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The effects of cold water immersion and active recovery on inflammation and cell stress responses in human skeletal muscle after resistance exercise

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Key points summary

- Cold water immersion and active recovery are common post-exercise recovery treatments. A key assumption about the benefits of cold water immersion is that it reduces inflammation in skeletal muscle. However, no data are available from humans to support this notion.
- We compared the effects of cold water immersion and active recovery on inflammatory and cellular stress responses in skeletal muscle from exercise-trained men 2, 24 and 48 h during recovery after acute resistance exercise.
- Exercise led to the infiltration of inflammatory cells, with increased mRNA expression of pro-inflammatory cytokines and neurotrophins, and the subcellular translocation of heat shock proteins in muscle. These responses did not differ significantly between cold water immersion and active recovery.
- Our results suggest that cold water immersion is no more effective than active recovery for minimizing the inflammatory and stress responses in muscle after resistance exercise.

ABSTRACT

Cold water immersion and active recovery are common post-exercise recovery treatments. However, little is known about whether these treatments influence inflammation and cellular stress in human skeletal muscle after exercise. We compared the effects of cold water immersion versus active recovery on inflammatory cells, pro-inflammatory cytokines, neurotrophins and heat shock proteins (HSPs) in skeletal muscle after intense resistance exercise. Nine active men performed unilateral lower-body resistance exercise on separate days, at least 1 wk apart. On one day, they immersed their lower body in cold water (10°C) for 10 min after exercise. On the other day, they cycled at a low intensity for 10 min after exercise. Muscle biopsies were collected from the exercised leg before, 2, 24, and 48 h after exercise in both trials. Exercise increased intramuscular neutrophil and macrophage counts *MAC1 and CD163* mRNA expression ($P<0.05$). Exercise also increased *IL1β*, *TNF*, *IL6*, *CCL2, CCL4, CXCL2, IL8 and LIF* mRNA expression ($P<0.05$). As evidence of hyperalgesia, the expression of *NGF* and *GDNF* mRNA increased after exercise ($P<0.05$). The cytosolic protein content of αB-crystallin and HSP70 protein content decreased after exercise ($P<0.05$). This response was accompanied by increases in the cytoskeletal protein content of αB-crystallin and the percentage of type II fibres stained for αB-crystallin. Changes in inflammatory cells, cytokines, neurotrophins, and HSPs did not differ significantly between the recovery treatments. These findings indicate that cold water immersion is no more effective than active recovery for reducing inflammation or cellular stress in muscle after a bout of resistance exercise.

Abbreviations

BSA, bovine serum albumin; CCL2, monocyte chemotactic protein 1; CCL4, macrophage inflammatory protein 1β); CD, cluster of differentiation; CXCL2 (macrophage inflammatory protein 2α); FoxO, forkhead transcription factor; GDNF, glial cell derived neurotrophic factor; HSP, heat shock protein; IGF, insulin-like growth factor; IL, interleukin; LIF, leukaemia inhibitory factor; MAC, macrophage integrin; mTOR, mammalian target of rapamycin; NGF, nerve growth factor; TBST, Tris-buffered saline–Tween 20; TGF, transforming growth factor; TNF, tumour necrosis factor.

33 **INTRODUCTION**

34 Our group has previously reported that, compared with active recovery, regular application
35 of cold water immersion after exercise reduces gains in muscle mass and strength following
36 3 months of resistance training (Roberts *et al.*, 2015b). Cold water immersion may have
37 attenuated long-term adaptive responses to resistance exercise by modulating
38 inflammation and cellular stress. There exists a long-standing belief that by reducing
39 temperature and blood flow in skeletal muscle, cryotherapy such as icing or cold water
40 immersion reduces the metabolic rate of and/or inflammation in tissues within and around
41 the injured site in skeletal muscle. This supposedly protects neighbouring cells against
42 ischaemia after injury, which is thought to reduce the risk of secondary cell injury or death
43 (Bleakley *et al.*, 2010). Animal studies demonstrate the effectiveness of ice massage (Puntel
44 *et al.*, 2011; Takagi *et al.*, 2011; Vieira Ramos *et al.*, 2016) or local infusion of cold saline (Lee
45 *et al.*, 2005; Schaser *et al.*, 2007) for reducing inflammation in muscle following injury.
46 However, no research has examined whether cold water immersion reduces local
47 inflammation in human skeletal muscle after resistance exercise.

48 Understanding the effects of treatments such as cold water immersion and active
49 recovery on inflammation within skeletal muscle after exercise is important. Cold water
50 immersion is a widespread practice among various sports, and a growing body of evidence
51 suggest that these strategies may affect muscle recovery from strenuous exercise. Repair of
52 skeletal muscle tissue following injury is complex. It involves interactions between
53 inflammatory cells, satellite cells, fibroblasts and endothelial cells, and a range of soluble
54 factors secreted by these cells (Chazaud, 2016). Reducing inflammation in muscle after
55 injury often impedes muscle repair (Urso, 2013). The notion that the anti-inflammatory
56 effects of cryotherapy such as icing or cold water immersion is beneficial for muscle repair
57 has underpinned sports medicine practice for many years (Meeusen & Lievens, 1986).
58 However, research directly supporting this notion in humans is currently lacking.

59 The aim of the current study was to investigate whether cold water immersion reduces
60 local inflammation in muscle following exercise compared with active recovery. To conduct
61 this analysis, we used muscle samples that we collected as part of a large study (Roberts *et*
62 *al.*, 2015b). In this large study, we compared cold water immersion with active recovery for

63 two reasons. First, active recovery in the form of a low-intensity ‘warm down’ is also a
64 common strategy that athletes use to recover after exercise (Reilly & Ekblom, 2005) in the
65 belief that it helps to reduce soreness and remove metabolic by-products in muscle after
66 exercise. Second, compared with remaining sedentary, active recovery after exercise
67 increases cardiac output and muscle blood flow, and reduces total peripheral resistance
68 (Bangsbo *et al.*, 1994; Journeay *et al.*, 2005). We have also previously demonstrated that
69 active recovery and cold water immersion cause divergent changes in cardiac output,
70 temperature, and microvascular blood flow in muscle after exercise (Roberts *et al.*, 2015a).
71 In the current study, we measured: neutrophil and macrophage infiltration in muscle
72 because these cells are important mediators of inflammation during muscle repair (Tidball &
73 Villalta, 2010); intramuscular gene expression of the cytokines and chemokines because
74 they recruit inflammatory cells to damaged muscle tissue (Peterson *et al.*, 2006; Shireman *et*
75 *al.*, 2007; Kohno *et al.*, 2011; Zhang *et al.*, 2013) and regulate muscle repair (Broussard *et*
76 *al.*, 2004; Chen *et al.*, 2007; Serrano *et al.*, 2008; Yahiaoui *et al.*, 2008; Zhang *et al.*, 2013);
77 intramuscular gene expression of the NGF and GDNF because they mediate pain and
78 nociceptor activity in muscle (Murase *et al.*, 2010; Murase *et al.*, 2013); and the heat shock
79 proteins HSP70 and αB-crystallin because they have a cytoprotective role, prevent
80 aggregation of denatured proteins and stabilize the cytoskeleton in cells (Morton *et al.*,
81 2009). We hypothesised that compared with active recovery, cold water immersion would
82 attenuate leucocyte infiltration and the expression of pro-inflammatory cytokines,
83 neurotrophins as mediators of muscle soreness, and heat shock proteins as mediators of
84 cellular stress in muscle after exercise.

85

86 **METHODS**

87 **Ethical approval**

88 Before providing their written informed consent, all participants were informed of the
89 requirements and potential risks of the study. The experimental procedures adhered to the
90 standards set by the latest revision of the Declaration of Helsinki, and were approved by the
91 Human Research Ethics Committee of The University of Queensland (project number
92 2012000662).

93 **Experimental design**

94 Nine physically active young men (mean ± SD age 22.1 ± 2.2 years, height 1.80 ± 0.06 m,
95 body mass 83.9 ± 15.9 kg) completed one bout of single-leg resistance exercise on two
96 separate days (using alternate legs). Each of the sessions was followed by either cold water
97 immersion or active recovery. Muscle biopsies were collected from the vastus lateralis of
98 the exercised leg before and after each training session. The order of the two trials was
99 randomized and counterbalanced to minimize any series order effects. Six of the nine men
100 completed the two trials 1 week apart, and the other three men completed their trials 4
101 weeks apart. This variation in the timing of the trials was unavoidable, unfortunately,
102 because the investigator who performed the muscle biopsies (T.R.) was not available to
103 perform the biopsies on all of the men at 1-week intervals. All participants had at least 12
104 months of experience in resistance training ≥3 times per week, and were familiar with all
105 exercise aspects of the study. The data presented herein are part of a large study, from
106 which we have previously published two papers containing separate findings, which are
107 described above (Roberts *et al.*, 2015b; Figueiredo *et al.*, 2016).

108 *Resistance exercises.* The resistance training sessions for the two experimental trials were
109 identical and involved single-leg exercises such as 45° leg press (six sets of 8–12 repetitions),
110 single-leg squats (three sets of 12 repetitions), knee extensions (six sets of 8–12 repetitions),
111 and walking lunges (three sets of 12 repetitions). The total duration of the session was ~45
112 min. All resistance training was supervised and performed at normal room temperature
113 (23–25°C).

114 *Recovery therapies.* Cold water immersion was initiated 5 min after the training session. For
115 the cold water immersion treatment, the participants sat in an inflatable bath (iCool iBody,
116 iCool, Miami, Australia) for 10 min with both legs immersed in water up to the waist. Water
117 was circulated continuously and maintained at 10.3 ± 0.5°C using a circulatory cooling unit
118 (iCool LITE, iCool, Miami, Australia). For the active recovery treatment, the participants
119 performed 10 min of active recovery at a self-selected low intensity on a stationary cycle
120 ergometer (Wattbike, Nottingham, UK). The mean power output during active recovery was
121 36.6 ± 13.8 W. The participants minimized any rewarming following cold water immersion
122 or cooling following active recovery by not showering or bathing for at least 2 h after the

123 recovery therapies. We have previously demonstrated that these recovery therapies
124 stimulate robust and distinct changes in muscle soreness and limb girth (Roberts *et al.*,
125 2014), cardiac output, muscle temperature, and microvascular perfusion (Roberts *et al.*,
126 2015a).

127 *Blood and muscle tissue collection.* Blood samples were collected before exercise,
128 immediately after exercise, immediately after the recovery therapies (i.e., 15 min after
129 exercise) and 30 min, 1, 2, 24, and 48 h after exercise. The blood samples were collected
130 from an antecubital vein into a serum separation tube (BD, Franklin Lakes, NJ). Serum tubes
131 were left to clot at room temperature for 30 min before centrifugation at 4°C at 3,000 *g* for
132 10 min to separate the serum, which was then stored at –80°C until the day of analysis.
133 Muscle biopsies were collected from the midportion of the vastus lateralis while the
134 participants were in a fed state before exercise and again at 2, 24, and 48 h after exercise.
135 Pre-exercise and 2 h post-exercise biopsies were collected from the same incision. The pre-
136 exercise biopsy was collected with the needle inserted in a distal direction, and the 2 h
137 biopsy was collected with the needle inserted in a proximal direction. Biopsies at 24 and 48
138 h were collected from separate incisions, each ~3 cm proximal from the previous incision,
139 with a proximal needle insertion. This method ensured that all biopsy sites were separated
140 by at least 3 cm to minimize any artefact related to inflammation resulting from multiple
141 biopsies. The same muscle tissue that was analysed in the acute study section of our
142 previous reports (Roberts *et al.*, 2015b; Figueiredo *et al.*, 2016) was used for the current
143 analyses.

144 *Control procedures.* We attempted to minimize potential variation in training responses by
145 providing standardized nutrition before and after each training session and by instructing
146 the participants to avoid performing any extra exercise for 72 h before and for 48 h after
147 each trial. On the morning of each trial, the participants consumed the same meal 2 h
148 before the pre-exercise muscle biopsy and a 30 g serve of a whey protein isolate drink after
149 exercise before each recovery treatment. They were then allowed to drink only water until
150 the 2 h biopsy was collected, at which time they were provided with another 30 g of whey
151 protein isolate to drink. The participants were instructed to consume their habitual diet for
152 2 d before each experimental trial and until the 48 h muscle biopsy. The participants were
153 instructed to avoid consuming any additional supplements of any kind between 4 d before

154 each pre-exercise biopsy and the 48 h post-exercise muscle biopsy. Dietary intake before
155 and during the first experimental trial was recorded in a food diary and replicated for the
156 second experimental trial.

157

158 **Blood and muscle tissue analysis**

159 *Creatine kinase.* Serum creatine kinase activity was measured using a spectrophotometric
160 assay on an automated analyser (Model 7450, Hitachi, Japan).

161 *Plasma cytokines.* Plasma cytokine concentrations were measured using commercial
162 enzyme-linked immunosorbent assays for IL-6, IL-10 and IL-1ra. These particular cytokines
163 were selected because they consistently show the greatest increase following exercise; the
164 plasma concentrations of IL-1β, TNF-α and MCP-1 do not increase to the same extent
165 (Peake *et al.*, 2015). IL-6 was measured using a Quantikine® High-Sensitivity Colorimetric
166 Sandwich ELISA (SS600B) from R&D Systems Inc. (Minneapolis, MN, USA). IL-10 was
167 measured using an OptEIA ELISA Kit II (BD-550613) from BD Biosciences (San Diego, CA,
168 USA). IL-1ra was measured using a Quantikine® Colorimetric Sandwich ELISA (SRA00B) from
169 R&D Systems, Inc. Measurements were made using a microplate reader (VERSAmax,
170 Molecular Devices, Sunnyvale, CA, USA).

171 *RT-PCR.* Total RNA was extracted from ~20 mg of muscle tissue using the AllPrep®
172 DNA/RNA/miRNA Universal Kit (QIAGEN GmbH, Hilden, Germany) according to the
173 manufacturer's instructions. cDNA was synthesized using High-Capacity RNA-to-cDNA™ kit
174 (Life Technologies, Carlsbad, CA). mRNA expression was then measured using RT-PCR on a
175 LightCycler 480 II (Roche Applied Science, Penzberg, Germany) using SYBR Green I Master
176 Mix (Roche Applied Science). The sequences for the primers used in this study are shown in
177 Table 1. The geometric mean of three housekeeping genes (i.e., chromosome 1 open
178 reading frame 43, charged multivesicular body protein 2A, and endoplasmic reticulum
179 membrane protein complex subunit 7) was used for normalization (Vandesompele *et al.*,
180 2002). Standard and melting curves were obtained for each target to establish primer
181 efficiency and single product amplification.

182 *Western blotting.* Pieces of muscle tissue weighing 45–55 mg were homogenized and
183 fractionated into cytosolic and cytoskeletal fractions using a commercial fractionation kit
184 (ProteoExtract Subcellular Proteome Extraction Kit, Cat#539790, Calbiochem, EMD
185 Biosciences, Germany) according to the manufacturer's procedures. The purity of the
186 fractions were confirmed by specific markers for the respective fractions (GAPDH (cytosol
187 and nuclear), PARP (nuclear), COX2 (membrane) and desmin (cytoskeletal). Protein
188 concentration was measured in triplicate using a commercial kit (DC Protein Microplate
189 assay, Cat#0113, Cat#0114, Cat#0115, Bio-Rad, Hercules, CA), a filter photometer (Expert
190 96, ASYS Hitech, UK), and the software provided (Kim, ver. 5.45.0.1, Daniel Kittrich, Prague,
191 Czech Republic).

192 Equal amounts of protein were loaded per well (16–50 μg) and were separated by
193 4–12% SDS-PAGE under denaturizing conditions for 35–45 min at 200 V in cold MES running
194 buffer (NuPAGE MES SDS Running Buffer, Invitrogen, Carlsbad, CA). All samples were run in
195 duplicate. After gel electrophoresis, the proteins were transferred onto a polyvinylidene
196 fluoride membrane for 90 min at 30 V using an XCell II Blot Module (Thermo Fisher
197 Scientific, Hemel Hempstead, UK) and NuPAGE transfer buffer (Invitrogen, Carlsbad, CA).
198 Membranes were blocked at room temperature for 2 h in a 5% fat-free skimmed milk and
199 0.1% Tris-buffered saline with Tween 20 (TBST) (Cat#170-6435, Bio-Rad; Tween-20,
200 Cat#437082Q, VWR International, Radnor, PA; skim milk, Cat#1.15363, Merck, Darmstadt,
201 Germany). Blocked membranes were incubated overnight at 4°C with a primary monoclonal
202 antibody against αB-crystallin (mouse anti-αB-crystallin, Cat#ADI-SPA-222, Enzo Life
203 Sciences, Farmingdale, NY) diluted 1:4000. After incubation, membranes were washed and
204 incubated with a secondary antibody at room temperature for 1 h. The membranes for αB-
205 crystallin immunoblotting were incubated with a secondary antibody diluted 1:30 000 (goat
206 anti-mouse, Cat#31430, Thermo Scientific/Pierce Biotechnology, Rockford, IL).

207 Membranes used for HSP70 quantification were incubated initially with another primary
208 antibody (anti-FoxO3a; data not shown) and secondary antibody. The primary and
209 secondary antibodies were then stripped from the membranes using Restore Western Blot
210 Stripping Buffer (Cat#21059, Thermo Fisher Scientific), blocked for 2 h at room temperature,
211 and incubated with the primary polyclonal antibody to HSP70 (rabbit anti-HSP70, Cat#ADI-

212 SPA-812, Enzo Life Sciences) diluted 1:4000 at 4°C overnight. The membranes were then
213 incubated in a secondary antibody (anti-rabbit IgG, HRP-linked antibody, Cat#7074, Cell
214 Signaling Technology, Danvers, MA). All antibodies were diluted in a 1% fat-free skimmed
215 milk and 0.1% TBST solution. Between stages, membranes were washed in 0.1% TBST. Bands
216 were visualized using an HRP detection system (Super Signal West Dura Extended Duration
217 Substrate, Cat#34076, Thermo Scientific/Pierce Biotechnology). Chemiluminescence was
218 measured using a ChemiDoc MP System (Bio-Rad Laboratories), and band intensities were
219 calculated with molecular imaging software (Image Lab, Bio-Rad Laboratories). All samples
220 were analysed in duplicate, and mean values were used for statistical analyses.

221 *Immunohistochemistry.* Eight-micrometre-thick cross-sections of muscle tissue were cut
222 using a microtome at –20°C (CM3050, Leica Biosystems, GmbH), mounted on microscope
223 slides (Superfrost Plus, Thermo Scientific, Boston, MA), air-dried, and stored at –80°C.
224 Muscle sections from each subject obtained at all time points before and after both trials
225 were mounted on the same microscope slide. Before immunostaining, frozen sections were
226 air-dried and blocked in 1% bovine serum albumin (BSA) in PBS for 30 min. Sections were
227 then incubated with primary antibodies (listed in Table 2) in 1% BSA overnight at 4°C. The
228 following primary antibodies were used: anti-laminin to stain the inner surface of myofibres
229 (#Z009701-2; DakoCytomation, Glostrup, Denmark; dilution 1:1000); CD66b to stain
230 granulocytes (#M1594; clone CLB-B13.9, Sanquin Reagents, Amsterdam, The Netherlands;
231 dilution 1:500); CD68 to stain macrophages and cells with bilobed nuclei (#M0718; clone
232 EBM-11, DakoCytomation; dilution 1:300); anti-αB-crystallin to stain αB-crystallin bound to
233 cytoskeletal/myofibrillar structures (#ADI-SPA-222, Enzo Life Sciences, Farmingdale, NY;
234 dilution 1:200), and SC-71 to quantify type IIa and IIx fibres (#SC-71, Developmental Studies
235 Hybridoma Bank, Iowa City, USA; dilution 1:500).

236 After overnight incubation, the slides were washed three times in PBS for 10 min.
237 Sections were then incubated for 1 h with secondary antibodies diluted 1:200 in 1% BSA at
238 room temperature. The secondary antibodies used were Alexa Fluor® 594 F(ab')2 fragment
239 of goat anti-rabbit IgG (#A-11072, Invitrogen, Eugene, OR), Alexa Fluor®488 anti-mouse IgG
240 ((#A-11029, Invitrogen, Eugene, OR), CF488A goat anti-mouse IgG (#20010, Biotium,
241 Hayward, CA), and CF594 goat anti-rabbit IgG (#20112, Biotium). The fluorochrome-stained
242 sections were washed three times in PBS for 10 min. After the last wash, the sections were

243 mounted with ProLong® Gold Antifade reagent with 4′,6-diamidino-2-phenylindole (DAPI;
244 Invitrogen, Eugene, OR).

245 Muscle sections were visualized using a high-resolution camera (DP72, Olympus, Japan)
246 mounted on a microscope (BX61, Olympus) with a fluorescent light source (X-Cite 120PCQ,
247 EXFO, Canada). For leucocyte analysis, the numbers of CD66b- and CD68-positive cells, and
248 the total number of muscle fibres from the area examined were counted (see Figures 2 and
249 3). The data are presented as the number of CD66b- or CD68-positively stained cells per 100
250 skeletal muscle fibres. For the αB-crystallin analysis, the numbers of αB-crystallin-positive
251 and -negative fibres were counted. A fibre was considered positive if the staining inside the
252 fibre was scattered and uneven, and negative if the staining was homogeneous. The data
253 are presented as the percentage of αB-crystallin-positive fibres. Areas of sections that
254 contained freeze damage or were folded during the cutting procedure were not included in
255 the analyses.

256

257 **Statistical analysis**

258 Before statistical analysis, all data were checked to determine if they were normally
259 distributed. Log transformations were applied to data that were not normally distributed
260 (i.e., macrophage cell counts; *MAC1, TNF*, and *NGF* mRNA; serum creatine kinase activity).
261 Normally distributed data (i.e., *GDNF* mRNA, HSP70, αB-crystallin and plasma cytokine
262 concentrations) were analysed using a 2×3 repeated-measures ANOVA to calculate the
263 main effects of time and time × trial interaction. When a significant main effect was evident
264 ($P < 0.05$), post hoc paired *t* tests were used to compare changes over time and differences
265 between the trials. Normally distributed data are presented as mean ± SD, and log-
266 transformed data are presented as the geometric mean ± 95% confidence interval of the
267 geometric mean. Data that were not normally distributed (i.e., neutrophil cell counts;
268 *CD163, IL1β, IL-6, CCL2*, and *HSP70* mRNA) were analysed using Friedman's test, followed by
269 Wilcoxon's signed-ranked tests to compare changes over time and differences between the
270 trials. Non-normally distributed data are presented as median ± interquartile range. The
271 false discovery rate was used to correct for multiple comparisons.

RESULTS

Inflammatory cell infiltration. Exercise induced a strong and sustained inflammatory response in muscle (Figure 1). The number of CD66b$^+$ neutrophils in muscle was higher than the pre-exercise number at 2 h after active recovery (9-fold difference; P = 0.015) and tended to be higher at 2 h after cold water immersion (3-fold difference; P = 0.086). mRNA expression of macrophage cell surface receptors increased in muscle after exercise. As a general marker of pro-inflammatory cells, *MAC1* expression was higher than the pre-exercise expression at 24 h (1.2-fold; P = 0.020) and 48 h (2.4-fold; P = 0.010) after active recovery, and 48 h after cold water immersion (1.8-fold; P = 0.036). The number of CD68$^+$ macrophages in muscle was higher than before exercise at 48 h after active recovery (1.5-fold P = 0.008) and tended to be higher 48 h after cold water immersion (1.7-fold; P = 0.071). As a marker of anti-inflammatory macrophages, *CD163* expression was higher than the pre-exercise expression at 24 h (6.7-fold; P = 0.008) and 48 h (3.2-fold; P = 0.011) after active recovery, and at 24 h after cold water immersion (3.2-fold; P = 0.008). *MAC1* and *CD163* mRNA expression and neutrophil and macrophage counts in muscle did not differ significantly between the trials. Representative images of staining for CD66b$^+$ neutrophils and CD68$^+$ macrophages are shown in Figures 2 and 3.

Cytokines and chemokines. Exercise induced the expression of several pro-inflammatory cytokine and chemokine genes in muscle (Figures 4 and 5). *IL1*β expression was higher than before exercise at 2 h after active recovery (9-fold; P = 0.011) and at 2 h after cold water immersion (27-fold; P = 0.021). *TNF* expression was higher than before exercise at 2 h (2.6-fold; P = 0.004) and 24 h (2.9-fold; P = 0.005) after active recovery, and at 2 h after cold water immersion (2.7-fold; P = 0.026). *IL6* expression was higher than before exercise at 2 h after active recovery (11-fold; P = 0.004) and at 2 h (8.6-fold; P = 0.008), 24 h (1.7-fold; P = 0.021), and 48 h (2.2-fold; P = 0.015) after cold water immersion. *CCL2* expression was higher than before exercise at 2 h after active recovery (21-fold; P = 0.008) and cold water immersion (30-fold; P = 0.008), and it remained higher at 24 h and 48 h after both trials. *CCL4* expression was higher than before exercise at 24 h after active recovery (2.8-fold; P = 0.019), and tended to be higher than before exercise at 24 h after cold water immersion

302 (1.7-fold; $P = 0.068$). *CCL5* expression showed a similar pattern of changes to *CCL4* (data not
303 shown). *CXCL2* expression was higher than before exercise at 2 h after active recovery (9.4-
304 fold; $P < 0.001$) and cold water immersion (17-fold; $P < 0.001$). It also tended to be higher
305 than before exercise at 24 h after active recovery (1.8-fold; $P = 0.065$) and was higher 24 h
306 after cold water immersion (1.6-fold; $P = 0.017$). *IL8* expression was higher than before
307 exercise at 2 h after active recovery (125-fold; $P < 0.001$) and cold water immersion (272-
308 fold; $P < 0.001$). It was also higher than before exercise at 24 h after active recovery (8.9-
309 fold; $P = 0.030$) and tended to be higher 24 h after cold water immersion (5.3-fold; $P =$
310 0.052). *LIF* expression was higher than before exercise at 2 h after active recovery (32-fold; P
311 < 0.001) and cold water immersion (37-fold; $P < 0.001$). It also tended to be higher than
312 before exercise at 24 h after active recovery (2.5-fold; $P = 0.065$) and was higher 24 h after
313 cold water immersion (2.2-fold; $P = 0.037$). Cytokine and chemokine mRNA expression in
314 muscle did not differ significantly between the trials.

315 *Neurotrophins.* Exercise stimulated the expression of two neurotrophins associated with
316 muscle soreness in muscle (Figure 5). *GDNF* and *NGF* expression increased in muscle after
317 exercise. *GDNF* expression was higher than before exercise at 2 h after active recovery (3.7-
318 fold; $P = 0.001$) and cold water immersion (4.3-fold; $P < 0.001$). *NGF* expression was higher
319 than before exercise at 24 h after active recovery (2.0-fold; $P = 0.040$), and at 2 h (1.2-fold; P
320 $= 0.040$), 24 h (2.1-fold; $P = 0.010$) and 48 h (1.5-fold; $P = 0.010$) after cold water immersion.
321 *GDNF* or *NGF* expression in muscle did not differ significantly between the trials.

322 *HSPs. HSP70* mRNA expression was higher than before exercise at 2 h after active recovery
323 (2.1-fold; $P = 0.013$) and cold water immersion (2.0-fold; $P = 0.028$) (Figure 6). The protein
324 content of HSP70 in the cytosol fraction of muscle homogenates was lower than before
325 exercise at 2 h (14%; $P = 0.032$) and 48 h (15%; $P = 0.034$) after active recovery, and at 2 h
326 after cold water immersion (18%; $P = 0.044$) (Figure 7). The protein content of HSP70 in the
327 cytoskeletal fraction was unchanged after both trials. The protein content of αB-crystallin in
328 the cytosol fraction of muscle homogenates was lower than before exercise at 2 h after both
329 active recovery (–33%; $P = 0.001$) and cold water immersion (–36%; $P = 0.003$) (Figure 8). It
330 remained lower than the pre-exercise value for the rest of the post-exercise recovery period
331 in both trials. Conversely, the protein content of αB-crystallin in the cytoskeletal fraction of
332 muscle homogenates showed a strong trend toward an increase after exercise ($P = 0.052$).

333 This response was accompanied by an increase in the percentage of αB-crystallin-positive
334 fibres (Figure 9). The median percentage of αB-crystallin-positive fibres was 26%
335 (interquartile range 3–77%) at 2 h after active recovery and 19% (interquartile range 2–43%)
336 at 2 h after cold water immersion. Staining for αB-crystallin was scattered and evident
337 mainly in type II fibres (Figure 9). The percentage of αB-crystallin-positive fibres did not
338 differ significantly between the trials.

339 *Creatine kinase and cytokines*. A systemic indirect marker of muscle damage, serum creatine
340 kinase activity increased moderately after both exercise trials ($P < 0.05$) (Figure 10). It
341 remained elevated up to 48 h after active recovery ($P < 0.05$). Plasma IL-6 concentration also
342 increased moderately after both exercise trials, and remained elevated up to 2 h after
343 exercise (Table 2). By contrast, the plasma concentrations of IL-10 ($P = 0.40$) and IL-1ra ($P =$
344 0.24) did not change after either trial (Table 2). The magnitude of the changes in creatine
345 kinase and cytokines was consistent with the intermittent nature and limited muscle mass
346 used for the single-leg resistance exercise. There were no significant differences in serum
347 creatine kinase activity or plasma cytokine concentrations between the trials.

348

349 **DISCUSSION**

350 To our knowledge, this is the first study to compare the effects of cold water immersion
351 versus active recovery on inflammation, neurotrophins, and HSPs within skeletal muscle
352 following exercise in humans. Exercise stimulated intramuscular inflammation, as
353 demonstrated by increased mRNA expression of *MAC1* and *CD163,* and increased the
354 numbers of neutrophils and macrophages. Intramuscular gene expression of cytokines and
355 neurotrophins also increased, and HSPs translocated from the cytosol to cytoskeletal
356 structures in muscle after exercise. Contrary to our hypothesis, these responses did not
357 differ substantially between cold water immersion and active recovery. These findings
358 provide evidence against the traditional notion that cryotherapy such as cold water
359 immersion helps to restrict inflammation and cellular stress responses in muscle following
360 exercise. Taking into account our previous observation that regular application of cold water
361 immersion attenuated long-term muscle adaptation compared with active recovery

362 (Roberts *et al.*, 2015b), the present findings suggest that this response was not due to a
363 reduction in inflammation and/or cellular stress after cold water immersion.

364 Animal studies have demonstrated that icing (Puntel *et al.*, 2011) or infusing cold saline
365 (Lee *et al.*, 2005; Schaser *et al.*, 2007) into injured muscle of rats reduces leucocyte rolling
366 and adhesion, and neutrophil infiltration and activation. By contrast, another study found
367 that cold water immersion did not reduce leucocyte counts in muscle of rats after exercise
368 (Camargo *et al.*, 2012). Icing reduces and/or delays macrophage infiltration in rat muscle
369 after muscle injury (Takagi *et al.*, 2011; Vieira Ramos *et al.*, 2016). In the present study,
370 there were no significant differences in the numbers of neutrophils and macrophages, or
371 mRNA expression of the cell surface receptors MAC1 and CD163 between cold water
372 immersion and active recovery (Figure 1).

373 Compared with research into the effects of cryotherapy on cell infiltration into muscle,
374 less is known about its effects on the intramuscular expression of cytokines. In the present
375 study, we focused on changes in the gene expression of IL-1β, TNF-α, IL-6, CCL2 (MCP-1),
376 CCL4 (MIP-1β), CXCL2 (MIP-2α), IL-8 and LIF in muscle after exercise because these cytokines
377 are responsive to mechanical loading associated with exercise (Peake *et al.*, 2015). They also
378 play important roles in recruiting inflammatory cells to damaged muscle tissue (Peterson *et*
379 *al.*, 2006; Shireman *et al.*, 2007; Kohno *et al.*, 2011; Zhang *et al.*, 2013) and regulating
380 muscle repair (Broussard *et al.*, 2004; Chen *et al.*, 2007; Serrano *et al.*, 2008; Yahiaoui *et al.*,
381 2008; Zhang *et al.*, 2013). Two animal studies have reported that icing reduces the
382 expression of *TGFβ* and *TNF* in the days following muscle injury (Takagi *et al.*, 2011; Vieira
383 Ramos *et al.*, 2016). In the present study, *IL1β*, *IL6*, *TNF, CCL2, CXCL2, IL8* and *LIF* mRNA
384 increased in skeletal muscle after both cold water immersion and active recovery (Figures 4
385 and 5). However, cytokine expression did not differ significantly between the cold water
386 immersion and active recovery trials. The effects of ice massage (Tseng *et al.*, 2013), cold
387 water immersion (Vaile *et al.*, 2008; Pointon *et al.*, 2012; Gonzalez *et al.*, 2014; Roberts *et*
388 *al.*, 2014), or exposure to –30°C air (Pournot *et al.*, 2011; Guilhem *et al.*, 2013) on systemic
389 inflammatory responses to intense eccentric exercise or resistance exercise are variable and
390 are relatively minor. We discovered that although plasma IL-6 concentration increased after
391 exercise, there was no significant difference between the two trials. Collectively, these

392 findings suggest that cryotherapy does not substantially alter local or systemic inflammatory
393 responses to exercise-induced muscle damage.

394 Several factors could (theoretically) account for the differences between the present
395 study and the animal studies described above. First, data from animal studies tend to
396 indicate that icing (Puntel *et al.*, 2011; Takagi *et al.*, 2011; Vieira Ramos *et al.*, 2016) is more
397 effective than cold water immersion (Camargo *et al.*, 2012) for reducing inflammation in
398 muscle. This difference could be related to differences in the temperature of ice compared
399 with cold water, which are in the range of 9–10°C. Second, we (Roberts *et al.*, 2015a) and
400 others (Ihsan *et al.*, 2013) have demonstrated that cold water immersion reduces muscle
401 temperature at a depth of 3 cm and microvascular perfusion at a depth of 1–2 cm. Because
402 animal muscles are smaller than human muscles, icing or cold water immersion may
403 produce more extensive changes in muscle temperature and blood flow throughout animal
404 muscles compared with human muscles. This may partly explain the greater anti-
405 inflammatory effects of icing in animal muscle (Puntel *et al.*, 2011; Takagi *et al.*, 2011; Vieira
406 Ramos *et al.*, 2016). Third, the animal studies described above induced muscle injury
407 through blunt impact trauma (Lee *et al.*, 2005; Schaser *et al.*, 2007; Puntel *et al.*, 2011),
408 freeze injury (Vieira Ramos *et al.*, 2016), or by crushing muscle with forceps (Takagi *et al.*,
409 2011). No research has directly compared these injury models with exercise. Yet it seems
410 reasonable to suggest that tissue injury and inflammation may be more severe and
411 prolonged after blunt impact trauma or freeze or crush injury compared with exercise
412 (Gayraud-Morel *et al.*, 2009). These differences may influence the efficacy of treatments for
413 muscle injury and inflammation. Finally, these studies compared the effects of cryotherapy
414 with no treatment, as opposed to active recovery.

415 Less muscle soreness after intense exercise may be the most consistent effect of cold
416 water immersion (Leeder *et al.*, 2011; Versey *et al.*, 2013). We did not assess muscle
417 soreness in the present study. However, we have previously demonstrated that the same
418 cold water immersion protocol (i.e., 10 min of cold water immersion at 10°C) significantly
419 reduced muscle soreness after intense resistance exercise (Roberts *et al.*, 2014). The
420 mechanisms through which cold water immersion reduces muscle soreness after exercise
421 are unknown. At rest (i.e., without prior exercise), topical icing of the ankle reduces nerve
422 conduction velocity, and increases pain threshold and pain tolerance (Algafly & George,

423 2007). These findings suggest that cryotherapy may influence the activity of nociceptors in
424 soft tissues. Pain and nociceptor activity in muscle are mediated, in part, by pro-
425 inflammatory cytokines (Schafers *et al.*, 2003; Hoheisel *et al.*, 2005), bradykinin, and the
426 neurotrophins NGF and GDNF (Murase *et al.*, 2010; Murase *et al.*, 2013). *NGF* and *GDNF*
427 mRNA expression increases in skeletal muscle following lengthening (eccentric) muscle
428 contractions in rats (Murase *et al.*, 2010; Murase *et al.*, 2013) and 60 min dynamic knee
429 extension exercise in humans (Romero *et al.*, 2016). Consistent with these responses, we
430 found that *GDNF* expression peaked at 2 h after exercise, whereas *NGF* expression peaked
431 at 24 h after exercise (Figure 5). *NGF* and *GDNF* expression did not differ significantly after
432 cold water immersion and active recovery. Therefore, these findings suggest that the
433 analgesic effects of cold water immersion after exercise do not involve changes in the
434 expression of these neurotrophins.

435 HSPs including HSP70 and αB-crystallin play important roles in cytoprotection and as
436 molecular chaperones to prevent aggregation of denatured proteins. They also regulate the
437 refolding of proteins and stabilize the cytoskeleton in cells (Morton *et al.*, 2009). We
438 observed that *HSP70* mRNA expression increased (Figure 6), whereas the cytosolic protein
439 content of HSP70 (Figure 7) and αB-crystallin (Figure 8) in muscle decreased acutely after
440 both cold water immersion and active recovery, and did not differ significantly between the
441 trials. The increase in *HSP70* mRNA expression is consistent with the findings of other
442 studies (Paulsen *et al.*, 2007). Previous studies have reported a delayed increase (Paulsen *et*
443 *al.*, 2007; Paulsen *et al.*, 2009) or no change (Cumming *et al.*, 2014) in cytosolic HSP70
444 content and an acute decrease (Paulsen *et al.*, 2009; Cumming *et al.*, 2014) in cytosolic αB-
445 crystallin content. The acute decrease in the cytosolic content of HSPs after exercise reflects
446 their mobilization to cytoskeletal structures, which was confirmed by the increased αB-
447 crystallin content in the cytoskeletal fraction, where they may help to stabilize and protect
448 stressed myofibrillar proteins (Paulsen *et al.*, 2007; Paulsen *et al.*, 2009).

449 The increase in the number of αB-crystallin-positive fibres (fibres with scattered and
450 uneven αB-crystallin staining) is further evidence that this stress protein binds to damaged
451 cytoskeletal or myofibrillar structures. We have previously reported a similar staining
452 pattern after high-force eccentric exercise, and more detailed observations with

453 immunogold staining and electron microscopy revealed accumulation of αB-crystallin in Z-
454 disks connected to disrupted sarcomeres (Paulsen *et al.*, 2009). Consistent with other
455 reports of the accumulation of another small HSP (HSP27) in type II fibres after resistance
456 exercise (Folkesson *et al.*, 2008), the scattered αB-crystallin staining was evident mainly in
457 type II fibres in the present study. This finding suggests that the mechanical strain on
458 myofibrillar structures was more pronounced in type II fibres. Collectively, the current
459 findings suggest that cold water immersion did not mitigate the stress-related signals that
460 stimulate cellular movement of HSPs in skeletal muscle after exercise. This may also partly
461 explain why cold water immersion did not significantly alter the infiltration of inflammatory
462 cells or cytokine gene expression in skeletal muscle following exercise.

463 Several methodological considerations relating to the present study warrant brief
464 discussion. First, several studies have reported that exercise with one leg induces adaptation
465 in the contralateral leg (Howatson & van Someren, 2007; Starbuck & Eston, 2012; Xin *et al.*,
466 2014). To address this issue, we analysed the changes in cellular infiltration and the
467 expression of cytokine mRNA, neurotrophin mRNA and heat shock proteins between the
468 first and second bout of exercise that each participant performed (independent of cold
469 water immersion or active recovery treatments). Indeed, there were no statistically
470 significant differences ($P < 0.05$) between the first and second bouts of exercise for any of
471 these variables, which suggests that no adaptation occurred in the contralateral leg
472 between the first and second bouts of exercise.

473 Second, some studies have reported that repeated muscle biopsies can cause injury and
474 inflammation in muscle (Guerra *et al.*, 2011; Van Thienen *et al.*, 2014). By contrast, we and
475 others have found that repeated muscle biopsies do not alter the expression of a wide array
476 of genes (Lundby *et al.*, 2005) or the infiltration of inflammatory cells in muscle (Paulsen *et*
477 *al.*, 2010). We aimed to minimize injury and inflammation arising from the muscle biopsies
478 in two ways: (1) for the pre-exercise and 2 h post-exercise biopsy, we inserted the biopsy
479 needle in opposite directions; (2) for the 24 h and 48 h biopsies, we inserted the biopsy
480 needle at two separate sites, 3 cm and 6 cm distal (respectively) from the previous incisions.

481 Third, we acknowledge that comparing cold water immersion with inactive recovery may
482 have been optimal for true experimental purposes. However, in reality, athletes are unlikely

483 to remain completely sedentary after exercise (Reilly & Ekblom, 2005). We contend that our
484 comparison between cold water immersion and active recovery is more reflective of typical
485 athletic practice. We also believe that the effects of active recovery itself were relatively
486 minor, because other research has demonstrated little or no difference in plasma creatine
487 kinase activity (Saxton & Donnelly, 1995) or circulating cytokines (including IL-6 and IL-10)
488 (Andersson *et al.*, 2010) between active recovery and inactive/sedentary recovery after
489 exercise. Notwithstanding possible differences between systemic versus intramuscular
490 markers of tissue damage/inflammation, the findings from these studies tend to suggest
491 that our results would have been similar if we had included inactive/sedentary recovery
492 rather than active recovery. Last, we did not include women in our study to minimize
493 variation arising from fluctuations in oestrogen as part of the menstrual cycle. Oestrogen is
494 known to influence inflammatory responses in muscle after exercise (Tiidus, 2003). We
495 acknowledge that our results may not be applicable to women.

496 In conclusion, contrary to popular anecdotal belief and the findings from preclinical
497 studies on cryotherapy treatments for muscle injury, we found that compared with active
498 recovery, cold water immersion did not significantly reduce inflammation or cellular stress
499 within muscle after exercise. It is important to consider the implications of these findings
500 within the broader context of understanding the factors that regulate inflammatory
501 responses in muscle after exercise, and managing athletic conditioning and recovery.
502 Considering the large differences in cardiac output, temperature, and microvascular blood
503 flow in muscle that occur after cold water immersion versus active recovery (Roberts *et al.*,
504 2015a), the present findings suggest that these physiological factors are not major
505 determinants of local inflammation and cellular stress in human muscle after exercise. Cold
506 water immersion consistently improves perceptions of fatigue and muscle soreness (Stanley
507 *et al.*, 2012) and enhances recovery of muscle function/performance following exercise
508 (Leeder *et al.*, 2011; Versey *et al.*, 2013; Roberts *et al.*, 2014). It also reduces clinical signs of
509 inflammation such as limb swelling/oedema after exercise (Yanagisawa *et al.*, 2003;
510 Yanagisawa *et al.*, 2004; Roberts *et al.*, 2014). Therefore, it would appear that cold water
511 immersion may still confer some short-term clinical and/or functional benefits for athletes,
512 without any changes in local inflammatory reactions within skeletal muscle during recovery
513 from exercise. Periodic use of cold water immersion may assist athletes when they need to

514 recovery quickly between training sessions or competitive events. However, in the long
515 term, regular cold water immersion appears to be detrimental for developing muscle
516 strength and hypertrophy.

517

518 **Competing interests**

519 The authors have no competing interests to declare.

520

521 **Author contributions**

522 J.M.P, L.A.R, V.C.F, J.F.M, J.C.S, D.C.-S. and T.R. contributed to the conception and design of
523 the research; L.A.R, V.C.F, I.E, S.K, S.N.A, K.S. and J.F.M performed the experiments; J.M.P
524 analyzed the data; J.M.P, V.C.F, S.K., K.S., J.F.M. and T.R. interpreted the results of the
525 experiments; J.M.P, L.A.R I.E., S.K., S.N.A. and T.R. prepared the figures; J.M.P drafted the
526 manuscript; J.M.P., L.A.R., V.C.F., I.E., S.K., S.N.A., K.S., J.F.M., J.S.C., D-C.-S. and T.R. edited
527 and revised the manuscript; J.M.P, L.A.R, V.C.F, I.E., S.K., S.N.A, K.S., J.F.M, J.S.C, D.C.-S.
528 approved the final version of the manuscript.

529

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536

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543

544

Table 1. mRNA primer sequences

Primer	Sequence
MAC1 (CD11b) forward	TCAGGTGGTGAAAGGCAAGG
MAC1 (CD11b) reverse	ATCTGTCCTTCTCTTAGCCGA
CD163 forward	GCGGCTTGCAGTTTCCTCAA
CD163 reverse	CTGAAATCAGCTGACTCATGGGA
NGF forward	GAGCGCAGCGAGTTTTGG
NGF reverse	TGGCCAGGATAGAAAGCTGC
GDNF forward	GAACTCTTGCCCCTGACCTG
GDNF reverse	GCGGCACCTCGGATCG
HSP70 forward	TGTTCCGTTTCCAGCCCCCAA
HSP70 reverse	GGGCTTGTCTCCGTCGTTGAT
IL6 forward	TCAATGAGGAGACTTGCCTGG
IL6 reverse	GGGTCAGGGGTGGTTATTGC
IL1β forward	TTCGAGGCACAAGGCACAA
IL1β reverse	TGGCTGCTTCAGACACTTGAG
IL8 forward	ACCGGAAGGAACCATCTCAC
IL8 reverse	GGCAAAACTGCACCTTCACAC
LIF forward	TGAAAACTGCCGGCATCTGA
LIF reverse	CACAACTCCTGCCGCCAA
CCL2 forward	GCAATCAATGCCCCAGTCAC
CCL2 reverse	CTTGAAGATCACAGCTTCTTTGGG
CCL4 forward	CTCCCAGCCAGCTGTGGTATTC
CCL4 reverse	CCAGGATTCACTGGGATCAGC
CXCL2 forward	GAAAGCTTGTCTCAACCCCG
CXCL2 reverse	TGGTCAGTTGGATTTGCCATTTT
TNF forward	AGCCCATGTTGTAGCAAACC
TNF reverse	TGAGGTACAGGCCCTCTGAT
EMC7, forward	GGGCTGGACAGACTTTCTAATG
EMC7, reverse	CTCCATTTCCCGTCTCATGTCAG
CHMP2A, forward	CGCTATGTGCGCAAGTTTGT

CHMP2A, reverse	GGGGCAACTTCAGCTGTCTG
C1orf43, forward	CTATGGGACAGGGGTCTTTGG
C1orf43, reverse	TTTGGCTGCTGACTGGTGAT

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546

547

Table 2. Plasma cytokine concentrations.

	Pre	Post	Rec	0.5 h	1 h	2 h	24 h	48 h
IL-6 (pg/ml)								
CWI	1.1 (0.5)	2.2 (1.2) *	3.0 (1.2) *	3.0 (1.3) *	3.6 (1.7) *	2.0 (1.8)	1.5 (0.5)	1.9 (1.7)
ACT	1.2 (0.6)	2.3 (0.8) *	3.0 (1.3) *	3.3 (1.6) *	2.7 (1.1) *	2.7 (1.4) *	1.3 (0.7)	1.2 (0.3)
IL-10 (pg/ml)								
CWI	8.4 (9.0)	33.0 (62.0)	10.0 (12.8)	9.1 (8.2)	18.7 (25.3)	15.2 (18.0)	8.8 (11.3)	7.7 (8.4)
ACT	11.5 (16.2)	8.9 (11.0)	9.1 (9.2)	5.4 (2.2)	9.8 (11.3)	11.6 (10.2)	7.8 (10.8)	8.1 (8.5)
IL-1ra (pg/ml)								
CWI	243 (145)	343 (240)	203 (158)	243 (148)	293 (197)	269 (167)	230 (138)	262 (165)
ACT	263 (203)	348 (234)	282 (217)	425 (379)	312 (216)	313 (242)	281 (166)	246 (138)

Data are mean (SD). n = 9. * $P < 0.05$ versus pre-exercise. Pre, pre-exercise; Post, immediately post-exercise; Rec, immediately after recovery therapies.

548

549

550 **Figure legends**

551 **Figure 1.** Post-exercise changes in CD66b$^+$ neutrophil infiltration, CD68$^+$ macrophage
552 infiltration, and *MAC1* and *CD163* mRNA expression. Data are presented as the change in
553 the median $\pm$ interquartile range for neutrophils and *CD163* mRNA, and the geometric mean
554 $\pm$ 95% confidence interval for macrophages and *MAC1* mRNA. ACT, active recovery; CWI,
555 cold water immersion. n = 9. * $P < 0.05$ versus pre-exercise value.

556

557 **Figure 2.** Representative image of immunofluorescence staining for CD66b$^+$ neutrophils.
558 Panel A shows red laminin staining of the sarcolemma; panel B shows blue DAPI staining of
559 nuclei; panel C shows green staining for CD66b; panel D shows merged images. Arrows
560 indicate CD66b$^+$ neutrophils. Scale bar represents 50 μm. n = 9.

561

562 **Figure 3.** Representative image of immunofluorescence staining for CD68$^+$ macrophages.
563 Panel A shows red laminin staining of the sarcolemma; panel B shows blue DAPI staining of
564 nuclei; panel C shows green staining for CD68; panel D shows merged images. Arrows
565 indicate CD68$^+$ macrophages. Scale bar represents 50 μm. n = 9.

566

567 **Figure 4.** Post-exercise changes in mRNA expression of IL-1β, TNF, IL-6 and CCL2. Data are
568 presented as changes in the median $\pm$ interquartile range for *IL1β*, *IL6*, and *CCL2* expression,
569 and the geometric mean $\pm$ 95% confidence interval for *TNF* expression. n = 9. * $P < 0.05$
570 versus pre-exercise value.

571

572 **Figure 5.** Post-exercise changes in mRNA expression of CCL4, CXCL2, IL-8 and LIF. Data are
573 presented as changes in the geometric mean $\pm$ 95% confidence interval. n = 9. * $P < 0.05$
574 versus pre-exercise value.

575

576 **Figure 6.** Post-exercise changes in mRNA expression of *GDNF* and *NGF* mRNA. Data are
577 presented as changes in the mean ± SD for *GDNF* and the geometric mean ± 95% confidence
578 interval for *NGF*. n = 9. * *P* < 0.05 versus pre-exercise value.

579

580 **Figure 7.** Post-exercise changes in expression of *HSP70* mRNA. Data are presented as the
581 change in the median ± interquartile range. n = 9. * *P* < 0.05 versus pre-exercise value.

582

583 **Figure 8.** Representative immunoblots and post-exercise changes in the protein content of
584 HSP70 and αB-crystallin in the cytosol and cytoskeletal fraction of muscle homogenates.
585 Data are presented as the mean ± SD. n = 9. * *P* < 0.05 versus pre-exercise value.

586

587 **Figure 9.** Intramuscular localisation of αB-crystallin. Upper panels show
588 immunohistochemistry staining for αB-crystallin in muscle fibres before exercise (Panel A)
589 and at 2 h after exercise (Panel B). A fibre was considered positive if the staining inside the
590 fibre was scattered and uneven (marked with red asterisks). Fibres were considered
591 negative if the staining was homogeneous (all fibres in the left image). Lower panels show
592 immunohistochemistry staining for myosin heavy chain IIA and IIX (SC71 antibody) in
593 neighbouring sections. Before exercise, there was more αB-crystallin protein present in type
594 I fibres (marked "I" in Panel C), whereas after exercise, the scattered αB-crystallin staining
595 was found mainly in type II fibres (Panel D). Scale bar represents 100 μm. n = 9.

596

597 **Figure 10.** Post-exercise changes in serum creatine kinase activity. Data are presented as the
598 geometric mean ± 95% confidence interval. n = 9. * *P* < 0.05 versus pre-exercise value.

599

600

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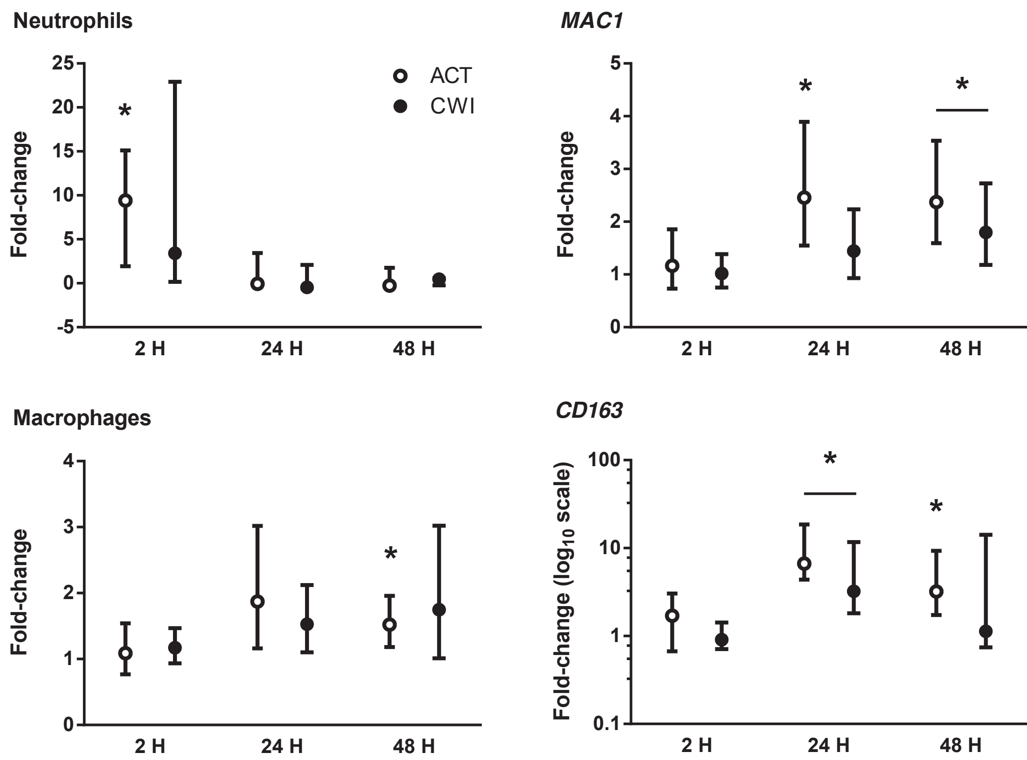
Figure 1



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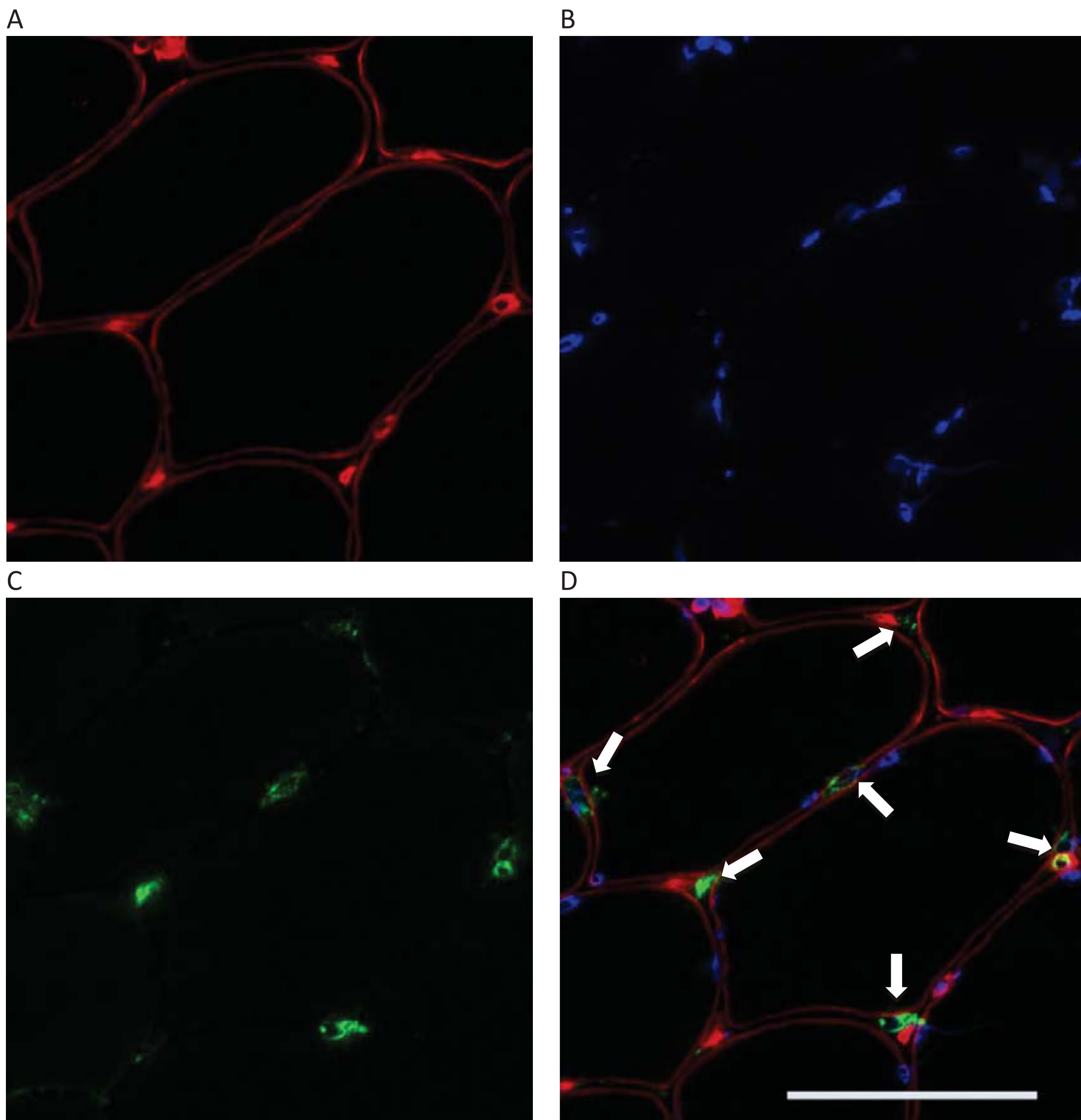

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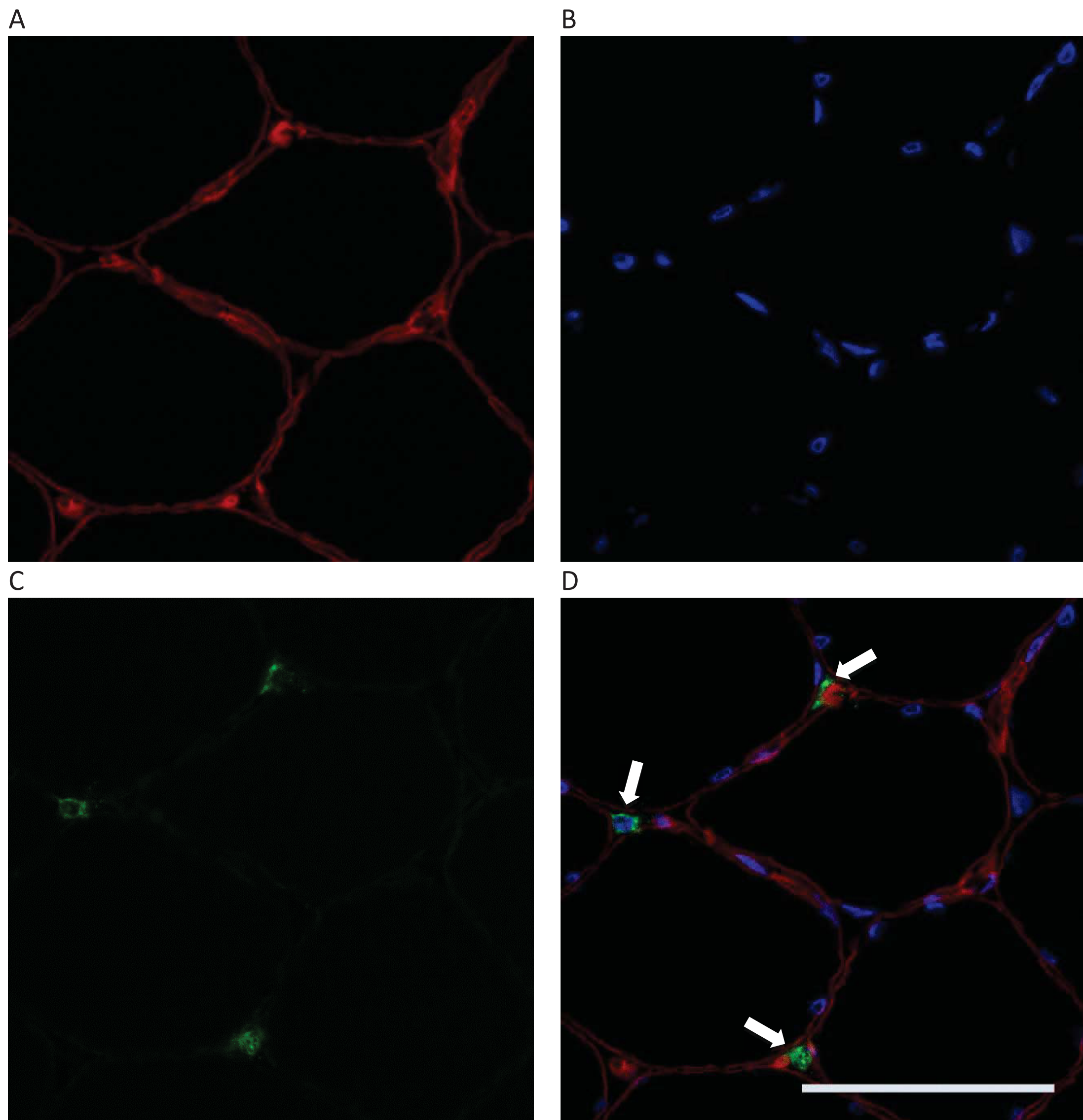

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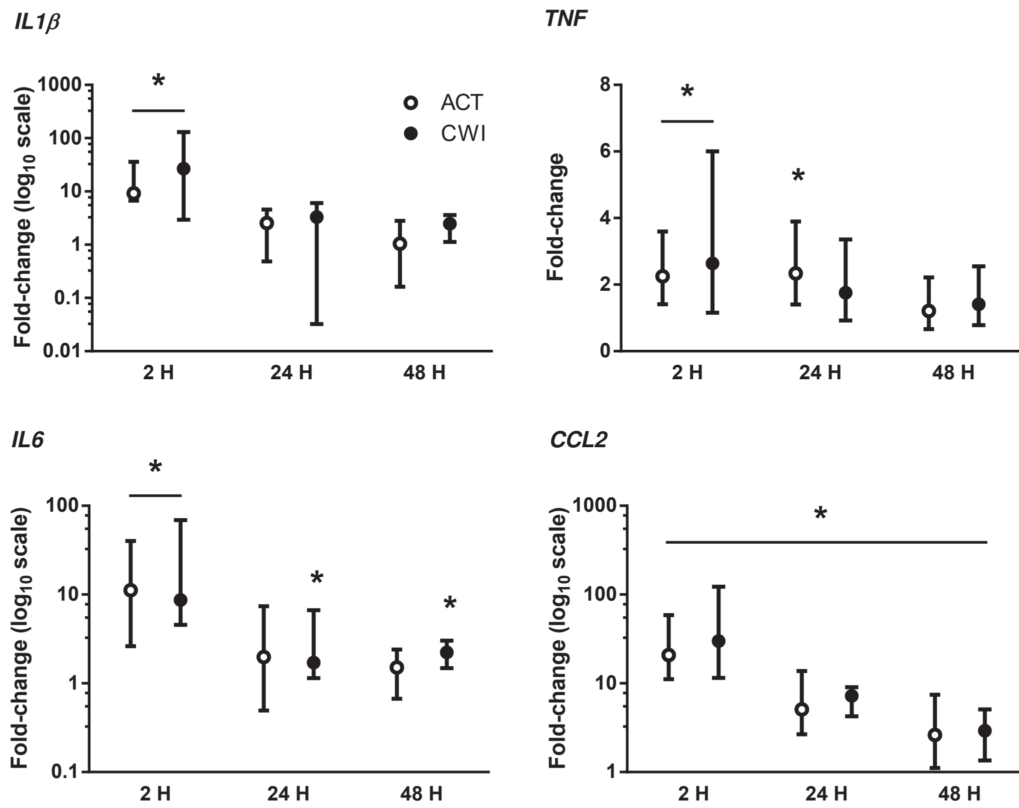

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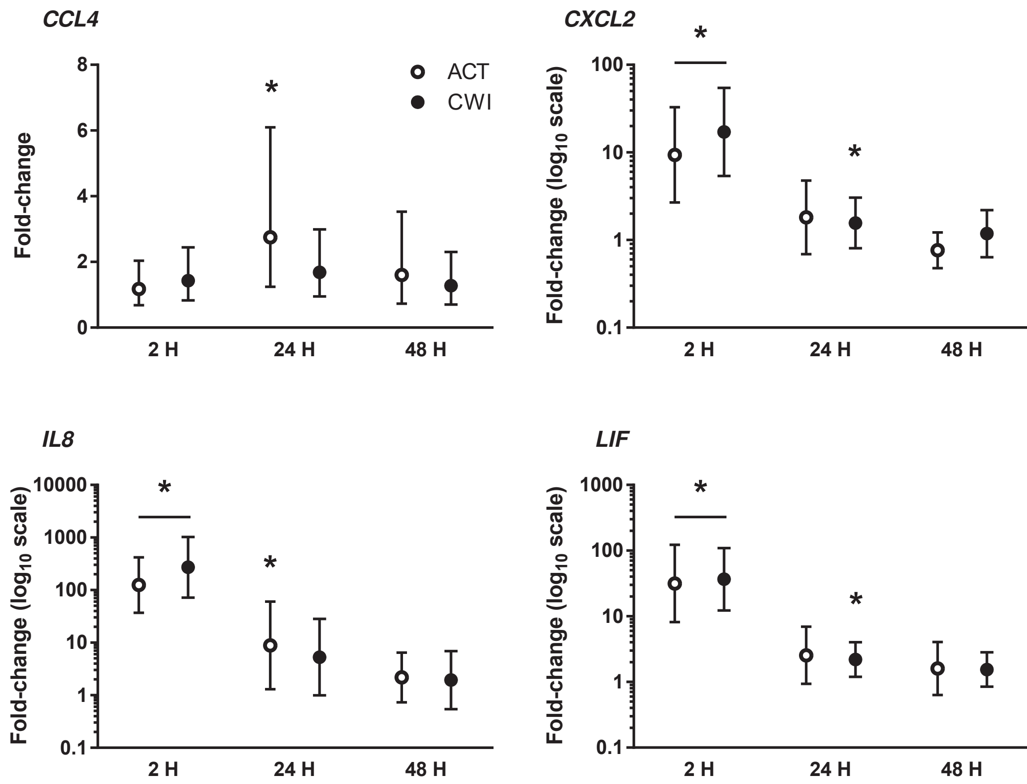

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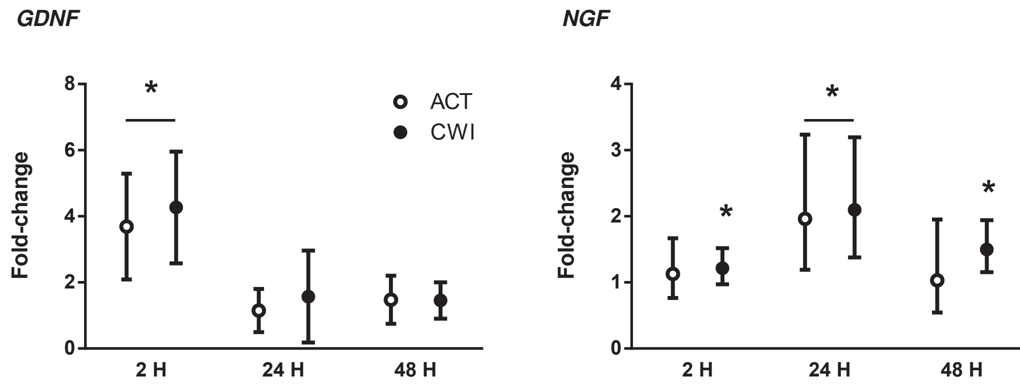

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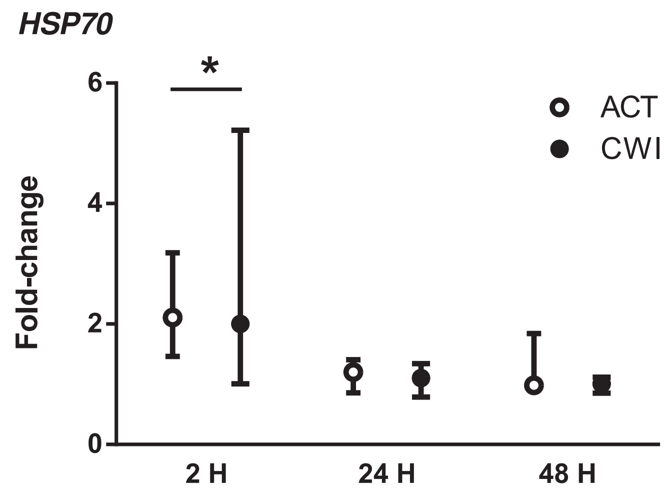

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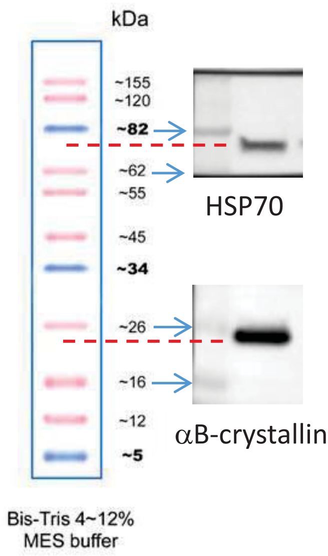

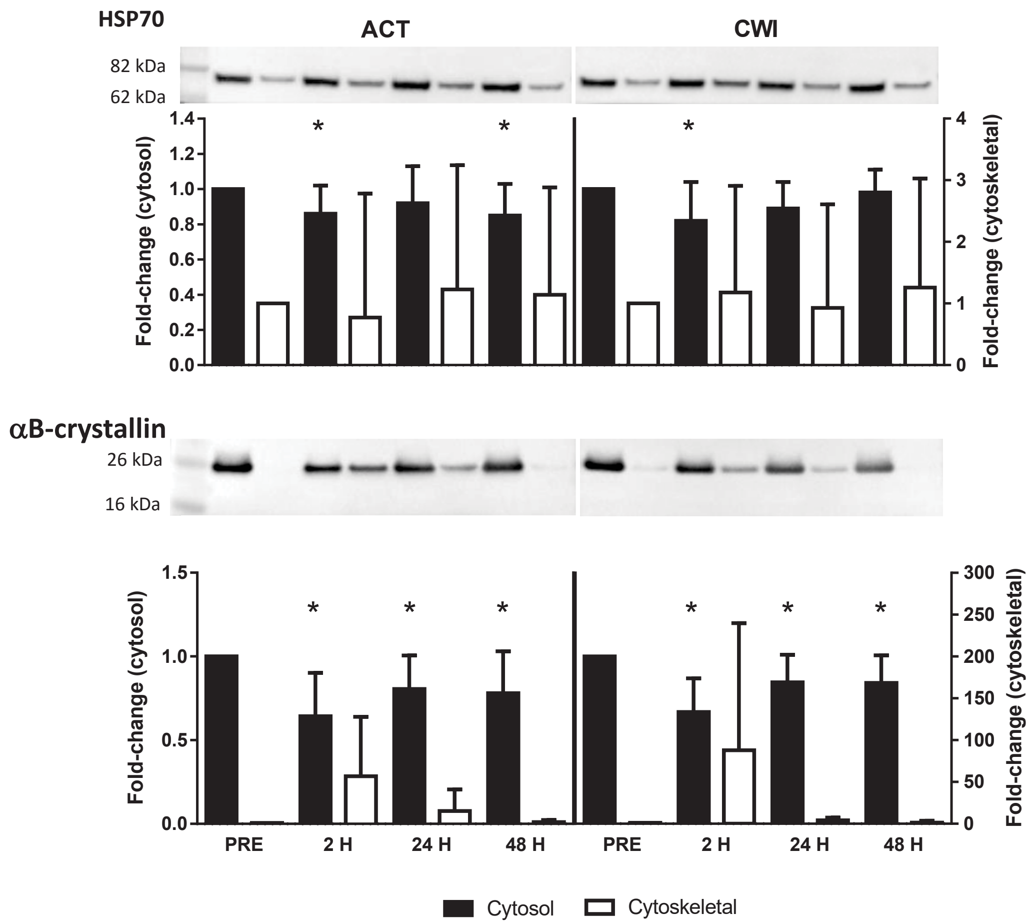

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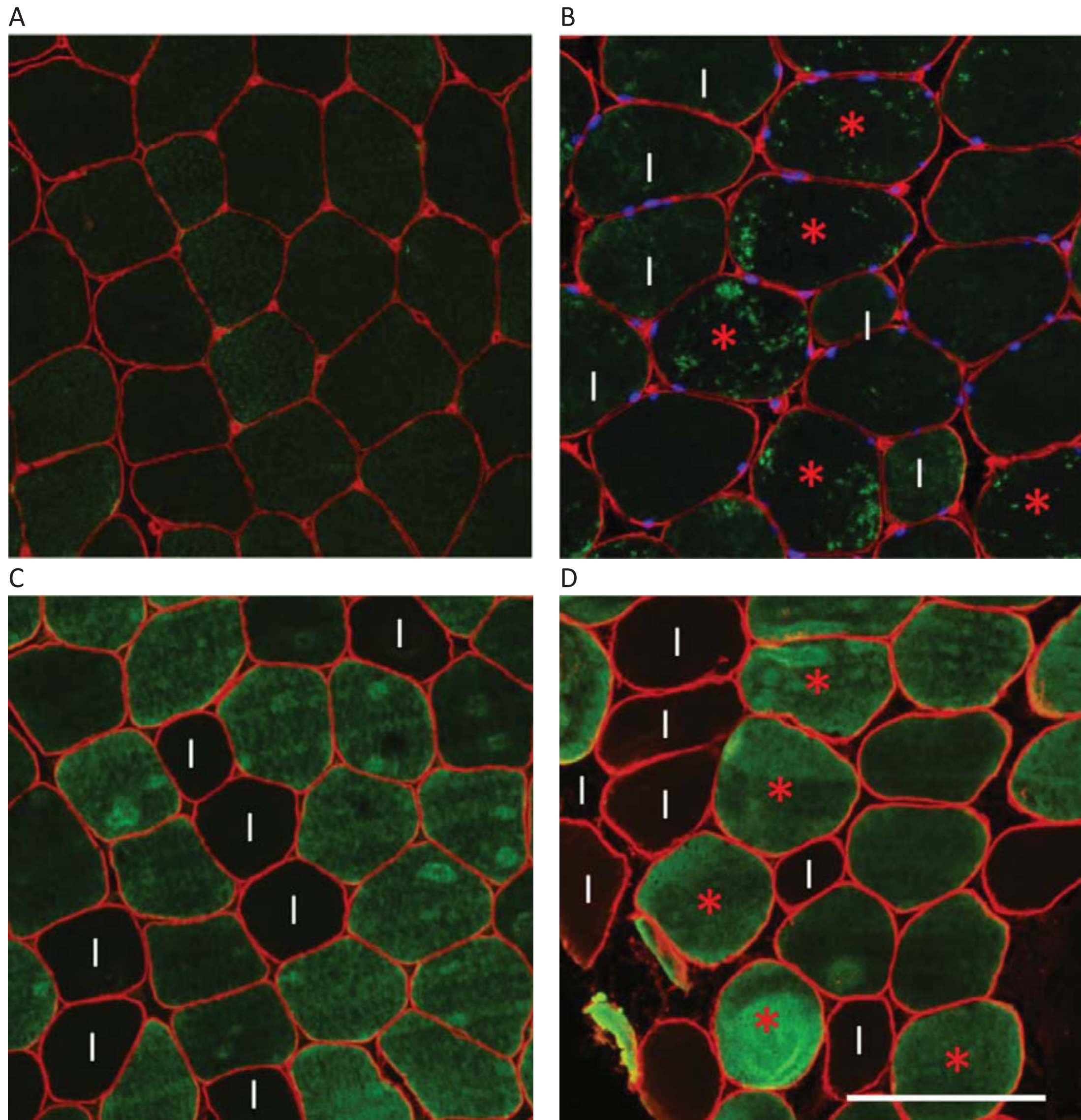

Figure 10