Excessive muscle oxidative stress causes abnormal myogenesis and spine curvature in adolescent mice

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INTRODUCTION: Adolescent Idiopathic Scoliosis (AIS) manifests lateral spinal deformity that progresses during adolescence with unclear etiology. Previously, we and others have reported an unbalanced paraspinal muscle activity in AIS and miscoordination of musculature at the apex of curvature. In another study, abnormal spinal curvature was observed in a *Sepn1* null mouse model under extensive swimming exercise, suggesting spine deformity might be linked to an inability of muscle to cope with oxidative stresses. Furthermore, rats depleted with intake of vitamin E, a potent free radical scavenger, displayed a severe kyphoscoliosis phenotype with necrotizing skeletal myopathies. We therefore hypothesized that excessive oxidative stress in paraspinal muscle during spine growth may lead to AIS. In this study, we investigated the effect of H_2O_2-induced oxidative stress on myogenesis and muscle fiber formation in C2C12 cells. Moreover, we explored if elevating oxidative stress in paraspinal muscle of adolescent mice by feeding them with a vitamin E deficient diet along with force swimming test (FST) could lead to spinal deformity.

METHODS: All animal experiment protocols were approved by local government agency (Department of Health, Hong Kong SAR) and institutional ethics committee (CULTAR). To study the influence of increase oxidative stress in paraspinal muscle on the spine growth, mice were fed with a vitamin E deficient diet (~5.2 IU/kg) (LabDiet 9GZA) or vitamin E sufficient diet (~50.1 IU/kg) (LabDiet 5755) and subjected them to 15 mins of FST every other day from P40 (postnatal 40 days) to P225. Longitudinal X-ray radiograph of the thoracolumbar region of spine were taken and input into an open-source software Surgimap (https://www.surgimap.com) for Cobb angle and Kyphotic Index (KI) measurements. Myogenesis of C2C12 cells were performed in DMEM-HG media with 2% horse serum and treated with 200μM H_2O_2 to induce oxidative stress. Quantitative PCR (QPCR) was performed using Power Sybr Green PCR mix (Thermo Fisher Scientific) with in-house primers to detect perinatal muscle fiber-associated genes: *Myh3*, *Myh8*; slow twitch muscle fiber-associated genes: *Myh7*; fast twitch muscle fiber-associated genes: *Myh2*, *Myh1*, *Myh4*; myogenic regulatory factor-associated genes: *Myod1*, *Myf5*, *Myog*; oxidative stress responsive p53 target genes: *p21*, *Gadd45a*, *Trp53inp1*, *Plk3*, *Btg2*, *Ddit4*, *Atf3*, *Gdf15*, *Ndrg1*, and normalized by *Gapdh* expressions. Protein expression of myogenic regulatory factors was detected by western blot analyses using antibody against Myod1 (Invitrogen) 1:1000, Myf5 (abcam) 1:1000, Myogenin (abcam) 1:500 respectively. Histological analyses of slow /fast twitch muscle fiber were performed by immunostaining of transverse paraspinal muscle sections from thoracolumbar region of spine with anti-slow twitch muscle related myosin heavy chain type I (MHC I) antibody (abcam) 1:100 and anti-fast twitch muscle related myosin heavy chain type II (MHC II) antibody (abcam) 1:100. Oxidative stress in paraspinal muscle was tracked by immunostaining of lipid peroxidation marker 4-hydroxynoneal (4-HNE) protein adducts using anti-4-HNE antibody (abcam) 1:100.

RESULTS: QPCR results showed 200μM H_2O_2 could promote an upregulation of a panel of known oxidative stress responsive p53 target genes, confirming H_2O_2 could elevate oxidative stress throughout the myogenesis of C2C12 cells. We observed a lower number and shorter diameter of myotubes in myogenesis of C2C12 cells cultured under H_2O_2 treatment (Fig, 1A). Interestingly, H_2O_2 could suppress the protein expression of Myod1 (myoblast determination protein 1) and Myogenin throughout the myogenesis. On the contrary, a sustained protein expression of Myf5, a myoblast proliferative transcription factor was evidenced in H_2O_2 treatment at Day 4 and 8 of myogenesis (Fig. 1B). H_2O_2 induced oxidative stress caused a reduced expression of perinatal muscle fiber-associated gene *Myh3* and slow twitch muscle fiber-associated gene *Myh7* (Fig. 1C), whereas the expression of fast twitch muscle fiber-associated gene *Myh4* was modestly upregulated. In vivo study showed a progressive lateral spinal curvature with Cobb angle ≥ 10º development in adolescent mice (from P60 to P225) subject to combined vitamin E deficiency and swimming with gender differences (4/10 in male and 1/7 in female) (Fig. 1D), but not in the control groups (basal diet and/or without swimming). No significant changes in KI were observed in all treatment groups, indicating the treatment mainly induced left-right asymmetry. Furthermore, a lower proportion of slow twitch myofiber (Fig. 1E) and an increased lipid peroxidation marker 4-HNE (Fig. 1F) were observed on the convex side of paraspinal muscle in the scoliotic but not the non-scoliotic mice. Body weight measurement indicated a faster growth rate in male mice than female mice.

DISCUSSION: Our findings showed that oxidative stress could impair myogenic differentiation and myotube formation with a dysregulation of myogenic regulatory factors. Under combined vitamin E deficiency and FST, adolescent mice could develop scoliosis along with a reduced proportion of slow twitch muscle fiber and increased lipid peroxidation in the convex side of the curvature. Slow twitch muscle fibers are related to endurance in long distance exercise. This might indicate the importance of slow twitch muscle tonicity to maintain the spinal alignment. The higher incidence rate of scoliosis in male (40%) than female (14%) mice might be associated with gender differences in growth velocity, muscle development and physical activity.

SIGNIFICANCE/CLINICAL RELEVANCE: This study implicates a previously unidentified impact of oxidative stress on adolescent spine alignment. Alleviating the oxidative stress such as using antioxidant supplements to prevent curve progression in AIS warrants further investigation.

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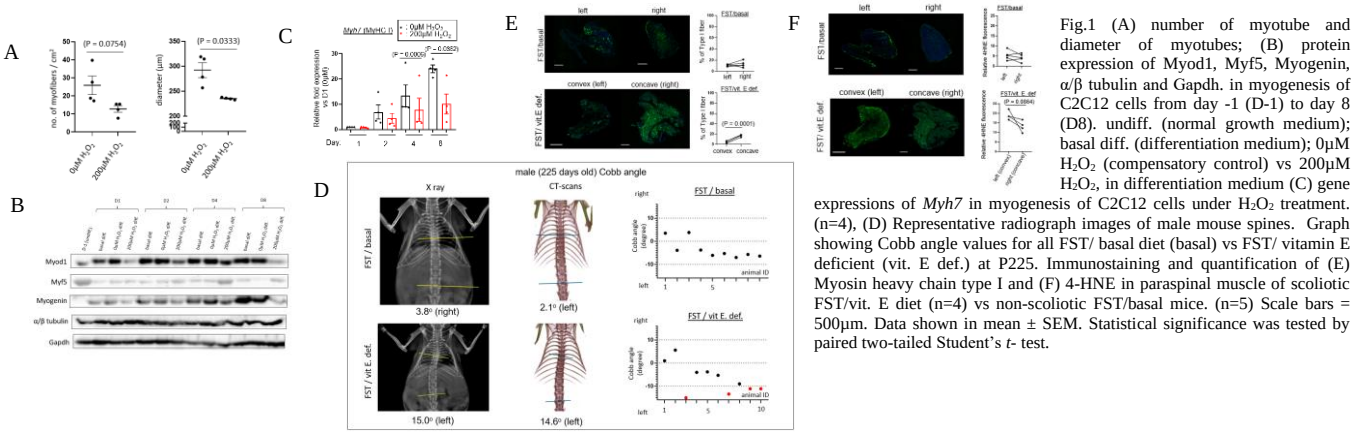

Fig.1 (A) number of myotube and diameter of myotubes; (B) protein expression of Myod1, Myf5, Myogenin, α/β tubulin and Gapdh. in myogenesis of C2C12 cells from day -1 (D-1) to day 8 (D8). undiff. (normal growth medium); basal diff. (differentiation medium); 0μM H_2O_2 (compensatory control) vs 200μM H_2O_2, in differentiation medium (C) gene expressions of *Myh7* in myogenesis of C2C12 cells under H_2O_2 treatment. (n=4), (D) Representative radiograph images of male mouse spines. Graph showing Cobb angle values for all FST/ basal diet (basal) vs FST/ vitamin E deficient (vit. E def.) at P225. Immunostaining and quantification of (E) Myosin heavy chain type I and (F) 4-HNE in paraspinal muscle of scoliotic FST/vit. E diet (n=4) vs non-scoliotic FST/basal mice. (n=5) Scale bars = 500μm. Data shown in mean ± SEM. Statistical significance was tested by paired two-tailed Student's *t*- test.