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# Examination of the possible effects of ozone application in Duchenne muscular dystrophy

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**ABSTRACT**

Duchenne muscular dystrophy (DMD), an X-linked disorder, has an incidence of one in 5000 boys and presents in early childhood with proximal muscle weakness. The disease is caused by mutations in DMD (encoding dystrophin) that abolish the production of dystrophin in muscle. In this study, we aimed to investigate the effect of ozone therapy (OT) on muscle oxygenation in DMD patients. For this purpose, DMD was developed in the primary skeletal muscle cell line. The changes that occurred by administering OT to the cell lines were observed. OT reduced mitochondrial ROS caused by DMD, partially improved the shape changes in myoblasts, but had no effect on dystrophin. OT to DMD patients may have a positive effect on muscle cells.

**Keywords:** Duchenne muscular dystrophy, mitochondrial ROS, ozone therapy

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## Introduction

Duchenne muscular dystrophy (DMD), considered a debilitating and fatal skeletal muscle disease, is characterized by muscle weakness, exercise intolerance, and progressive deterioration of skeletal muscle (Blau et al., 1983). DMD is caused by large deletions and other minor forms of mutations (deletions, duplications, and inversions) in the dystrophin gene located on the short arm of the X chromosome. Lack of functional dystrophin disrupts the structure and function of muscle fibers necessary for the physiological growth of muscle tissue (Muntoni et al., 2003). The dystrophin (dp427) protein, which connects the actin cytoskeleton of muscle fibers to the extracellular matrix and is therefore an integral part of the contractile structure of muscle, is encoded by the dystrophin gene (DMD) on the X chromosome. Therefore, while DMD predominantly affects boys, girls are likely to be asymptomatic "healthy carriers". DMD is also characterized by a progressive degeneration of skeletal muscles, with symptoms appearing early in life, around 3 years, leading to loss of ambulation from the 13th year onwards, followed by cardiac complications and respiratory disorders such as chronic respiratory failure (Hoffman et al., 1987). Untreated, most DMD patients become wheelchair-bound by age 11-12. Muscle histology in DMD patients is nearly normal before the onset of clinical symptoms at 3–5 years of age. The initial stage of the disease is characterized by the presence of focal groups of necrotic myofibrils, muscle hypertrophy, and abnormally high levels of muscle creatine kinase. In the second (pathological) phase, repeated cycles of degeneration exhaust the regenerative capacity of muscle-specific stem cells (satellite cells) and fibrotic mechanisms cause the replacement of muscle tissue by collagenous connective tissue (Anthony et al., 2011).

Deficiencies or changes in dystrophin protein structure disrupt the stability of the cell membrane, induce oxidative stress, and also lead to increased turnover of calcium ions in the cytosol, all of which can lead to cell death. The resulting cell membrane lesions allow calcium ions to enter through the gaps, which trigger protease enzymes that promote premature cell death (Liew and Kang, 2013). Following cell membrane disruption, creatine kinase effluxes, leading to loss of stored energy for contraction. Although the underlying pathomechanisms still require further investigation, some studies have reported that a systemic metabolic disorder is central to the etiology of the disease. According to these studies, DMD; involved in glycolysis, nucleotide synthesis, tricarboxylic acid (TCA) cycle and electron transport chain (ETC). It causes structural changes in enzymes (Aslesh et al., 2021). This results in dysfunction of the contractile apparatus leading to decreased muscle strength, dysregulation of intracellular Ca2+ buffering, loss of homeostasis, and Ca2+-induced degeneration. It is assumed that the dysregulation of intracellular Ca2+ homeostasis caused by DMD is associated with mitochondria dysfunction (Falzarano et al., 2015). Mitochondria are important regulators of Ca 2+ ions in skeletal muscle, working synchronously with the sarcoplasmic reticulum (SR) to maintain a Ca 2+ ion potential of approximately 50 nM at rest and handling 100-fold functional oscillations up to 5 µM during stimulation. It is thought that increased Ca2+ ion during activity provides functional benefits to the muscle and thus oxidative ATP production can be matched with the demand at the cross-bridge level (Salmaninejad et al., 2021).

Ozone has been used medically for over 150 years, and over these years it has been applied either as a method of disinfection or as a treatment option for a variety of diseases (Bocci, 1999). It is an unstable molecule consisting of three oxygen atoms that can quickly convert to oxygen and a single oxygen atom that acts as a strong oxidant to kill microorganisms. At appropriate concentrations it also serves as an ideal medicine. It has been reported that ozone shows its protective effect by stimulating the endogenous antioxidant system and reducing glycogen consumption and lactate production. Although ozone has been applied for therapy since the end of the 18th century, the cellular mechanisms that explain the positive effects of ozone therapy, especially at low concentrations, are still largely unexplored. Three basic forms of topical ozone application have been identified: ozonated water, ozonated oil, and oxygen/ozone gas. Additionally, ozone activates the protein synthesis mechanism. Increase the amount of ribosomes and mitochondria in cells (Clavo et al., 2003). These changes at the cellular level; Stimulates the regeneration potential in tissues and organs by increasing functional activities. In this study, we aimed to investigate the effect of ozone therapy (OT) on muscle oxygenation in DMD patients. Despite recent clinical studies on OT, further biological and biochemical studies are needed to understand the limitations and possible side effects of its use in humans.

Within the scope of this study, we aimed to determine whether ozone can increase the amount of mitochondria in the dystrophic skeletal muscle cell line and to determine the cellular mechanisms responsible for regeneration abilities, considering its possible therapeutic properties based on dystrophin synthesis modulation by dystrophic skeletal muscle cells. In this study, the effects of low ozone concentrations on structural and functional cell properties

were investigated using morphological, morphometric, cytochemical and immunocytochemical techniques in brightfield and fluorescence.

**Material and methods**

**Cell culture**

Primary skeletal muscle cell line (SkMC)-Primary Skeletal Muscle Cells (PCS-950-010)) was obtained from ATCC. PCS were maintained in skeletal muscle growth medium (ATCC PCS-950-040) at 37°C, 5% CO2, and 95% humidity before transfection.

**Transfection**

Short interfering, Cas9 nuclease and sgRNA 2 were transfected into primary skeletal muscle cell line. Cells were plated at 60% density in six-well culture dishes the day before transfection.

Immediately before transfection, cells were washed with phosphate-buffered saline and 1 ml of serum-free SkMC growth medium was added. Transfection was performed according to the manufacturer's protocol using Genesilencer (PeqLab, Erlangen). It was carried out using Germany. Briefly, 5 µl of Genesilencer reagent was mixed with 25 µl of serum-free medium.

1000 ng of siRNA was diluted in a mixture of 25 µl of siRNA diluent and 15 µl of serum-free medium and incubated for 5 min at room temperature. The siRNA solution was added to the diluted Genesilencer and incubated at room temperature for 5 min before adding to the cells.

Serum was removed from the medium for the first 4 hours after transfection. Next, 1 volume of medium with a serum concentration of 10% was added to a final concentration of 5%. Twenty-four hours after transfection, the medium was transferred to SkMC differentiation medium containing 2.5% fetal bovine serum. (Promocell) has been changed.

**Cell viability and proliferation**

Cell Counting Kit-8 (CCK-8) was used to determine cell viability. The assay was performed in a 96-well plate according to the manufacturer's protocol. 10 µl of Cell Counting Kit-8 solution was added to each well containing 100 µl of fresh medium. After 2 h of incubation in humidified atmosphere, absorbance was measured at 450 nm using a microplate reader (Anthos HTII, Anthos Labtec, Wals, Austria). Results given as proportion of vital cells were calculated as follows: After transfection then OD 450 nm 5x103 SkMC was divided into OD 450nm 5x103 SkMC before transfection. Cell proliferation was measured using a colorimetric BrdU cell proliferation enzyme-linked immunosorbent assay kit (Roche, Mannheim, Germany). OD, maximum adjustable microplate reader

Measured at 450 nm using (Molecular Devices, Ismaning/Munich, Germany). Estimating cell viability and proliferation between five and nine samples was measured at 18 and 48 h or 72 h after transfection. Metabolic effects of ASO and siRNA on proliferation Time points were chosen based on their maximum effect, which differed across pathways and was determined in separate experiments.

**Application of ozone therapy (OT)**

Ozone gas was dissolved in PBS solution to maintain a final concentration of 0.3 mg/L. Ozonated phosphate buffer was added to the medium and the cells were incubated for another 30 minutes.

cultured and the medium was changed. This procedure was performed once a day for 3 days (Yiu & Kornberg, 2008).

**Assessment of mitochondrial function**

Cells or intact mitochondria were incubated with 5 µmol/L MitoSOX Red, 2 µmol/L 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolylcarbocyanineiodide (JC-1), or 20-fold diluted fluorescein isothiocyanate (FITC)–conjugated Annexin V to determine changes in mitochondrial ROS. ROS levels in cytoplasmic fractions of cells were determined by using dichlorofluorescein diacetate (10 µmol/L) staining.

**Dystrophin Staining**

Cells were incubated with 4% paraformaldehyde in TBS (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 2mM EGTA, 2mM MgCl2) for 20 min at room temperature, three times with TBS and 0.5% Triton X-100/TBS. Following exposure to a blocking solution (containing TBS-0.5% bovine serum albumin; Sigma) for 10 min, fixed cells were incubated with monoclonal mouse anti-inflammatory for 1 h and 30 min. Dystrophin (NCL-DYS2, Novocastra, Newcastle Tyne, UK) and adhalin (NCL-50DAG, Novocastra) with Cy3-conjugated goat anti-mouse antibody (Jackson Immunoresearch, West Grove, PA, USA) diluted 1:200 for 1 h. was treated. Samples Vectashield mounting medium (Vector, Burlingame, CA, USA). Immunolabeled samples were examined by confocal

laser scanning microscopy.

**Results**

**Cell viability and proliferation**

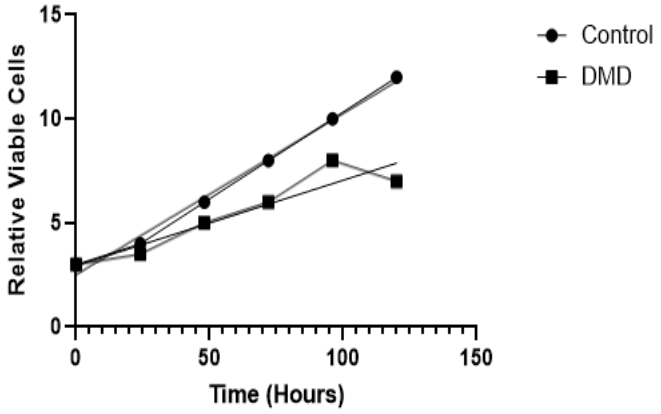


**Figure 1.** Growth curve analysis of iPSCs cells.

**Cell culture and transfection**

Generation of the disease-specific iPSC lines proceeded routinely, and characterization of iPSCs revealed no major differences compared with DMD generated from healthy cells. Efficient myogenic differentiation of the DMD-specific iPSCs proceeded only under optimized conditions.

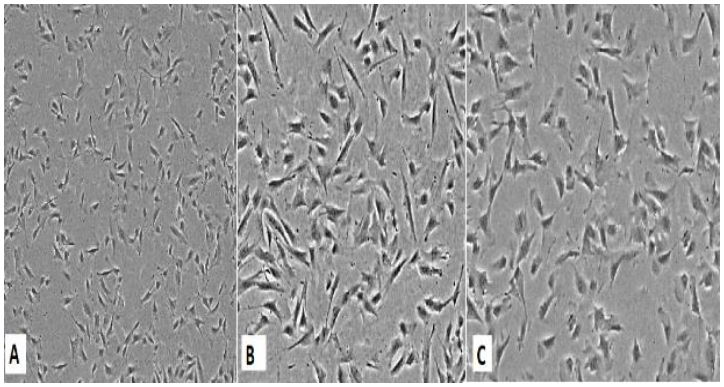


**Figure 2.** Generation of DMD specific iPSC lines. (A) control myoblasts; Myoblasts display typical compact morphology, (B) DMD myoblasts; malformed myoblasts and (C) DMD miyoblast+OT; skeletal muscle cells recovered after TO therapy.

**Effect of OT on mitochondrial dysfunction**

Mitochondrial ROS levels were measured in control cells and compared with DMD cell culture. DMD increased the levels of mitochondrial ROS in cell culture in a concentration-dependent manner (Fig 3), an effect partially prevented by OT (Fig 3).

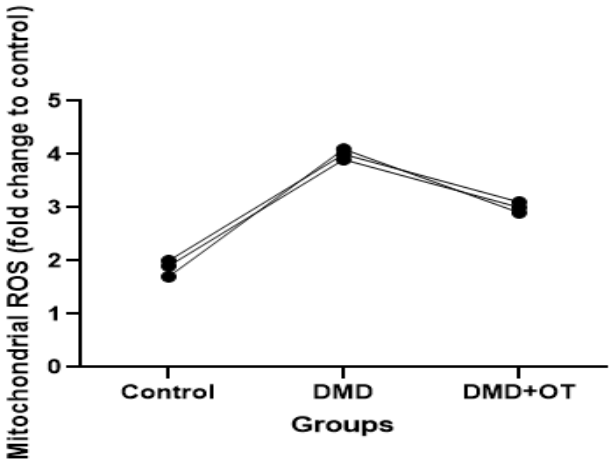


**Figure 3.** Mitochondrial ROS levels

**Dystrophin**

Dystrophin quantification in cell models is a valuable tool to characterize diseased versus non-diseased cells and measure dystrophin restoration in DMD cells, as potential therapeutic approach. Dystrophin was stained positive in the control group (Fig. 4A). DMD tissues, as expected, completely lacked dystrophin (Fig.4B). Similarly, dystrophin negative staining was observed in the DMD+OT group (Fig. 4C).

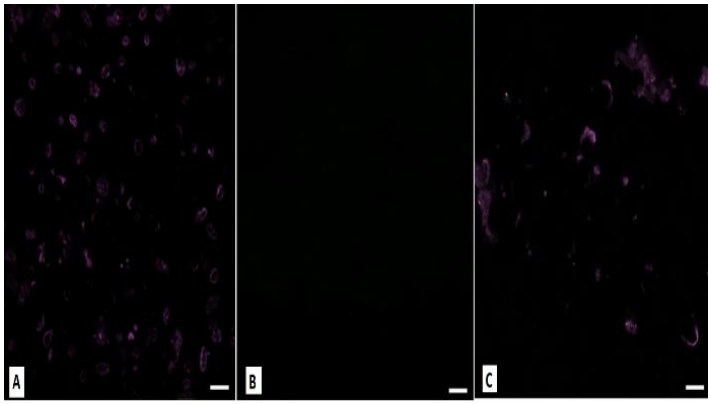


**Figure 4.** Representative images of dystrophin in control (A), DMD (B) and DMD+OT (C) Scale bar, 100 µm.

**Discussion**

Much research on the pathogenesis of Duchenne muscular dystrophy (DMD) is based on the hypothesis that there may be a defect in the sarcolemma of the dystrophic muscle fibre and that this in turn may allow an influx of calcium ions that cause hypercontraction and overload mitochondria (Rus, 2006).

There are several promising monotherapies for DMD, but there is no treatment yet that can reverse or completely halt the progression of all pathophysiological consequences of dystrophin deficiency in patients. Therefore, studies on DMD continue.

In this study, we aimed to identify suitable, fast-responsive and accessible molecular biomarkers of DMD to help determine the best route, frequency and

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