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Mitigation of pathological parameters under Jagged1 influence in DMD knockout zebrafish and patient-derived myoblast cultures

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Duchenne muscular dystrophy (DMD) is an X-linked, degenerative disease mainly affecting male children, with progressive weakness of whole-body skeletal muscles and the heart. There is a gradual loss of ambulation, heart weakness, and breathing capacity by late teens. Heart or lung dysfunction causes early death in patients during the second or third decade. Steroid treatment delays disease progression by 2-3 years, albeit with serious side effects. The few FDA-approved gene therapies are mutation-specific and exorbitantly priced. There is an unmet medical need for the children affected with DMD. Interestingly, a previous study showed that single nucleotide change caused Jagged1 overexpression, which resulted in avoidance of early death and ambulatory loss in 1-1.5-year-old golden retriever dogs severely affected with muscular dystrophy. Identifying the pathological processes mitigated by Jagged1 overexpression might help understand the mechanism of this rescue. Hence, we generated DMD knockout in zebrafish, another severe model of DMD with overexpression of the human Jagged1 (JAG1). Pathological aspects like cell death, cell proliferation, cytoplasmic and mitochondrial oxidative stress were compared between dystrophic, rescued, and control groups. Surprisingly, JAG1 increased mitochondrial oxidative stress during rescue, while reducing other pathological processes. Similarly, increased mitochondrial ROS production occurred with Jag1 peptide treatment in *in vitro* differentiated patient-derived myotubes, suggesting a conserved mechanism involved in the rescue.

Keywords: Duchenne Muscular Dystrophy, Golden retriever muscular dystrophy (GRMD) model, Jagged1 peptide, Myotubes, Zebrafish

Duchenne muscular dystrophy (DMD) is one of the most common childhood onset degenerative diseases globally and in India, resulting from mutations in the X-linked gene DMD/DYS[1]. 1 in 3000-6000 live male births is the estimated global occurrence rate, while some hotspots in India have reported rates as high as 1 in 1400 live male births[2,3]. DMD encodes for multiple transcripts, generating isoforms of a protein called dystrophin which are designated by the size as Dp427, Dp116, Dp140 *etc*[4-7]. Mutations resulting in nonfunction of the longest isoform, Dp427 alone, are associated with DMD, while partially functional Dp427 is associated with late-onset, milder Becker's muscular dystrophy (BMD). The full-length Dp427 is expressed in skeletal muscle, heart, and brain regions. Its absence results in progressive weakness and dysfunction of cardiac and whole-body skeletal muscles. There is a non-progressive cognitive delay or deficit in some of the DMD patients. Heart or lung failure during the second to third decade causes premature death in patients. The advancements in the medical management of this disease, like steroids, corrective spine surgeries, electric wheelchairs, and ventilators, have extended ambulatory and life span in patients, but no cure is still available[8,9].

Extensive studies spanning more than a century, based on animal models like mouse (mdx), golden retriever muscular dystrophy (GRMD) dogs, and zebrafish (*Sapje*), have provided molecular details of pathology. Muscle formation and excitation-contraction coupling (ECC) usually occur in the absence of Dystrophin protein[10,11]. Nevertheless, once the muscle contractions begin, the ECC-associated processes go haywire along with lateral transduction of the sarcomeric force towards membranes[12]. Excessive membrane lesions increase ion incursion and reactive oxygen species (ROS), further affecting mitochondrial ATP production. Calcium, though very important to the mechanism of contraction, is kept at low levels during rest, rather, transients of calcium influx and reabsorption are required for proper muscle

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function. The calcium persistence is likely due to the reduced activity of endoplasmic reticulum located calcium pump SERCA that reabsorbs calcium in an ATP-dependent manner[13]. The mitochondria also contain several calcium channels to regulate calcium metabolism, especially the pyruvate dehydrogenase complex, which is sensitive to calcium. The persistence of calcium at a higher level reduces mitochondrial energy generation and increases mitochondrial ROS production[14,15]. In the cytoplasmic compartment, the calcium and ROS have a positive feedback loop which is overactive in DMD[16]. The calcium-activable proteases have been thought to initiate myofiber death via multiple mechanisms[17]. On the other hand, a master regulator of stress in all cells – the p38MAPK is attributed to activate Bax-mediated apoptosis during the early stages of disease[18]. In healthy contracting muscles, p38MAPK activation occurs via several mechanisms, resulting in PGC-α stability and activation[19]. However, why p38MAPK activations turn pathological in dystrophic conditions is poorly understood. In later stages, most myofibers are lost by necroptosis due to heightened immune response[20]. The inflammatory cytokines and their receptor-signalling has also been implicated in myofiber death, yet pathology initiation in knockouts of these genes in *mdx* background suggests that inflammation is most likely outcome of ongoing myofiber damage[21-23].

A recent study showed that myoblast proliferation and fusion during regeneration could also contribute to membrane damage seen in DMD, as mechanical properties of the membrane need to change to allow myoblast fusion[24]. The activated satellite cells dividing asymmetrically and symmetrically express dystrophin transiently before generating committed myoblasts[25,26]. Hence, the absence of dystrophin can accelerate the loss of stem cells and thus exhaust the myogenic potential in patients.

Vieira and co-workers reported two escapers from GRMD colony as they did not show disease manifestation due to a mutation that increased Jagged1 protein levels in a muscle-specific manner[27]. The same study also showed that Jagged1 mRNA injection in *Sapje* larvae increased survival at one month. Additionally, higher levels of muscle fibrosis and increased rate of myoblast proliferation from escapers were reported[27,28]. Nevertheless, the mechanism of rescue is not known. Which pathological processes were improved by Jagged1 overexpression (OE) also remain unknown. Such information can help direct the discovery of therapeutic agents better.

Here, human Jagged1/JAG1 CDS containing plasmid was co-injected with CRISPR reagents to recapitulate the rescue. The pathological processes like cytoplasmic and mitochondrial ROS, and cell death were compared in the dystrophic and rescued zebrafish larvae. The immortalized myoblasts have been invaluable as they retain patient-specific features and have been used for drug screening[29]. These immortalized or induced pluripotent cell-derived myoblasts differentiate in *in vitro* cultures normally yet show increased oxidative stress, premature differentiation, and poor calcium handling hence, they are susceptible to osmotic stress and increased cell death[29-33]. The mini-Jagged1 peptide has been shown to act like a Notch receptor agonist, though disputed[34,35]. However, the advantage of using the Jagged1 peptide on human cell lines has improved feasibility for translational research and was adopted in the current study.

Materials and Methods

Ethical approval

Protocols were approved by the Institutional Animal Ethics Committee (IAEC No. Z/IAEC-3/10-2019). All the methods are reported in accordance with Animal Research: Reporting of *in vivo* Experiments (ARRIVE) guidelines.

Zebrafish knockout and Jagged1 overexpression

Zebrafishes (*Danio rerio*) procured from a local aquarium were raised with the standard protocol described in the Zebrafish book at zfin.org. The annotated zebrafish DMD gene sequence (Ensembl: ENSDARG00000008487) was used to find NGG pam sequences in exons 4 to 8 with CasDesigner and CHOPCHOP tools. The sgRNAs consisting of pam adjacent 20-23 nucleotide and constitutive region were *in vitro* synthesized according to the manufacturer's instructions (AbmGeneCraft-R classic sgRNA synthesis kit #G952). The pCS2-Cas9 (from *S. pyogenes*) under CMV promoter - a gift from Alex Schier (Addgene plasmid #47322; http://n2t.net/addgene:47322;RRID: Addgene_47322). The pcDNA3.1 human Jagged1 CDS plasmid with CMV promoter was a kind gift from Prof. Rajan Dighe and Prof. Annapoorni Rangarajan, IISc, Bangalore. The single-cell embryos were collected and injected with pCS2:Cas9 plasmid and a mixture of 3 guide RNAs with or without pcDNA3.1 –h

Jagged1 formed DMD and DMD+Jag1 rescue groups. The final concentration of ~ 30 ng/embryo after dilution with 1:1 phenol red dye was injected till embryos reached the two-cell stage. The control larvae were injected with only phenol red dye. The Jagged1 plasmid injected control embryos were used for comparison. The Femtojet microinjector and glass pulled needles were used for injection.

Cytoplasmic oxidative stress

The cell-permeable reagent 2′, 7′-dichlorofluoresce in diacetate (DCFDA, also known as H2DCFDA, DCFH-DA, and DCFH) is useful to quantitatively assess reactive oxygen species in live cell samples. It measures hydroxyl, peroxyl and other reactive oxygen species activity within the cell. After absorption in cells, DCFDA is deacetylated by cellular esterases to a non-fluorescent compound, which can be oxidized by ROS into 2′, 7′-dichlorofluoresce in (DCF). DCF is highly fluorescent and is detected by fluorescence spectroscopy with excitation/emission at 485 nm / 535 nm. Five larvae from each injection group were taken. The DCFDA dye was added at a final concentration of 1.8 µM, incubated in the dark for 20 min, then washed with PBS three times in the dark and imaged under a fluorescent confocal microscope. The fluorescence intensities were calculated by ImageJ software as arbitrary units (a.u.). The group mean and SD of three larvae were used for single replicate.

Mitochondrial oxidative stress

JC1 is a lipophilic cationic carbocyanine dye that accumulates in mitochondria. It emits a red fluorescence at 590 nm when it is in dimeric form. In the monomeric form, it emits green fluorescence at 488 nm. When oxidative stress is high in mitochondria, it reacts with this dye, causing monomerization, thereby increasing green emission. The Green/Red emission ratio is dependent on the membrane potential and thus indicates health in any disease condition. The absorption significantly reduced after 4 dpf in larvae, hence *in vivo* mitochondrial potential measurements from muscles directly under a confocal microscope were unsuccessful. Hence Green and Red emission was measured on larval muscle suspensions by FACS (Fluorescence Activated Cell Sorter). Ten larvae from each injection group were quickly euthanized in cold water. The head till pouch was removed, so the major tissue that remains is the muscles. These were digested in 1X trypsin EDTA and Collagenase IV (1 mg/mL) for 5 min. The CO_2-free complete media was used to neutralize the enzymes (trypsin and collagenase IV) and reduce the stress of cells in a single-cell suspension. The JC1 dye was added to this media at 5 µM final concentration for 10 min, and emission was measured in BD Accuri™ machine. The data acquired were analyzed using BD Accuri™ C6 software and Prism software to generate graphs.

Cell cycle analysis

Propidium iodide (PI) is not permeable to live cells. It is absorbed only by dead cells. Once inside cells, it binds to DNA by intercalating between the nitrogen bases. Once it binds to DNA, it is stable and emits red fluorescence, which can be assessed by fluorescent microscopy or FACS. In FACS, the inherent properties of cells and nucleus diffract the light that is captured as forward scatter and side scatter depending on the size and content of cytoplasm and nucleus. The cell cycle analysis was carried out on BD Accuri™ FACS machine based on PI staining in fixed cells. Five larvae from each group were fixed in 4% PFA overnight at 4°C. The head and fat pouches were cut off. Then larvae tails were digested with trypsin and collagenase-IV for 5 min at 37°C. The digestion was stopped by washing three times with PBS. The pipetting was done to further disrupt the tissue into suspension. This suspension was passed through a 45 µM membrane filter to remove debris. The single cell suspension was treated with 30-40 µM PI final concentration for 20 min in the dark and used for cell cycle analysis.

Culture of Patient-Derived Immortalized Myoblast lines

The immortalized myoblast cell lines AB1023DMD11Q clone1 (mutation stop exon 59: c.8713C>T, p.Arg2905X; 47.82 division number) and age-matched control cell line AB1190 clone 1 (48.7 division number) were established by Dr. Anne Bigot and Dr. Vincent Mouly at the Institut de Myologie, Paris, France. The myoblasts were grown in complete skeletal muscle cell growth medium (Takara, C-23060), 1% Glutamax (Invitrogen ref 35050-038) and 1% Gentamicin (Invitrogen ref 15750-037) according to protocol shared by senders. For experiments, similar myoblasts at similar division numbers were seeded at 2×10^5 cells initially, rinsed with 10 mL PBS, trypsinized (with 2 mL of trypsin for 5-10 min at 37°C, 5% CO_2, collected cells in 5 mL growth media, centrifuged at 1200 g and resuspended in

3 mL growth media) and transferred to Matrigel GFR coated six well plates. At 70-80% confluency, growth media was replaced with a differentiation medium containing DMEM + Gentamycin 50 µg/mL + 10 µg/mL of insulin. To recapitulate the effect of Jagged1 expression during the myogenin window, 22 h post-differentiation, synthetic Jagged1 mini peptide CDDYYYGFGCNKFCRPR final dilution in PBS or only PBS was added[34]. 24 h post-treatment, which is ~ 48 h post-differentiation, JC1 dye (1 µM) was added and incubated in the dark for 20 min. The images were taken in Zeiss LSM 880 microscope. Fluorescence intensities of emission at 488 nm (Green) and 590 nm (Red) were quantified with ImageJ as arbitrary units (a.u.). The Green/Red ratios from three independent replicates were statistically analysed.

Statistical analysis

The groups were uninjected controls, DMD, and DMD + Jagged1 plasmid (rescue) and Control + Jagged1 plasmid. Similarly, DMD + PBS, DMD + Jagged1 peptide, Control + PBS and Control + Jagged1 peptide treatment groups were compared in human cell culture studies. Mean and SD data for three independent experiments were analysed in GraphPad Prism 7.0 for two-way ANOVA with Tukey's multiple comparison test. A p value of less than or equal to 0.05 was considered significant.

Results

Jagged1 overexpression reduces cytoplasmic oxidative stress in dystrophic zebrafish larvae

The intensity of DCFDA stained fluorescence (arbitrary units) was taken as a read-out for oxidative stress. Oxidative stress was found to be higher in the DMD group than in control but was reduced with Jagged1 OE, as seen in the rescued group (Fig. 1A). The fluorescence intensity from stained larval tail muscles of the DMD group was higher than the control group. The rescue group showed a statistically significant reduction in intensity compared to DMD (Fig. 1B).

Jagged1 overexpression reduces the percentage of apoptotic cells and increases the proliferating cells

Figure 2A shows representative graphs of FACS gating used to quantify percent cell populations in different stages of the cell cycle. The M4 gate shows apoptotic cells, while M1, M2, and M3 represent the G0/G1, S, and G2/M phases of the cell cycle. Only

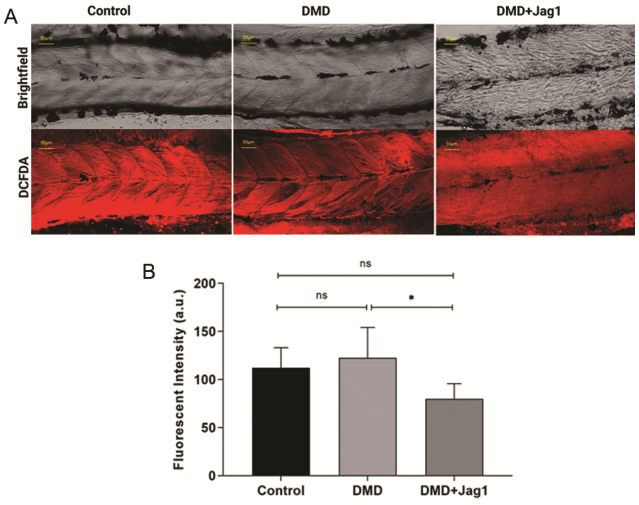

Fig.1 — Cytoplasmic ROS level in the larval tail muscle. (A) Representative images of bright-field and corresponding DCFDA stained images of Control, DMD and DMD+Jag1 (rescue) groups (scale bar in yellow: 50 µM); and (B) Graph showing statistical analysis of fluorescence intensity following DCFDA staining in Control, DMD and DMD+Jag1. n=9, N=3; p value ≤0.05*; ns non-significant

apoptotic and dividing cell percentages were compared between the control, DMD, and DMD+Jag1 (rescue) groups. The statistical analysis showed that the percent apoptotic cells are higher in the DMD compared to the control, which reduced in the rescued group (Fig. 2B). There was also a trend of increase in the G2/M percent (dividing cells) population in the rescue compared to the control and dystrophic, but statistically non-significant (Fig. 2C).

Jagged1 overexpression increases mitochondrial ROS production in rescued muscles

Figure 3A shows how gating was applied to JC1 stained cells from which data was obtained. The ratio of Green/Red percentage cells from the population was calculated in Excel sheets. The statistical analysis of which is given in the (Fig. 3B), which showed a higher Green/Red ratio in the rescue group as compared to the control and DMD groups. In the control background also, Jagged1 overexpression increases mitochondrial ROS production, but this effect seems more pronounced in the dystrophic condition.

Jagged1 mini-peptide treatment increases mitochondrial ROS in 48 h human dystrophic myotubes

Mitochondrial oxidative stress measured as Green/Red intensity ratio was similar to control myoblasts in DMD myoblasts during 22-48 h of differentiation time during which myogeninis expected

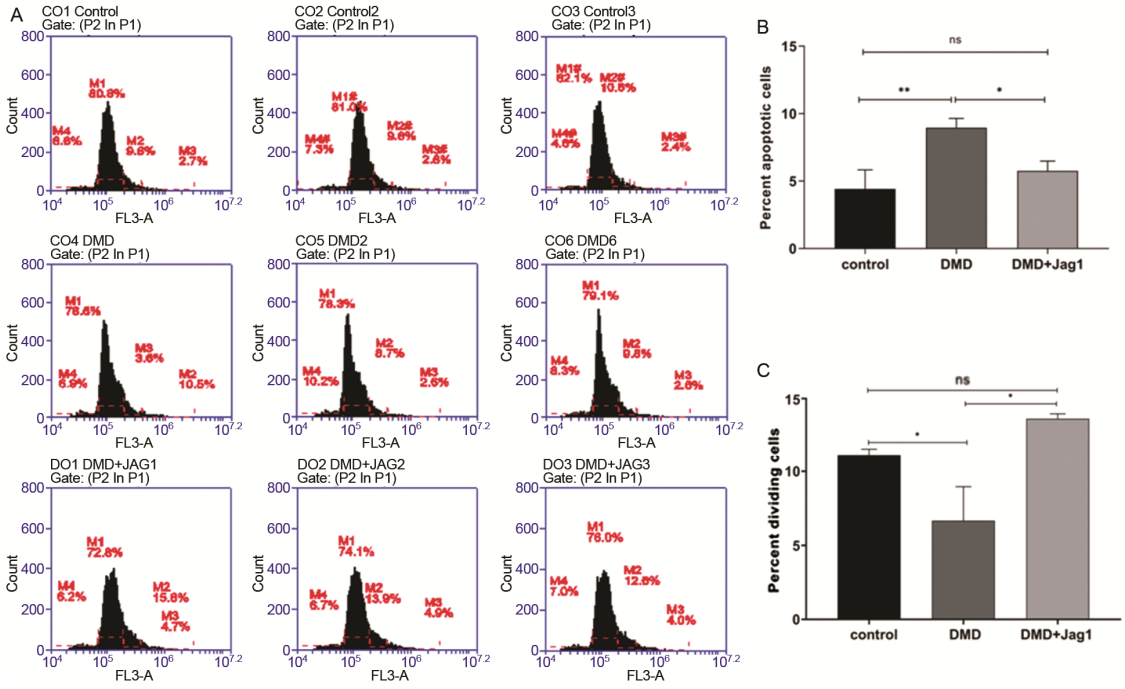

Fig. 2 —The cell cycle analysis of larval tail tissue. (A) Representative FACS graphs showing cell population in M4-apoptotic stage, M1-G0/G1, M2-S-phase, M3-G2/M stage after gating; (B) Graph of statistical analysis of percent apoptotic cells; and (C) Graph of statistical analysis of percent dividing cells in S-phase. n=30, N=3; p value ≤0.05 *, ≤0.01 **; ns non-significant

to begin sharp upregulation in expression (Fig. 4A). Treatment with mini-Jagged1 peptide during this period showed increased mitochondrial oxidative stress significantly only in DMD myoblasts, not in control myoblasts (Fig.4B).

Discussion

The muscle activity-associated processes like calcium ingress, Na^+/H^+ exchanger activity, and oxidative stress turn pathological in dystrophic conditions. The activation of master stress regulator p38MAPK also causes apoptosis instead of usual PGC1-α activation for metabolic adaptation in healthy muscles in response to exercise[18,19]. Thus, dystrophic muscles' inherent poor stress response at structural, osmotic, signalling, and metabolic levels results in perpetual damage during the low-intensity daily activity in patients. The damage-activated immune response is also required for proper regeneration in healthy muscles, but in DMD, it does not reach the resolution phase due to ongoing damage. The continuous cycles of degeneration-regeneration and heightened inflammatory milieu cause exhaustion of myogenic potential at a young age, making disease progression non-reversible[10].

In accordance with the literature, we found a higher percentage of dying cells in addition to increased cytoplasmic and mitochondrial oxidative stress in dystrophic larval tail muscles[13]. There was a significant reduction in dividing cells from the dystrophic larval tail. In zebrafish (*Sapje* and *Sapje*-like) models of DMD, damage by 3-4 days post-fertilization (dpf) and regeneration during 4-10 dpf have been shown[36,37]. The larval tail is composed of 80% of muscles, while a small percentage of other cells make up the rest of it. The immune cells and endothelial cells are expected to have higher proliferating cells in the inflammatory condition of dystrophy. Hence, the steep reduction of proliferating cells found in dystrophic larval tails here (Fig. 2B) probably represents a loss of myogenic potential at 8 days of age. The rescue group shows a significantly higher percentage of proliferating cells than the dystrophic and control groups (Fig. 2B). This correlates with the preservation of myogenic potential from original escaper GRMD animals, suggesting the rescuing mechanism is conserved[27]. The higher percentage of cells in the S-phase can also indicate a hindrance to cell cycle progression. It has been shown that Jagged1 expression in patient-derived myoblasts interferes with the mitogenic action of IL-1β[38]. Cell cycle inhibition can induce apoptosis in proliferating cells, but the reduction in apoptotic cells in the rescue group does not support this possibility. Moreover,

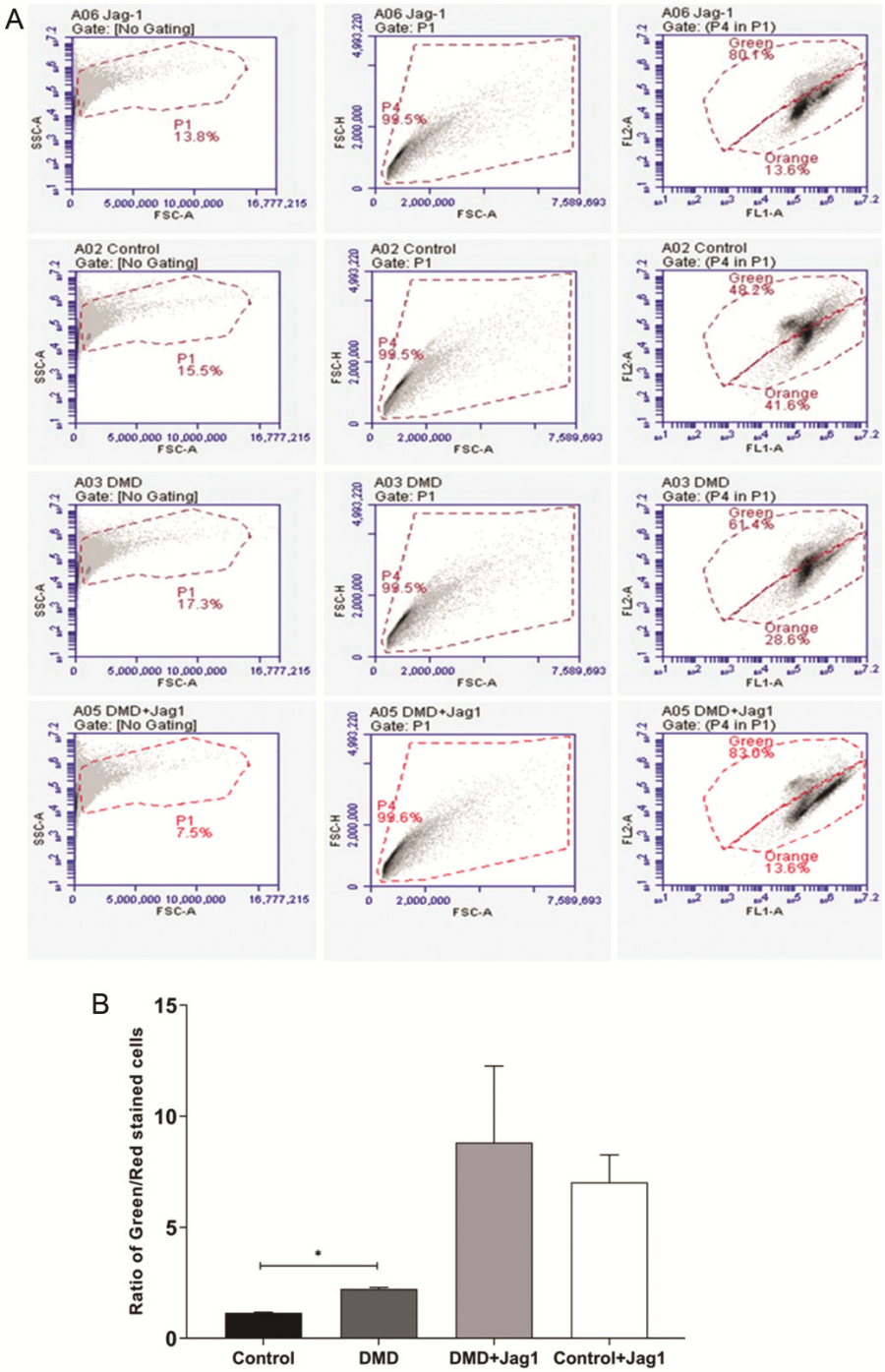

Fig. 3 — JC1 dye staining for mitochondrial ROS with FACS. (A) Representative images showing gating applied to quantify the ratio of Green/Red fluorescent emitting percent of cells from the larval tail; and (B) Graph showing statistical analysis of ratio Green/Red fluorescent emitting percent of cells in Control, DMD and DMD+Jag1. n=20, N=3; p value ≤0.05*

increased myoblast death during regeneration would result in disease exacerbation, not the functional rescue reported in the escaper GRMD or in 8-day old zebrafish larvae that we observed. One drawback in this study is the lack of myoblast-specific antibodies, which would have confirmed the identity of cells in apoptotic and proliferating populations.

The decrease in DCFDA dye intensity indicates reduced cytoplasmic oxidative stress in the rescue group compared to the dystrophic group (Fig. 1B).

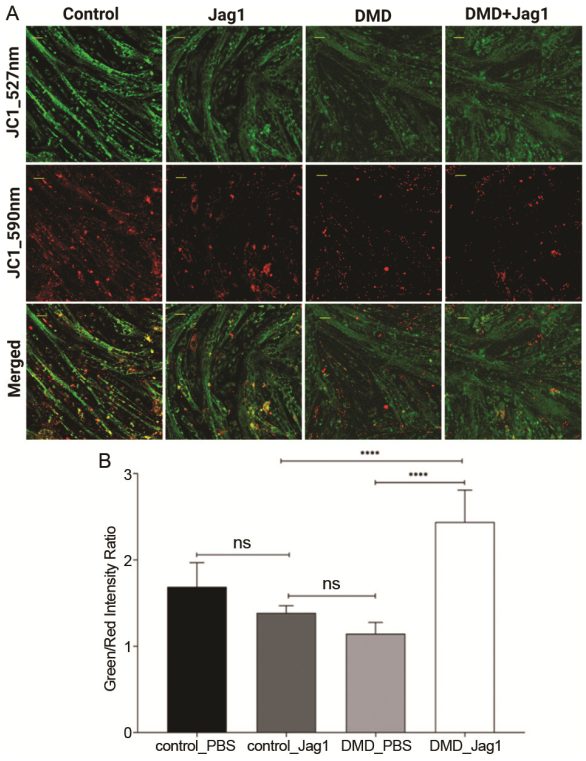

Fig. 4 —Mitochondrial ROS in human dystrophic myotubes post Jag1-peptide treatment. (A) Representative images of JC1 dye staining (in 527 nm/Green and 590 nm/Red channels) at 48 h of differentiation and 24 h of mini-Jag1peptide treatment (Scale bar in yellow: 200 µM); and (B) Quantification of the ratio of Green/Red intensities (arbitrary unit) shows a significant increase in the DMD +Jag1 peptide group compared to Control +Jag1 and DMD+PBS groups. n=20, N=3; p value ≤0.0001 ****; ns non-significant

Though the role of oxidative stress in pathogenicity is known in great detail, it is not considered as a direct initiator of myofiber death. The antioxidants like N-acetyl cysteine (NAC), or glutathione supplementation or selenium, which improves glutathione assimilation, did not improve the patient's condition[39]. One of the master transcriptional regulators of oxidative stress response is NRF2. When NRF2 was knocked out in heterozygous condition ($NRF2^{-/+}$), it did not exacerbate injury in mdx background[40]. Although activators of NRF2 are being tested in the mdx model[41], the findings cast doubt on its potential as DMD therapy. Even though ROS production and apoptosis were reduced in this study, it may not be a direct cause-effect of JAG1 overexpression ROS production is more likely pathogenic in DMD than a poor response to oxidative stress. Studies from different cancers suggest that Notch signalling helps survive oxidative stress, especially in progenitor cells independently of NRF or AP1[42,43]. Notch intracellular domain (NICD) also has been shown to bind and inhibit an apoptotic effector called ASK1 downstream of p38MAPK signalling[44]. Hence, there are several mechanisms by which Jag1-Notch signalling can improve oxidative stress response in dystrophic muscles, but the exact mechanism requires further study.

In addition to sarcolemma-associated NOC and xanthine oxidase (XO), mitochondria are considered the major source of ROS in DMD. The persistence of calcium or cytoplasmic oxidative stress is thought to cause increased mitochondrial ROS production[14,15]. Dystrophic mitochondria also consume 50% less oxygen during rest and exercise, which results in half of ATP production compared to healthy muscles[14,45-47]. The reduced activity of complex-I of the electron transport chain (ETC) is thought to be the reason behind increased ROS production and reduced ATP production[14]. Though overactive uncoupling protein-2 (UCP-2) shunting electron potential towards heat production and accumulated α-ketoglutarate inhibiting ATP Synthase also contribute to lower ATP production from dystrophic mitochondria[48-50]. It is unknown whether mitochondrial ROS production is causal in lower ATP synthesis or a result of lower TCA and ETC in the case of DMD. Here, unexpectedly we find increased mitochondrial ROS production in the rescue group as compared to the control or DMD group. Given the known severe energy starvation in the dystrophic muscles, and the structural-functional rescue seen at 8 dpf larvae, it is very likely this increased ROS is linked to increased mitochondrial ATP production but needs further confirmatory study. The increased ROS production could also increase fibrosis via inflammatory modulation, though not checked here, but was described in the escaper GRMD muscles[28,51]. The mitochondrial ROS production also seems to increase with Jagged1 mini peptide in iDMD myoblasts undergoing differentiation. The combination of reduced cytoplasmic and increased mitochondrial ROS can potentially reduce calcium, microtubule, and energy starvation-induced pathology in DMD[16, 52] but needs further confirmatory studies.

Conclusion

However, further studies are required to confirm rescue in dystrophic muscle cultures and mechanistic details of this rescue, this study provides pathological processes mitigated by Jagged1 overexpression. It also suggests that Jagged1-based

therapies should be pursued further to cure this debilitating condition.

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Conflict of interest

All authors declare no conflict of interest.

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