACATGGACAGTGGATGC	162

Primer sequences were designed using NCBI Primer-BLAST using sequences accessed through GenBank and checked for specificity using Nucleotide–Nucleotide BLAST search.
Rplp1, ribosomal protein, large, P1; Hprt, hypoxanthine guanine phosphoribosyl transferase; Acta2, actin, alpha 2, smooth muscle.

Instruments, Australia) on ice for 10 s then centrifuged at 5500 g for 10 min at 4 °C. SERCA activity in the supernatant was performed as described previously [9].

2.7. Histology

Serial sections (7 μm) were cut transversely through the diaphragm using a refrigerated (−20 °C) cryostat (CTI Cryostat; IEC, Needham Heights, MA, USA). Sections were stained with haematoxylin and eosin (H&E) to determine general muscle architecture; Van Gieson's stain for assessment of collagen infiltration [9]; and CD68 (Ab201845, Abcam., Cambridge, UK) for determination of macrophage infiltration [18]. Digital images of stained sections were obtained using an upright microscope with camera (Axio Imager D1; Carl Zeiss), controlled by AxioVision AC software (AxioVision AC Rel. 4.8.2; Carl Zeiss Imaging Solutions). Images were quantified with AxioVision 4.8.2 software. Digitally captured images (×120 magnification) with a minimum of three fields-of-view per muscle cross-section were processed and analysed as described previously [9,18].

2.8. Statistics

All values are presented as mean ± S.E.M. Unpaired Student's t-tests were used to compare between groups. P-values were reported for both significant differences ($P < 0.05$) and trends ($P < 0.10$) for all data.

3. Results

There was no difference in food consumption, fasted (2 h) blood glucose or change in body mass between the groups (Table 2). As *mdx* mice consumed 2.7 g feed per day, average choline intake was 13.5 mg per day. Consequently, there was a small 15% ($P = 0.10$) increase in circulating choline and a two-fold increase in trimethylamine oxide (TMAO; a choline metabolite) concentration in the choline-fed mice ($P < 0.05$, Table 2).

Choline supplementation did not affect grip strength or daily voluntary running, but there was a tendency for choline to improve latency to fall (Rotarod analyses; $P = 0.074$, Table 2). There was a significant reduction in inflammation and fibrosis in the diaphragm, as assessed by macrophage (CD68 -33%, $P < 0.05$; Fig. 1A and B) and collagen infiltration (Van Gieson's staining, −34%, $P < 0.05$) respectively (Fig. 1A and C) in CHL compared to CON treated mice. There was a tendency for choline treatment to reduce mRNA expression of *Tnfα* ($P = 0.091$), *F4/80* ($P = 0.10$), *Cd206* ($P = 0.057$, Fig. 1D), *Tgfβ3* ($P < 0.05$) and *Col1a1* ($P = 0.053$, Fig. 1E).

Choline treatment did not affect protein expression of SERCA 1 or SERCA 2a (Fig. 2A and B), but increased maximal SERCA activity in quadriceps muscle from CHL treated mice compared to control (Fig. 2C, $P < 0.05$). A decrease in *Tnfα* ($P < 0.05$), *F4/80* ($P < 0.05$), *Cd68* ($P = 0.053$) and *Cd206* ($P < 0.05$)

Table 2
Functional assessments and biochemical analysis of liver and serum collected from *mdx* mice with or without choline supplementation.

	CON	CHL	P-value
Δ Body mass (g)	14.9 ± 0.3	14.4 ± 0.5	0.375
Food consumption (g/day)	2.5 ± 0.1	2.4 ± 0.3	0.712
Fasted glucose (mmol/L)	10.4 ± 0.9	8.9 ± 0.3	0.121
Whole-body functional measurements			
Peak strength/body mass (g/g)	4.7 ± 0.2	5.2 ± 0.3	0.125
Daily voluntary running (km)	3.1 ± 0.2	3.2 ± 0.4	0.763
Latency to fall (sec)	114.8 ± 8.4	144.7 ± 15.1	0.074
Biochemical analysis			
Choline (relative to CON)	1.0 ± 0.0	1.2 ± 0.1	0.106
TMAO (relative to CON)	1.0 ± 0.2	2.2 ± 0.2[a]	0.000
ALT (nmol NADH/min/ml)	133.4 ± 35.5	20.0 ± 4.4[a]	0.009
AST (nmol NADH/min/ml)	250.3 ± 74.6	237.2 ± 37.0	0.875
Triglycerides (umol/g)	7.4 ± 0.9	8.3 ± 0.9	0.451
Liver mRNA analysis (fold change to CON)			
Tnfα	1.0 ± 0.3	1.0 ± 0.2	0.975
F4/80	1.0 ± 0.1	1.0 ± 0.1	0.846
Cd68	1.0 ± 0.1	0.9 ± 0.1	0.215
Cd206	1.0 ± 0.1	0.8 ± 0.1	0.207
Acta2	1.0 ± 0.2	0.6 ± 0.1[a]	0.038
Tgfβ1	1.0 ± 0.1	0.8 ± 0.1	0.112
Tgfβ3	1.0 ± 0.2	0.7 ± 0.1	0.252
Col1a1	1.0 ± 0.1	0.9 ± 0.2	0.580
Col3a1	1.0 ± 0.1	0.8 ± 0.1	0.248
Mmp2	1.0 ± 0.2	0.7 ± 0.1	0.120

Choline treatment did not affect the growth of the mice (change in body mass) from week 0–4, average daily food consumption for the treatment period, or blood glucose levels following a 2-h fast. Grip strength and running capacity were unaffected by choline supplementation, but there was an improvement in rotarod performance (n = 15–20). Dietary choline increased serum levels of TMAO and reduced ALT levels (n = 8–15). In the liver, there was a reduction in *Acta2* mRNA (n = 14–15). qPCR and choline/TMAO data are presented as relative to CON for ease of comparison. Values are mean ± SEM.
AST, aspartate aminotransferase; ALT, alanine aminotransferase; TMAO, trimethylamine oxide.
[a] denotes significant difference ($P < 0.05$) between treatment groups.

mRNA expression (Fig. 2D) was also demonstrated in this muscle, alongside a tendency for a reduction in *Col3a1* ($P = 0.1$, Fig. 2E).

Choline supplementation reduced serum ALT concentration ($P < 0.01$) but had no effect on AST or liver triglycerides (Table 2). In the liver, choline treatment reduced *Acta2* mRNA ($P < 0.05$) but had no effect on the expression of other genes associated with inflammatory or fibrotic processes (*Tnfα, F4/80, Cd68, Cd206, Tgfβ1, Tgfβ3, Col1a1, Col3a1* and *Mmp2*; Table 2).

4. Discussion

Choline plays a role in multiple biological processes and during periods of abnormal local and systemic inflammation, choline demand tends to outweigh availability [14]. As choline levels are reduced in skeletal muscle of *mdx* mice [15], we tested the hypothesis that choline supplementation would improve the dystrophic pathology. Choline-treated *mdx* mice had significantly less fibrosis and inflammatory cell infiltration in the diaphragm muscle compared with untreated mice. Although 4 weeks of choline treatment did not alter functional performance (measured by rotarod, running distance and grip strength), the reduction in fibrosis is important for increasing the efficacy of any therapy for DMD.

Fibrosis is a hallmark of the dystrophic pathology, which usually occurs because of inflammation, excessive ROS generation and chronic elevations of intracellular Ca^{2+} [4]. We have not assessed the direct mechanism by which choline decreased fibrosis but showed that choline can attenuate inflammation, an important regulator of collagen deposition. Choline, and the activity of the phosphatidylcholine synthesis pathway, regulate cytokine production in macrophages [16] and attenuate

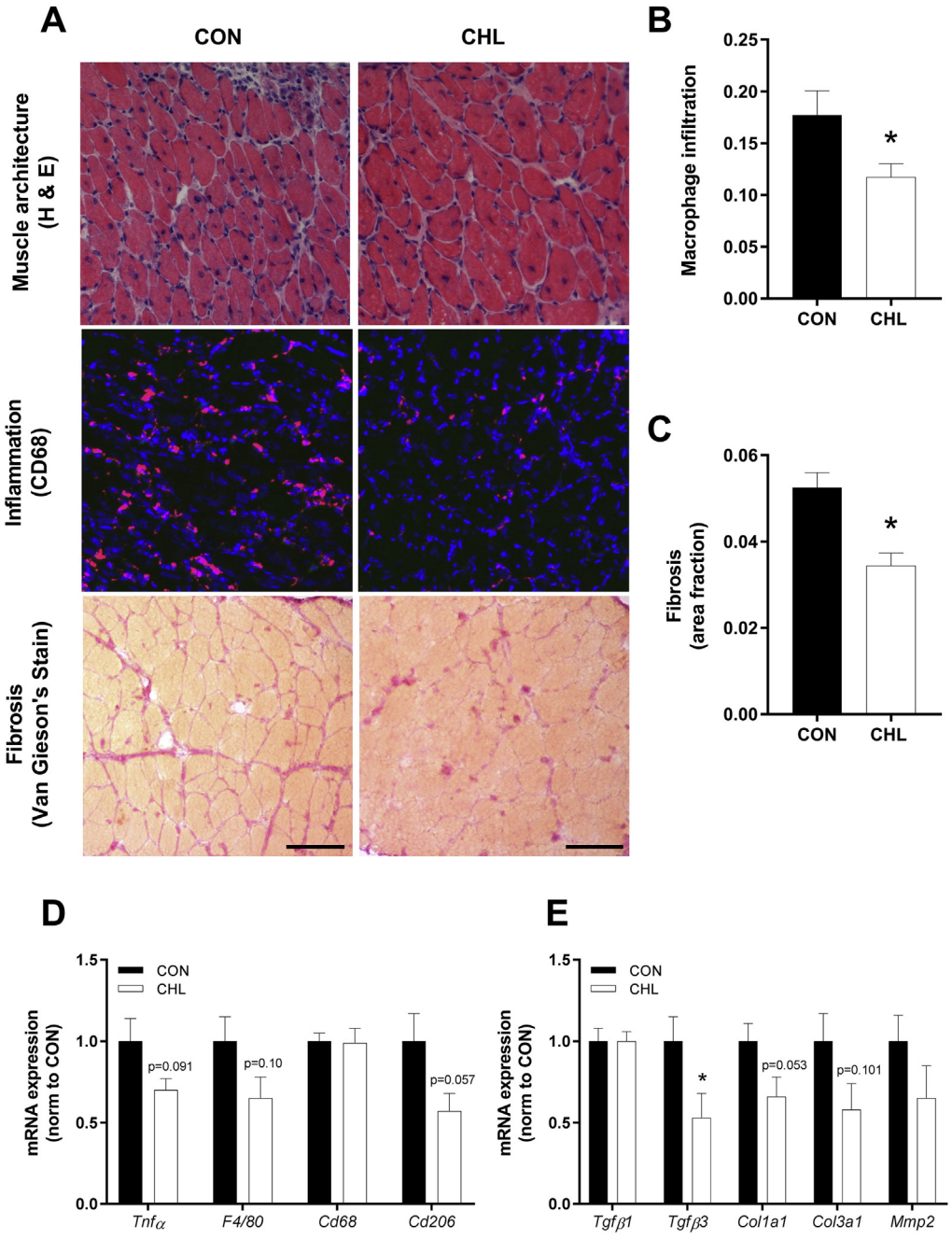

Fig. 1. Choline improves diaphragm muscle histology in *mdx* mice.To investigate muscle structure, we examined: general structure (Haematoxylin and eosin; H & E), inflammation via a macrophage infiltration marker (CD68) and fibrosis (Van Gieson's staining; expressed as area fraction [fibrosis area/total muscle area]) (A). There was a significant decrease in macrophage infiltration (B) and fibrosis (C) in CHL compared to CON treated *mdx* mice. Complementary qPCR data (n = 12–20) revealed decreased markers of inflammation (D, *Tnfα*, *F4/80* and *Cd206*) and fibrosis (E, *Tgfβ3* and *Col1a1*). In (A) pink represents macrophage infiltration (CD68) or fibrosis (Van Gieson's), with nuclei stained blue. Values are mean ± SEM (n = 10 CON and n = 10 CHL). * denotes significant difference ($P < 0.05$) between treatment groups. Scale bar is 100 μm.

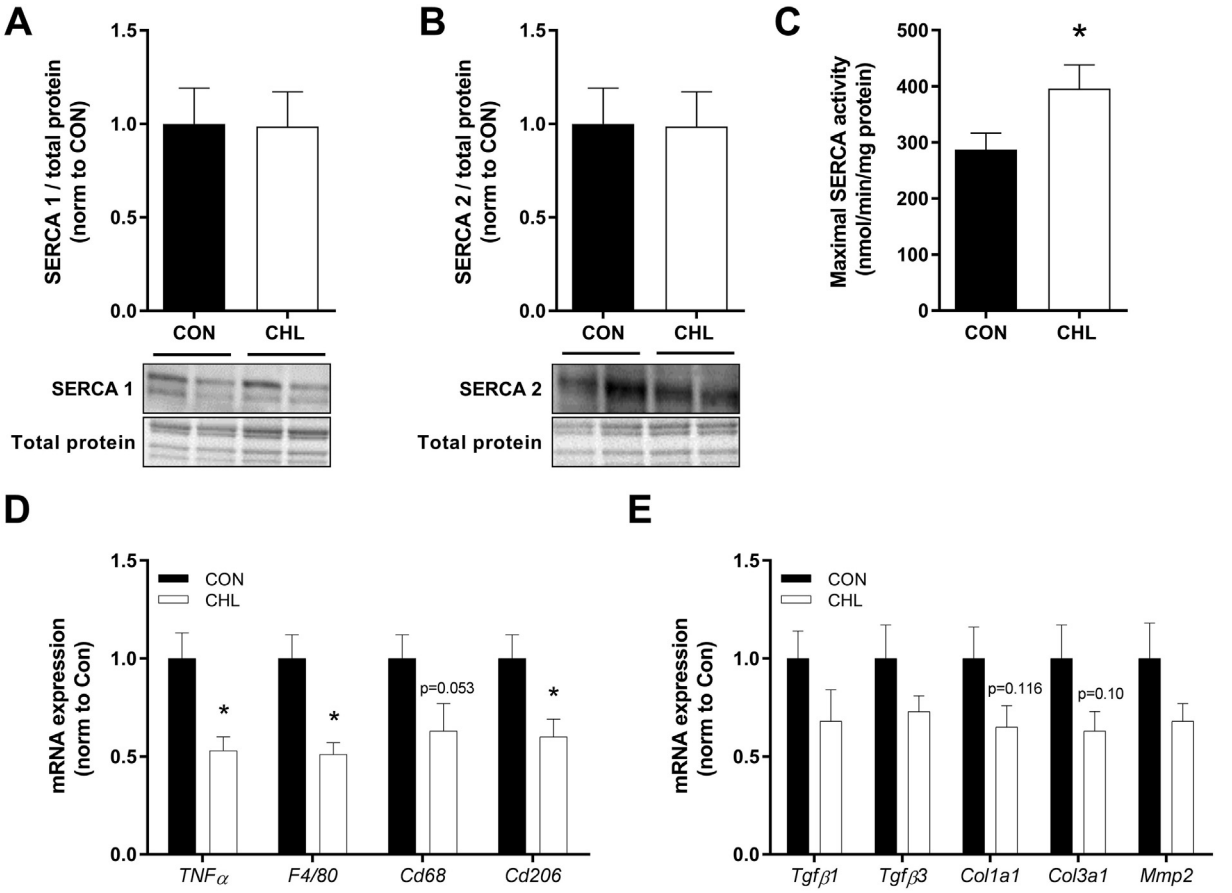

Fig. 2. Choline treatment increases maximal SERCA activity in quadriceps muscles of *mdx* mice.Choline treatment did not affect protein expression of SERCA 1 (A) or SERCA 2a (B; n = 14), but maximal SERCA activity was increased in CHL treated mice compared to control (C; n = 10). Complementary qPCR data conducted in the quadriceps muscles (n = 12–20) revealed decreased markers of inflammation (D, *Tnfα*, *F4/80*, *Cd68* and *Cd206*) and fibrosis (E, *Col3a1*). Data were normalised to CON for ease of visualisation. Values are mean ± SEM. * denotes significant difference ($P < 0.05$).

LPS induced increases in intracellular Ca^{2+} and the subsequent activation of macrophages and production of inflammatory cytokines [14]. Our results provide evidence for the anti-inflammatory properties of choline based on treated *mdx* mice having 30% less macrophage infiltration in the diaphragm muscle. Complementary qPCR data confirmed decreased expression of inflammatory cytokines (*Tnfα*) and macrophage markers (*F4/80* and *Cd206*) in quadriceps muscle and trending towards a decrease in the diaphragm. Our observations are consistent with choline's reported systemic anti-inflammatory effects in animal models of endotoxin shock [14] and neurodegenerative diseases [11].

Prolonged elevated intracellular $[Ca^{2+}]$ is a key feature of the dystrophic pathology in skeletal muscles of *mdx* mice. Increased $[Ca^{2+}]_{IC}$ results in numerous adverse downstream effects, including activation of NF-κβ, cytokine production, and activation of degenerative pathways involving Ca^{2+}-sensitive calpains, resulting in abnormal inflammation, loss of membrane integrity and eventually cell death [3]. This study is the first to demonstrate that choline supplementation can improve Ca^{2+}handling as evidenced by enhanced maximal SERCA activity in the quadriceps muscles of treated *mdx* mice. SERCA is responsible for the transport of Ca^{2+} from the cytosol into the sarcoplasmic reticulum and is therefore crucial for cellular homeostasis. The improvement in maximal SERCA activity in choline treated mice was not due to changes in SERCA protein expression suggesting that choline, likely via its metabolite phosphatidylcholine, results in conformational changes in SERCA that enhance its activity. These observations are consistent with previous studies from our laboratory where Hsp70 overexpression in muscles of dystrophic mice enhanced SERCA activity, without changing SERCA expression, and ameliorated aspects of the dystrophic pathology [9]. As such, choline's enhancement of SERCA may be a potential mechanism of attenuating inflammation, fibrosis and overall pathology [3].

The absence of dystrophin in skeletal muscle of *mdx* mice leads to changes in whole-body metabolism and inflammation. We have previously reported impaired glucose tolerance and decreased liver glycogen in *mdx* mice [17]. Since choline can modulate lipid metabolism [23] we also assessed the effect of choline supplementation on markers of liver metabolism and damage. Choline supplementation did not affect liver triglyceride content or the expression of pro-inflammatory genes but did reduce serum ALT levels and liver *Acta2* mRNA expression, a reliable marker of the early stages of liver fibrosis [7]. As serum ALT levels may reflect both liver and muscle damage [12], reduced ALT concentration suggest improvements in both liver and muscle homeostasis.

In the present study the improvements in Ca^{2+}handling in the quadriceps muscle and attenuation of fibrosis in the diaphragm did not improve whole-body function. As *mdx* mice have a slow progression of the pathology in the limb muscles at this relatively early age, the absence of change in grip-strength and rotarod performance following treatment is not surprising. In addition, similar improvements in fibrotic infiltration do not always similarly enhance muscle function, as we have shown in previous studies in *mdx* mice using other pharmacological or nutritional interventions [19]. While a nutritional intervention alone would be unlikely to cure a severe genetic disorder, our results suggest that based on its anti-fibrotic effects, choline treatment could potentially prolong the therapeutic window and enhance the efficacy of gene- and cell-based therapies.

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Conflicts of interest

All authors declare that they do not have any conflict of interest.

Statement of authorship

FA, MKC, GSL and RK conception and design of research. FA, MKC, JT, TN, GSL, RK and MKM performed experiments. FA and MKC analysed data. FA, MKC, GSL, RK and MKM interpreted Results. FA and

MKC drafted manuscript. FA, MKC, MKM, MJW, GSL and RK edited and revised manuscript. FA, MKC, JT, TN, MKM, MJW, GSL and RK approved final version of manuscript.

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