Properties of regenerated EDL mouse muscle following notexin injury

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Introduction: We have previously proposed a two-stage model of muscle necrosis in muscular dystrophy in which the branched muscle fibres (that are formed as a consequence of repeated rounds of regeneration) play a major role in the terminal stages of the disease (Chan & Head, 2011). In our model it is the branching and not the absence of dystrophin *per se* which renders fibres susceptible to contraction induced injury. Our earlier studies suggest that there is a tipping point which is reached when greater than 40% of the muscle fibres within a muscle have significant branching deformities (Chan & Head, 2011). Once this point is reached we hypothesise that even genetic rescue of the missing dystrophin protein will not rescue the dystrophic muscle. In order to further test this hypothesis we have generated dystrophin-positive branched fibres from a single round of notexin induced muscle regeneration.

Methods: Use of animals was approved by the University of New South Wales Animal Care Ethics Committee For each mouse, the right extensor digitorum longus (EDL) muscle was given a single injection of notexin, while the left EDL muscle was given a single injection of saline and served as a control. The experiments were then performed 21 days post-injection. Mice were anaesthetised with halothane and euthanased by cervical dislocation. The EDL muscles were dissected out and placed in an organ bath superfused with oxygenated Krebs. The muscle was given 6 eccentric contractions of 20% strain, with 5 mins rest between each contraction. The muscle was then removed, weighed and digested at 37°C in oxygenated Krebs solution containing 3 mg/mL collagenase Type I (Sigma) and 1 mg/mL trypsin inhibitor (Sigma). The muscle was gently agitated using pipette suction, releasing some individual fibres from the muscle mass. Fibres were examined with a light microscope.

Results: *Contractile properties.* Notexin-injected EDL muscles had significantly higher mass, higher cross-sectional area and lower specific force than contralateral saline-injected controls. Notexin-injected muscles had 12% higher mass (14.6 ± 0.5 mg vs. 13.0 ± 0.4 mg, $P = 0.0014$, paired *t*-test, $n = 13$ in each group) and 11% higher cross-sectional area (0.92 ± 0.03 mm^2 vs. 0.83 ± 0.02 mm^2, $P = 0.0025$) than controls. Maximum absolute muscle force did not differ between notexin-injected muscles and controls; however, due to the higher cross-sectional area, specific force was 9% lower in notexin-injected muscles (214 ± 11 mN/mm^2 vs. 234 ± 9 mN/mm^2, $P = 0.0362$). Following 6 eccentric contractions of 20% strain, the resultant decrease in muscle maximum force (force deficit) was not significantly different between notexin-injected muscles (16% ± 1.1%) and controls (18% ± 2.5%). *Fibre morphology.* 29% of the fibres from notexin-injected EDL muscles were branched (303 fibres examined). None of the fibres from saline-injected muscles were branched. Central nuclei were found in 93% of fibres from notexin-injected muscles (1,574 fibres examined) and in 10% of fibres from saline-injected muscles (905 fibres examined). Branching patterns were typically simple; the main patterns observed were: (1) a small branch emerging from the main fibre, (2) a bifurcation occurring at the end of a fibre, and (3) a branch emerging from the main fibre, then rejoining.

Conclusion: We have shown that a single round of regeneration induces fibre branching in 29% of muscle fibres. This is equivalent to the branched muscle fibre composition in young *mdx* mice up to 20 weeks of age (Head *et al.* 1992). Similar to these young *mdx* mice (Carberry *et al.* 2013, Chan *et al.* 2007), muscle mass has increased and the muscle has hypertrophied, and muscle specific force has fallen. In support of our hypothesis and the results we have published in young *mdx* mice (Chan *et al.* 2007), these muscles are not more susceptible to mild eccentric contraction-induced damage than non-regenerated muscles. In our model this is because the number and complexity of the fibre branching is below tipping point. We would like to suggest these wild-type regenerated muscles would make the ideal control for young *mdx* muscles.

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