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Remdesivir induces persistent mitochondrial and structural damage in human induced pluripotent stem cell derived cardiomyocytes

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Abstract

Aims: Remdesivir is a prodrug of an adenosine triphosphate analogue and is currently the only drug formally approved for the treatment of hospitalised COVID-19 patients. Nucleoside/nucleotide analogues have been shown to induce mitochondrial damage and cardiotoxicity, and this may be exacerbated by hypoxia, which frequently occurs in severe COVID-19 patients. Although there have been few reports of adverse cardiovascular events associated with remdesivir, clinical data are limited. Here, we investigated whether remdesivir induced cardiotoxicity using an *in vitro* human cardiac model.

Methods and Results: Human induced pluripotent stem cell-derived cardiomyocytes (hiPSC-CMs) were exposed to remdesivir under normoxic and hypoxic conditions to simulate mild and severe COVID-19 respectively. Remdesivir induced mitochondrial fragmentation, reduced redox potential and suppressed mitochondrial respiration at levels below the estimated plasma concentration under both normoxic and hypoxic conditions. Non-mitochondrial damage such as electrophysiological alterations and sarcomere disarray were also observed. Importantly, some of these changes persisted after the cessation of treatment, culminating in increased cell death. Mechanistically, we found that inhibition of DRP1, a regulator of mitochondrial fission, ameliorated the cardiotoxic effects of remdesivir, showing that remdesivir-induced cardiotoxicity was preventable and excessive mitochondrial fission might contribute to this phenotype.

Conclusions: Using an *in vitro* model, we demonstrated that remdesivir can induce cardiotoxicity in hiPSC-CMs at clinically relevant concentrations. These results reveal previously unknown potential side-effects of remdesivir and highlight the importance of further investigations with *in vivo* animal models and active clinical monitoring to prevent lasting cardiac damage to patients.

Translational perspective:

Adult cardiomyocytes have limited ability to regenerate, thus treatment-induced cardiotoxicity can potentially cause irreparable harm. Remdesivir is currently the only FDA approved treatment for COVID-19 but clinical safety data are limited. Using human pluripotent stem cell-derived cardiomyocytes, we revealed that remdesivir induced persistent mitochondrial and structural abnormalities at clinically relevant concentrations. We advise confirmatory experiments in *in vivo* animal models, investigations of cardioprotective strategies, and closer patient monitoring such that treatment-induced cardiotoxicity does not contribute to the long term sequelae of COVID-19 patients.

1. Introduction

Coronavirus disease of 2019 (COVID-19), which is caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), has claimed millions of casualties worldwide. Although COVID-19 primarily affects the respiratory system, cardiac damage is prevalent, occurring in 20-30% of hospitalised patients and contributing to 40% of deaths.[1] The urgency of the COVID-19 pandemic has demanded the rapid development of therapeutic strategies. To this end, remdesivir, a compound originally designed for the treatment of Ebola virus disease, has been repurposed for use against COVID-19. Remdesivir has broad spectrum activity against multiple RNA viruses and inhibits SARS-CoV, MERS-CoV, and SARS-CoV-2 *in vitro* and *in vivo*.[2-4] In a double-blind, placebo-controlled multicentre clinical trial, 1062 persons with COVID-19 were randomised to receive a 10-day treatment of remdesivir or placebo.[5] Patients who received remdesivir experienced a statistically significant decline in the median recovery time to 10 days vs 15 days in the placebo group. Mortality was reduced to 6.7% (remdesivir) vs 11.9% (placebo) by day 15, and 11.4% (remdesivir) vs 15.2% (placebo) by day 29 (hazard ratio, 0.73; 95% CI, 0.52 to 1.03).[5] These results are consistent with a smaller placebo-controlled trial involving 237 patients which showed a statistically non-significant faster time to clinical improvement in patients who received remdesivir compared to control (21 vs 23 days), particularly in those who received the drug within 10 days of onset of symptoms (16 vs 23 days)[6]. These results are contradictory to the larger open-label WHO Solidarity trial, in which no clinical or survival benefit was observed.[7] Among patient subgroups, those with moderately severe disease (requiring supplemental oxygen) responded better than those with mild (not requiring oxygen) and severe disease (requiring high flow oxygen or mechanical ventilation).[5] Serious adverse events were detected at similar rates in the remdesivir treatment vs placebo group.[5] Remdesivir was first granted emergency use authorisation for the treatment of COVID-19 patients on the May 1, 2020 and was the first and, at the time of writing, the only drug to

receive formal approval from the US Food and Drug Administration (FDA) on the Oct 22, 2020 for use in adults and children hospitalised with suspected or laboratory confirmed COVID-19.

Remdesivir is a prodrug of an adenosine triphosphate analogue which binds to RNA-dependent RNA polymerase to inhibit viral replication.[8] Nucleotide/nucleoside analogues are important treatment against RNA viruses but their use have been associated with increased incidences of mitochondrial toxicity, which mainly affects tissues with high energy demand such as the heart.[9] The mitochondria comprise ~30% of the volume of adult cardiomyocytes (CMs), and are critical for cardiac metabolism and apoptosis. Damage to this organelle can therefore severely impair cardiac function. For instance, the clinical development of the nucleotide/nucleoside analogue BMS-986094 was terminated due to lethal cardiotoxicity.[10] Toxicity is largely attributed to inhibition of Pol-γ[11] and POLRMT[12], the DNA and RNA polymerases responsible for the synthesis and transcription of mitochondria DNA. Other mechanisms of mitochondrial interference, such as inhibition of and alterations in the expression of mitochondrial genes and proteins[13], enhanced production of mitochondrial reactive oxygen species[14] have been proposed. Although there have been few reports of adverse cardiac effects related to the use of remdesivir, clinical data are currently limited.

Patients with COVID-19 are vulnerable to treatment induced cardiotoxicity due to the high prevalence of cardiovascular damage among these individuals.[15] Many factors may contribute to heart injury including pre-existing cardiovascular co-morbidities[16, 17], direct viral infection of CMs[18-22], acute inflammation and myocarditis[23], and hypoxia-induced cardiac damage[24]. Acute inflammation in the lungs can compromise respiratory function, leading to low oxygen saturation in the blood and hypoxia in the heart. Dypsnea and low oxygen saturation are observed in 50-60% of hospitalised patients with COVID-19, and are associated with greater risk of cardiac damage and poor prognosis.[25, 26] The heart is metabolically demanding, and is reliant on oxygen to drive oxidative phosphorylation in the mitochondria. Increased cardiometabolic demand associated with systemic infection, coupled with hypoxia caused by acute respiratory illness can disturb the balance between myocardial oxygen demand and supply to result in mitochondrial dysfunction and injury. Remdesivir

was initially indicated for use in patients with moderate/severe COVID-19 who requires supplemental oxygen, although this was later broadened to all hospitalised patients. In moderate/severe patients, systemic hypoxemia may damage the heart, and thereby increases susceptibility to the potential adverse effects of remdesivir.

Human induced pluripotent stem cells (hiPSCs) can self-renew in culture; their differentiation to the cardiac lineage represents a potentially unlimited source of CMs for disease modelling and cardiotoxicity testing.[27-29]. Human iPSC derived CMs (hiPSC-CMs) spontaneously contract, express genes/proteins associated with cardiac identity and recapitulate key aspects of human cardiac physiology[27, 30]. Specifically, hiPSC-CMs have been shown to respond to agents which damage the mitochondria and are now an important component of cardiotoxicity testing.[28, 29, 31] In the context of COVID-19, hiPSC-CMs were recently used to demonstrate direct infection of CMs by SARS-CoV-2.[18, 19, 22, 32, 33] Sarcomeric disarray, cessation of beating, electrical and contractile disturbances, and apoptosis were observed after infection.[18, 33] Transcriptional analysis further revealed significant downregulation of genes important for mitochondrial function and oxidative phosphorylation in infected hPSC-CMs.[18] Human iPSC-CMs are therefore a suitable platform for the evaluation of the adverse cardiac effects of COVID-19 treatment.

Here, we investigated the cardiotoxic effects of remdesivir by exposing hiPSC-CMs to clinically relevant concentrations of this drug under normoxic and hypoxic conditions to simulate (i) prophylactic use in healthy individuals and treatment for patients with mild COVID-19, and (ii) therapeutic use in patients with severe COVID-19 suffering from pneumonia induced hypoxemia respectively. Remdesivir induced mitochondrial dysfunction in the form of reduced redox potential and respiration, mitochondrial fragmentation, as well as structural abnormalities at concentrations several folds below Cmax under both normoxic and hypoxic conditions. Importantly, some of these changes persisted after the withdrawal of this drug. Inhibition of mitochondrial fission ameliorated remdesivir induced damage, showing that disturbed mitochondrial dynamics was a mechanistic contributor to the cardiotoxic effects of remdesivir.

2. Methods

2.1 Human hPSC culture and cardiac differentiation

We used the hiPSC line AICS-0060-027 (Allen Cell Collection) for our experiments unless otherwise indicated. The cell line is a derivative of the parental line (WTC-11), and contains a mono-allelic mEGFP-tagged MYL2 modification, and was cultured as per instructions from Allen Cell Collection. Cardiac differentiation was performed via the modulation of the WNT signalling pathway.

2.2 Evaluation of mitochondrial function

Mitochondrial redox activity was measured using the resazurin-based PrestoBlue assay (Thermo Fisher Scientific, Waltham, MA). Mitochondrial respiration was monitored using a Seahorse extracellular flux analyzer (XFe-96) (Agilent Technologies, CA, USA) using the mito stress assay. For evaluation of mitochondrial and nuclear morphology, cells were incubated with the MitoTracker Deep Red FM dye (Thermo Fisher Scientific) and Hoescht 33342 dye (Thermo Fisher Scientific), and scored. Mitochondrial superoxide (O_2^-) levels were assayed using the MitoSOX™ Red reagent (Thermo Fisher Scientific). Mitochondrial membrane potential ($\Delta\psi m$) was measured using the tetramethylrhodamine, ethyl ester (TMRE) (Thermo Fisher Scientific).

2.3 Action potential measurements

Action potential was measured with ruptured whole-cell patch-clamp.

Please see supplemental methods for details.

3. Results

3.1 Remdesivir induced mitochondrial abnormalities in hiPSC-CMs

We utilised an hiPSC line (AICS-0060-027) in which the cardiac ventricular marker, MLC2V, is tagged with the eGFP fluorescent reporter. Human iPSC-CMs were generated using an established monolayer differentiation protocol via modulation of the WNT signalling pathway, followed by metabolic selection (Fig. S1A)[34]. Spontaneous contractions could be observed by days 7-8 of differentiation. Flow cytometry experiments showed that hiPSC cardiac cultures were >95% positive for cardiac Troponin-T, an established marker of CMs, and MLC2V-eGFP$^+$ ventricular CMs comprised ~80% of the population at the time of assay (Fig. S1B). CMs were used after day 40 of differentiation, when they assumed an oblong, elongated morphology, with α-actinin and MLC2V-eGFP localised to the Z-disks and A-band respectively (Fig. S1C). Human iPSC-CMs expressed low/high levels of MYH6 and MYH7, commonly associated with structural immaturity/maturity respectively (Fig. S1D).[35] These results showed that the hiPSC-CMs had a high degree of structural organisation and were suitable for the modelling of human cardiac dysfunction.

Human iPSC-CMs were exposed to remdesivir under normal and hypoxic conditions for three days. Since remdesivir is thought to be most beneficial to patients who receive supplemental oxygen (i.e. mild hypoxia), but not high flow oxygen (severe hypoxia), we chose an oxygen (O_2) concentration of 2.5% to simulate mild hypoxia that did not directly cause cell death but may instead potentiate damage induced by external stimuli. Human iPSC-CMs exposed to 2.5% O_2 were viable, beat and were morphologically indistinguishable from hiPSC-CMs cultured under normoxic (20% O_2) conditions.

To determine if remdesivir induced mitochondrial dysfunction, we first compared the redox potential of hiPSC-CMs treated with different doses of this drug under normoxic and hypoxic conditions. The 50% effective concentration (EC50) of remdesivir *in vitro* is in the (sub)micromolar range while the Cmax of remdesivir in healthy volunteers is 9.0 and 4.3 μM on day 1 and 5 of treatment respectively.[36] A dose range of 0.1-12.5μM was chosen to represent clinically relevant concentrations of remdesivir for cardiotoxicity evaluations. Redox potential is mostly driven by the

proton gradient in the mitochondria and is often used as an indicator of metabolic activity in this organelle. Remdesivir dose-dependently reduced redox potential under normoxic and hypoxic conditions (Fig. 1A). While remdesivir had negligible effects at low doses (0.1 μM and 0.5 μM), it significantly decreased redox activity at 2.5 μM and 12.5 μM by 21.0±3.9% and 35.9±4.0% under normoxic, and by 22.5±5.4% and 28.7±6.9% under hypoxic conditions respectively.

Next, we tested the effect of remdesivir on mitochondrial respiration, which is critical for cardiac metabolism. Seahorse metabolic flux assay showed that remdesivir repressed respiration in a dose-dependent manner (Fig. 1B). Under normoxic conditions, remdesivir significantly reduced basal respiration and ATP production by 59.8±5.7% and 68.3±8.3% respectively when applied at 2.5 μM. Under hypoxic conditions, remdesivir significantly reduced basal respiration by 33.3±6.1% and 36.3±8.7% at 0.5 μM and 2.5 μM; maximal respiration at 37.7±8.0% at 2.5 μM; and ATP production at 24.9±5.9% and 37.5±8.4% at 0.1 μM and 0.5 μM. Remdesivir at 12.5 μM produced highly variable effects of oxygen consumption.

Mitochondrial dynamics constitutes part of the quality control process of this organelle and is maintained by a balance between fusion and fission, which promote the elongation and fragmentation of mitochondria, respectively.[37, 38] Since mitochondrial dynamics is frequently disturbed by cardiotoxins, we investigated whether remdesivir altered mitochondrial morphology. Fluorescence imaging of mitochondrial dye revealed three patterns of mitochondrial morphologies among hiPSC-CMs (Fig. 1C and S2A). The first consisted of densely organised, elongated mitochondrial networks spread throughout the cytoplasm and this is considered to represent a healthy balance of fission and fusion. The second had punctate, fragmented mitochondria consistent with increased fission. The last had mitochondria located around the nucleus, also suggestive of an imbalance towards increased fission. All hiPSC-CM samples exhibited a mixture of the three phenotypes, but in different proportions. Control, normoxic hiPSC-CMs primarily had an elongated mitochondrial network while remdesivir induced mitochondrial fragmentation in a dose-dependent manner (Fig 1C). Under normoxic conditions, the proportion of cells with elongated mitochondria significantly decreased

from 65.1±6.8% in control hiPSC-CMs to 43.9±7.5%, 27.3±6.0%, and 13.4±5.0% upon treatment with increasing doses of remdesivir at 0.5, 2.5 μM and 12.5 μM respectively, and this was accompanied by a corresponding increase in the proportion of cells with punctate, fragmented mitochondria from 21.0±5.9% to 44.4±7.1, 57.3±7.2 and 64.4±9.5%. Similarly, hypoxia decreased the prevalence of elongated mitochondria in favour of punctate mitochondria and this worsened with increasing doses of remdesivir. The proportion of elongated mitochondria significantly decreased from 45.1±7.8% in control hPSC-CMs to 18.7±3.0% and 17.8±4.2% upon treatment with 2.5 μM and 12.5 μM of remdesivir, while the proportion of punctate mitochondria rose from 37.3±6.1% in control, to 64.4±4.1% and 58.9±8% at the same doses. The proportion of cells with perinuclear mitochondria was similar in all samples. Consistent with these results, immunostaining for TOM20, a protein present in the outer mitochondrial membrane that is commonly used as a marker of mitochondria, also revealed mitochondrial fragmentation in hiPSC-CMs treated with remdesivir (Fig. S3A). The mitochondrial mass of hiPSC-CMs, as measured by the intensity of mitochondrial dye staining, was not statistically different among different groups (Fig. S4A), showing that remdesivir promoted mitochondrial fragmentation but did not alter mitochondrial mass in hiPSC-CMs.

To determine if increased oxidative stress contributed to the toxic effects of remdesivir, the level of reactive oxygen species (ROS) in the form of mitochondrial superoxide was measured using the MitoSox red dye and was found to be similar in control and treated hiPSC-CMs (Fig. S4B). These results were further confirmed using the CellRox dye, an indicator of ROS in the cytoplasm as well as the mitochondria (data not shown).

We next tested whether remdesivir induced the depolarisation of the mitochondrial membrane potential ($\Delta\psi m$), which is commonly considered to be a prelude to irreversible apoptosis and cell death. No statistically significant decrease in $\Delta\psi m$ was detected (Fig. S4C).

To investigate if remdesivir disturbed the expression of genes important for mitochondrial function, we measured the mRNA levels of genes encoding components of electron transport chain (ETC) encompassing the mitochondrial complex I (MT-ND1 and MT-ND5), II (SDHA), IV

(COX6A2) and V (ATP6), as well as a non-ETC gene encoding a mediator of fatty acid β-oxidation in the mitochondria (ACADVL) (Fig. 2). With the exception of SDHA, remdesivir dose-dependently and significantly reduced the expression of ETC genes including ND1, ND5, COX6A2 and ATP6, while ACADVL mRNA levels were similar among all samples. Of the ETC genes, MT-ND1, MT-ND5 and ATP6 are encoded by the mitochondrial genome, while COX6A2 and SDHA are encoded by the nuclear genome. Thus, the effect of remdesivir was not limited to mitochondrial- or nuclear-encoded genes. Overall, the general repression of ETC gene transcripts was consistent with and might contribute to the reduced redox potential and mitochondrial respiration detected (Fig 1A and B).

3.2 Remdesivir induced structural abnormalities but not cell death

In addition to mitochondrial dysfunction, non-mitochondrial damage was observed in remdesivir-treated cells and manifested as perturbations in sarcomeric arrangement. In control hiPSC-CMs, eGFP-tagged MLC2V assumed a densely-packed striated appearance along myofilaments which spanned the entire cell, reflecting its localisation to the thick filaments of sarcomeres (Fig S1C and 3A). Low doses of remdesivir (0.1 and 0.5 μM) did not have any noticeable effect under normoxic conditions, but 0.5 μM of remdesivir under hypoxia induced the thinning and truncation of myofibrils. Human iPSC-CMs treated with 2.5 and 12.5 μM of remdesivir had greatly disorganised myofibrils under both normoxic and hypoxic conditions. While cells with striated structures could still be found, sparse and truncated myofibrils, and patchy MLC2V-eGFP signal with no or poorly discernible organisation were common. Similarly, immunostaining against α-actinin, a protein located at the Z-lines of CMs, also revealed severe disorganisation and areas of barely detectable signal in hiPSC-CMs treated with 2.5 and 12.5 μM of remdesivir (Fig. S3B).

We next tested if remdesivir induced apoptosis or cell death in hiPSC-CM cultures. Nuclear condensation is a sign of apoptosis and manifest as brightly-stained, small and ‘condensed’ nuclei (Fig. S2B). Staining with the Hoescht nuclear dye did not reveal any significant increase in the number of condensed nuclei following remdesivir treatment (Fig 3B). Consistently, nuclear area and

staining intensity were similar in control and remdesivir treatment groups irrespective of dose and normoxia/hypoxia (Fig. S5). No significant difference in cell number was observed among all samples, demonstrating the absence of significant cell detachment (Fig. S5). We thus concluded that remdesivir did not significantly induce cell death in hiPSC-CM cultures.

3.3 Remdesivir altered the electrophysiological properties of hiPSC-CMs

In addition to mitochondrial toxicity, we also tested if remdesivir disturbed the electrophysiological properties of hiPSC-CMs. Single cell patch clamp analysis showed that remdesivir (2.5 μM) significantly reduced spontaneous firing frequency (Control 0.89±0.14 vs remdesivir 0.36±0.07 Hz) and the diastolic depolarisation rate (Control 20.32±3.77 vs remdesivir 7.74±1.19 mV/s), and tended to decrease the maximum depolarisation rate (Control 40.94±7.42 vs remdesivir 21.7±6.38 mV/s) (Fig. 4). The action potential amplitude, and maximal diastolic potential were similar in control and hiPSC-CMs treated with remdesivir.

3.4 Remdesivir induced persistent cardiac damage

Our results so far demonstrated that remdesivir caused mitochondrial, structural and electrophysiological abnormalities, but significant changes associated with irreversible damage such as $\Delta\psi m$ depolarisation and nuclear condensation were not observed. We therefore asked if damage induced by remdesivir was transient and reversible. To address this, hiPSC-CMs were exposed to remdesivir as described previously, followed by 'recovery' for 3 days in media devoid of this drug under normoxic conditions to simulate patient recovery after the cessation of remdesivir treatment (Fig. 5A). Remdesivir dose-dependently altered redox potential under normoxic and hypoxic conditions. It significantly decreased redox potential by 31.1±5.3% and 44.1±8.1% at 2.5 μM and 12.5 μM under normoxic condition, and by 41.0±8.7% and 54.6±8.5% under hypoxic conditions (Fig. 5B).

We also evaluated whether remdesivir-induced mitochondrial fragmentation was reversed upon recovery. While low dose of remdesivir (0.5 μM) altered mitochondrial morphology immediately after treatment (Fig. 1C), no significant difference was detected between control and treated cells upon recovery (Fig. 5C). By contrast, the mitochondria of hiPSC-CMs treated with higher doses of this drug (2.5 μM and 12.5 μM) remained more fragmented than control. The proportion of cells with elongated mitochondria decreased from 40.1±4.5% in control CMs to 17.2±6.6% and 7.3±3.8% in hiPSC-CMs treated with 2.5 μM and 12.5 μM of remdesivir respectively. Similar trends were observed under hypoxic conditions: showing a decrease in elongated mitochondria from 47.4±4.9% in control to 19.7±2.5% and 4.6±2.1% with 2.5 μM and 12.5 μM remdesivir, and this was accompanied by a corresponding increase in the proportion of cells with punctate mitochondria from 46.1±3.8% to 66.1±1.1% and 65.5±6.6%. The proportion of cells with perinuclear mitochondria increased from 6.5±1.5% in control to 29.9±5.7% with 12.5 μM remdesivir, demonstrating persistent mitochondrial damage (Fig. 5C). Apart from mitochondrial changes, structural alterations also persisted beyond the three-day treatment period. Cells treated with 2.5 μM and 12.5 μM of remdesivir showed disorganised sarcomeric arrangement analogous to the patterns seen immediately after treatment (Fig. 3A and 5D).

We evaluated the prevalence of nuclear condensation as a surrogate for apoptosis and cell death. Although no significant increase in nuclear condensation was seen immediately after three days of treatment (Fig. 3B), a dose dependent increase in the proportion of condensed nuclei was observed in hiPSC-CMs three days after they were treated with 2.5 μM and 12.5 μM of remdesivir (Fig. 5E). The same trend was also observed with the TUNEL assay, which detects DNA breaks, further confirming that remdesivir-induced cardiotoxicity can ultimately lead to increased apoptosis (Fig. S6).

Lastly, we extended our ‘recovery’ period to 14 days and asked if mitochondrial and structural abnormalities could still be observed. Human iPSC-CMs treated with remdesivir exhibited significantly reduced redox potential of 35.4±6.4% and 43.0±5.6% at 2.5 μM and 12.5 μM under

normoxic and 22.7±3.8% at 2.5 μM under hypoxic conditions, compared to control (Fig. S7A). Mitochondrial fragmentation and sarcomeric disarray were also apparent, showing that remdesivir treatment had a long term detrimental impact on hiPSC-CMs (Fig. S7B and C).

In summary, remdesivir induced mitochondrial and structural damage which persisted beyond the treatment period, culminating in increased cell death.

3.5 Remdesivir-induced cardiotoxicity could be ameliorated by an inhibitor of mitochondrial fission

Given the extensive mitochondrial fragmentation observed in remdesivir treated hiPSC-CMs, we proposed that excessive mitochondrial fission might underlie the cardiotoxic effects induced by this drug, and that the inhibition of DRP1[38, 39], a master regulator of mitochondrial fission, could protect against remdesivir. We tested this by applying mdivi-1, which is a well-established inhibitor of DRP1[40], to hiPSC-CMs. Human iPSC-CMs treated with mdivi-1 alone had similar redox potential, exhibited elongated mitochondria and organised sarcomeres comparable to control cells (Fig. 6A and S8A). Mdivi-1 has been shown to have an IC50 of 10 μM for mitochondrial fragmentation in human cells[41]. We therefore tested the ability of 5 μM, 15 μM, 50 μM of mdivi-1 to repress remdesivir induced mitochondrial fragmentation. 15 μM of remdesivir was found to be most consistently protective and was used for subsequent experiments (Fig. S8B). Co-treatment of mdivi-1 with 2.5 μM remdesivir significantly promoted the formation of elongated mitochondria under normoxic conditions compared to cells treated with remdesivir alone (Fig. 6B). Mdivi-1 treatment also produced a corresponding decrease in the proportion of cells with punctate mitochondria under hypoxia. Similar observations were made using immunostaining with anti-TOM20 antibody (Fig. S3A), further confirming the protective effects of mdivi-1. However, at 12.5 μM remdesivir, mdivi-1 could only significantly protect hiPSC-CMs under hypoxic but not normoxic conditions.

We next tested if preventing mitochondrial fragmentation with mdivi-1 could normalise redox potential in hiPSC-CMs. Under both normoxic and hypoxic conditions, mdivi-1 restored redox

potential in hiPSC-CMs treated with 2.5 μM of remdesivir to near control levels (Fig. 6A). However, the same trend was not observed at 12.5 μM of remdesivir, at which mdivi-1 did not have noticeable effect.

The structural abnormalities induced by remdesivir could also be ameliorated by mdivi-1. Mdivi-1 improved the sarcomere arrangement of CMs treated with 2.5 μM, and 12.5 μM of remdesivir (Fig. 6C). Myofibrils were more densely packed, and MLC2V-eGFP signal was more striated with mdivi-1 co-treatment than remdesivir alone.

Lastly, we measured the level of DRP1 mRNA and protein in control and remdesivir-treated cells to test if remdesivir directly altered DRP1 expression. Although DRP1 mRNA showed a dose-dependent and significant increase in cells treated with remdesivir (Fig. S8C), there was no noticeable change in protein expression (Fig. S8D). These results showed that while DRP1 participated in the pathogenesis of remdesivir induced cardiotoxicity, remdesivir did not promote mitochondrial fission via upregulation of this protein.

In summary, our results showed that remdesivir induced cardiotoxicity was preventable and could be ameliorated by mdivi-1 *in vitro*, and identified excessive mitochondrial fission to be a mechanistic contributor to this phenotype.

3.6 The cardiotoxic effect of remdesivir is not cell-line specific

Key findings were validated in a second hiPSC line, MDI-C16, showing that our results are not cell line dependent. These experiments confirmed reduced redox potential (Fig. S9A) and mitochondrial fragmentation (Fig. S9B) upon remdesivir treatment. These changes persisted for at least three days after remdesivir treatment (Fig. S10) and mdivi-1 ameliorated the toxicity induced by remdesivir (Fig. S9C).

4. Discussion

Remdesivir is a nucleotide analogue effective against COVID-19, but its potential to cause cardiotoxicity is unclear. Using an *in vitro* hiPSC-CM model, we showed that remdesivir induced cardiotoxicity at clinically relevant concentrations under normoxic and hypoxic conditions. Mitochondrial dysfunctions and sarcomere disarray were detected, and they persisted after cessation of treatment. Consistent with recent clinical reports of serious bradycardia, remdesivir also perturbed electrophysiological properties of hiPSC-CMs. This is the first report of adverse and persistent cardiac effects associated with remdesivir at clinically relevant concentrations and highlights the importance of cardiac monitoring in patients treated with this drug. Importantly, inhibition of mitochondrial fission protected against mitochondrial fragmentation, normalised redox potential and prevented sarcomere disarray, showing that remdesivir induced cardiotoxicity can be ameliorated.

The efficacy of remdesivir against COVID-19 has been demonstrated in many *in vitro* and *in vivo* studies.[2, 3] Remdesivir is effective *in vitro* in the sub-micromolar range: it blocked SARS-CoV-2 infection in African green monkey kidney Vero E6 cells with an EC50 and EC90 of 0.77 μM and 1.76 μM respectively,[3] and in human airway epithelial cells, at an IC50 of 0.069 μM.[42] Remdesivir is also efficacious in randomised clinical trials and shortened time to recovery.[5] In light of these findings, remdesivir received authorisation from the US FDA for use in hospitalised patients with COVID-19 with a standard dose of 200 mg, followed by daily doses of 100 mg for 4 or 9 days. In healthy adult volunteers receiving a similar dose regimen, and the peak plasma concentrations were 5.4 μg/mL (9.0 μM) on day 1 and 2.6 μg/mL (4.3 μM) on day 5.[36] Thus both the EC50 and the Cmax of remdesivir are within the dosage range tested in this study (0.1-12.5 μM). We first detected increased mitochondrial fragmentation at 0.5 μM but these perturbations did not persist. On the other hand, multiple features of mitochondrial and sarcomeric abnormalities were observed at ≥ 2.5 μM under normoxic and hypoxic conditions, which is 3.6 and 1.7 fold below Cmax in patients on day 1 and 5 of treatment. Importantly, these abnormalities persisted after cessation of treatment, culminating in increased cell death.

Mitochondrial toxicity of remdesivir has been demonstrated in two human intestinal (HT29 and HCT116) and two human liver cell lines (HepG2 and PLC/PRF/5) at high doses of 10 or 20 μM.[43] Conversely, a recent report evaluated the effect of remdesivir using a panel of cell lines and primary cells, and concluded that remdesivir had a low potential to elicit off-target toxicity, although significant cell line specific differences were noted.[44] Neither of the two reports included CMs. Ours is the first report of persistent mitochondrial toxicity in CMs at clinically relevant concentrations. By systematically evaluating the mitochondrial phenotype of remdesivir treated cells, we showed that remdesivir primarily induced mitochondrial fragmentation, suppressed mitochondrial respiration and reduced redox potential. Mitochondrial damage may occur via various mechanisms. The toxicity of ribonucleoside/nucleotide analogues has largely been attributed to inhibition of POLRMT[11], the RNA polymerase responsible for the transcription of mitochondria DNA. However, purified human mitochondrial RNA polymerase has been shown to effectively discriminate against remdesivir-triphosphate with a selectivity value of ~500-fold,[45] and remdesivir triphosphate was found to have negligible effect on key human DNA and RNA polymerases[44], suggesting that inhibition of the latter is unlikely to be a major contributor of the cardiotoxic effects of remdesivir. Instead, we identified excessive mitochondrial fission to be a critical driver of the remdesivir induced abnormalities, since the inhibition of the former with a small molecule inhibitor, mdivi-1, reduced mitochondrial fragmentation, normalised redox potential and improved sarcomeric arrangement in cells treated with 2.5 μM of remdesivir. Excessive mitochondrial fragmentation has previously been shown to contribute to a range of cardiac disorders and mdivi-1 is cardioprotective in the settings of doxorubicin induced cardiotoxicity,[46] ischemia-reperfusion injury,[47, 48] and pressure overload induced heart failure.[49] Here we provide important proof-of-principle that cardioprotective strategies (such as mdivi-1) can be employed to at least partially protect CMs against the cardiotoxic effects of remdesivir. It is possible that additional factors contribute to the cardiotoxicity of remdesivir at extremely high doses and this may explain the lesser ability of mdivi-1 to protect against remdesivir at 12.5 μM.

We initially reasoned that hypoxia might potentiate the mitochondrial toxicity induced by remdesivir; instead remdesivir induced similar level of toxicity under normoxic and hypoxic conditions. These unexpected findings may relate to the severity of hypoxia used in the study. There is a paucity of data that directly correlates oxygen saturation *in vivo* with oxygen concentration *in vitro*. Furthermore, COVID-19 patients exhibit highly diverse oxygen saturation ranging from normal (>95%), to very low (<70%).[50] It is therefore difficult to choose an O_2 concentration *in vitro* to represent all patients. Since remdesivir was found to be most effective in patients with mild hypoxia (i.e. requiring oxygen supplementation but not mechanical ventilation)[5], we chose an oxygen (O_2) concentration of 2.5% to simulate mild hypoxia that did not directly cause cell death. It is possible that the relatively mild conditions used was not enough to exacerbate the effects of remdesivir.

In addition to mitochondrial damage, single cell patch clamp analysis revealed electrophysiological alterations in hiPSC-CMs treated with 2.5 μM of remdesivir. Specifically, remdesivir decreased firing frequency and this is consistent with a previous examination of remdesivir using multi-electrode array analysis, which showed prolonged field potential duration and reduced beating rates.[32] Both our observations are in line with recent reports of bradycardia observed in patients.[51, 52]

Remdesivir was previously considered to be safe in animal studies and in clinical trials. Remdesivir has been associated with a slight increase in the risk of cardiac arrest (1.9% vs 1.4%) and serious atrial fibrillation (0.9% vs 0.2%) compared to placebo in one clinical study[5], but the Solidarity trial by the WHO showed similar rates of cardiac death (0.3% vs 0.4%).[7] Adverse cardiovascular events and evaluations of cardiac stress markers were not reported in two other clinical studies.[53, 54] Due to the low numbers of cardiovascular incidences reported, it is unclear whether the difference in cardiac outcome observed between remdesivir and placebo groups are significant, but immediate, overt cardiotoxicity was not apparent. There are potentially multiple reasons for the dichotomy between our study and currently available clinical data. Firstly, we utilised *in vitro* derived hiPSC-CMs, which may be immature and may not respond in the same manner as human adult CMs.[55-58]

Secondly, the cardiotoxicity of remdesivir initially manifests as mitochondrial and structural abnormalities without overt cell death during the treatment period, and may not be immediately apparent in clinical trials, which primarily report acute clinical adverse events. Thirdly and importantly, pre-existing cardiac co-morbidities, and direct cardiac damage caused by COVID-19 may mask any adverse effects of remdesivir. Further investigations in animal models or in patients would be critical to confirm cardiotoxicity *in vivo*. For clinical monitoring, high sensitivity troponin measurements, which can detect the death of CMs, may be informative during and after treatment. Cardiovascular magnetic resonance have already been used to reveal lower left ventricular ejection fraction, higher left ventricle volumes, and raised native T1 and T2 in patients recovered from COVID-19[59] and may aid in the detection of subtle changes in cardiac function induced by remdesivir.

Remdesivir is currently used clinically at a concentration that far exceeds its effective concentration *in vitro*. Remdesivir can inhibit SARS-CoV-2 in African green monkey kidney Vero E6 and human airway epithelial cells with an EC/IC50 of 0.77 μM and 0.069 μM. respectively[3, 42]. Similarly, recent studies in hPSC-CMs also demonstrated efficacy of remdesivir at 0.2-0.6 μM.[22, 32] While remdesivir induced cardiotoxicity ≥2.5 μM, we did not observe persistent functional alterations at low concentration of this drug closer to its *in vitro* effective dose (0.1 μM and 0.5 μM). It may be beneficial to assess the *in vivo* efficacy of this drug at lower doses to minimise adverse effects. Consistent with this, clinical trials comparing 5 or 10 days of remdesivir treatment either showed that there was no statistically significant difference in clinical outcome, or that the 5-day regimen was slightly superior.[53, 54] A careful titration of dose and duration, and alternate formulations of remdesivir[60] may help to balance antiviral efficacy with cardiotoxic risks.

Despite the limitations of our model, the demonstration of persistent cardiotoxicity at clinically relevant concentrations highlight the need for further investigations of cardiotoxicity in *in vivo* animal models, and clinical studies. Adult CMs have limited ability to regenerate, thus treatment-induced cardiotoxicity can potentially cause irreparable harm to patients already made vulnerable by

cardiovascular co-morbidities and cardiac damage caused by viral infection. Functional cardiac monitoring in the clinic are warranted such that cardiotoxicity does not contribute to the long term sequelae of COVID-19 patients.

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Data availability

The data underlying this article are available in the article and in its online supplementary material.

Author Contribution Statement

E.N.P conceived the study. M.K. maintained and differentiated hiPSCs, and performed most of the cell biology experiments. C.L performed immunostaining, microscopy and analysis. C.T and R.D. performed image analysis. H.S.L. and K.T.L. performed qPCR and Western blotting experiments. Q.D and S.Y.T performed electrophysiological experiments. All members including M.K., C.L., H.S.L., C.T., R.D., Q.D, S.Y.T, K.T.L, B.P.Y and E.N.P prepared and wrote the manuscript.

Conflict of Interest

None declared.

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Figure Legends

Figure 1. Remdesivir induced mitochondrial dysfunction in hiPSC-CMs.

Human iPSC-CMs were treated with indicated doses of remdesivir under normoxic or hypoxic conditions for 3 days. (A) Mitochondrial redox activity was measured using the PrestoBlue assay, n=11. (B) Seahorse metabolic flux analysis revealed altered metabolic profiles upon remdesivir treatment. Oxygen consumption rate=OCR, OGN=oligomycin, FCCP=Carbonyl cyanide p-trifluoro-methoxyphenyl hydrazone, Rot/AA=Rotenone+actimycin. Key parameters are summarised and graphed: Normoxia: n=5, Hypoxia: n=6. (C) Confocal fluorescence images of mitotracker dye staining (red) revealed disturbed mitochondrial organisation in hiPSC-CMs treated with remdesivir. Hoescht nuclear staining is in blue. The proportion of cells with elongated (asterick), punctate (arrowhead) and perinuclear (arrow) mitochondria were quantified, n=7. Statistical significance was calculated relative to control cells using the one-way ANOVA with Dunnett's multiple comparisons test for (A, B), and two-way ANOVA with Sidak's multiple comparisons test for (C), *$p<0.05$, **$p<0.01$, ***$p<0.001$, ****$p<0.0001$. Scale bar = 10μm.

Figure 2. Remdesivir perturbed the expression of genes important for mitochondrial function.

Human iPSC-CMs were treated with indicated doses of remdesivir under normoxic or hypoxic conditions for 3 days. The expression of selected genes were measured by qRT-PCR, normalised to B2M, n=5. Statistical significance was calculated using the one-way ANOVA with Dunnett's multiple comparisons relative to control cells *$p<0.05$, **$p<0.01$, ***$p<0.001$.

Figure 3. Remdesivir induced sarcomeric disarray in hiPSC-CMs.

(A) Confocal images of hiPSC-CMs showing MLC2V-eGFP signal in green, Hoescht nuclear staining in blue. Representative images of 6 independent batches of cells are shown. Control hiPSC-CMs have densely-packed, striated MLC2V-eGFP signal, reflecting its localization to the thick

filaments of sarcomeres, and was absent from the Z-disk and I-band. Sparse and truncated sarcomeres (arrowheads), patchy MLC2V-eGFP signal with no or poorly discernible organisation (arrows) could be detected in cells treated with high doses of remdesivir. (B) The proportion of cells with condensed nuclei was assessed among control cells and cells treated with the indicated doses of remdesivir. Statistical significance was examined using the one-way ANOVA test and no statistically significant difference was detected among the samples, n=8.

Figure 4. Remdesivir altered the electrophysiological properties of hiPSC-CMs.

Human iPSC-CMs were treated with/without 2.5μM of remdesivir under normoxic conditions for 3 days. The electrophysiological properties were measured using patch-clamp analysis. (A) Representative tracings and (B) action potential parameters of control and remdesivir-treated cells are shown. Statistical significance was calculated relative to control cells using the student's T-test, n=14 *$p<0.05$, **$p<0.01$.

Figure 5. Remdesivir induced persistent mitochondrial and structural abnormalities in hiPSC-CMs.

(A) Human iPSC-CMs were treated with indicated doses of remdesivir under normoxic (blue) or hypoxic (yellow) conditions for 3 days, and allowed to recover in the absence of remdesivir for 3 more days under normoxic conditions. (B) Mitochondrial redox activity was measured using the PrestoBlue assay, n=8. (C) Confocal fluorescence images of mitotracker dye staining (red) revealed elongated mitochondria (asterick) in most control cells, while the majority of remdesivir treated cells had punctate mitochondria (arrowhead). Perinuclear mitochondria (arrow) were enriched in cells treated with 12.5 μM of remdesivir, n=5. Hoescht nuclear staining is in blue. (D) Confocal images of hiPSC-CMs showing MLC2V-eGFP signal in green, Hoescht nuclear staining in blue. Sparse and truncated sarcomeres (arrowheads), patchy MLC2V-eGFP signal with no or poorly discernible organisation (arrows) could be detected in cells treated with remdesivir. Dotted arrow indicates a cell

with brightly stained, condensed nuclei. (E) Quantification of cells with condensed nuclei, n=6. Statistical significance was calculated relative to control cells using the one-way ANOVA with Dunnett's multiple comparisons test for (B) and (E), and two-way ANOVA with Sidak's multiple comparisons test for (C), *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001. Scale bar = 10μm.

Figure 6. An inhibitor of mitochondrial fission protected against remdesivir induced cardiotoxicity.

Human iPSC-CMs were treated with indicated doses of remdesivir under normoxic or hypoxic conditions, in the presence/absence of mdivi-1 (MD, 15 μM). (A) Mitochondrial redox activity was measured using the PrestoBlue assay, n=7. (B) Mitochondrial morphology was assessed using mitotracker staining in red, Hoescht nuclear staining in blue, n=6 for normoxia and n=5 for hypoxia. MD co-treatment with 2.5 μM remdesivir increased the proportion of cells with elongated mitochondria (asterisk), while cells treated with remdesivir alone displayed mostly punctate (arrowhead) and perinuclear (arrow) mitochondria. (C) Confocal images of hiPSC-CMs showing MLC2V-eGFP signal in green. Sparse and truncated sarcomeres (arrowheads), patchy MLC2V-eGFP signal with no or poorly discernible organisation (arrows) were enriched in cells with remdesivir alone, while MD co-treatment resulted in more striated sarcomeres. Statistical significance was calculated using the (A) one-way ANOVA with Sidak's multiple comparisons test against cells treated with remdesivir alone without MD, and two-way ANOVA with Sidak's multiple comparisons test against cells treated with remdesivir alone without MD for (B). *p<0.05, **p<0.01. Scale bar = 10μm.

Figure 1

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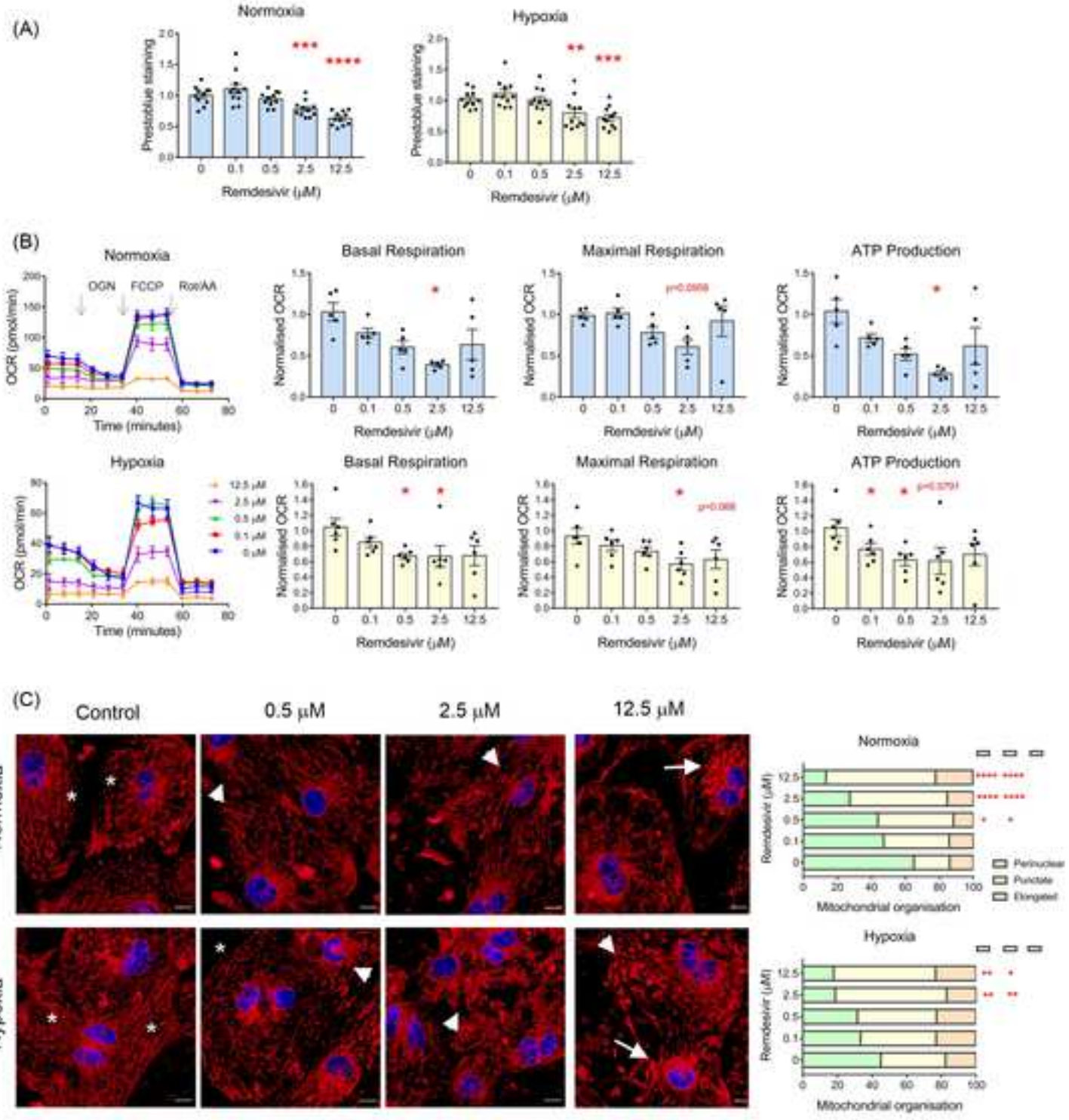
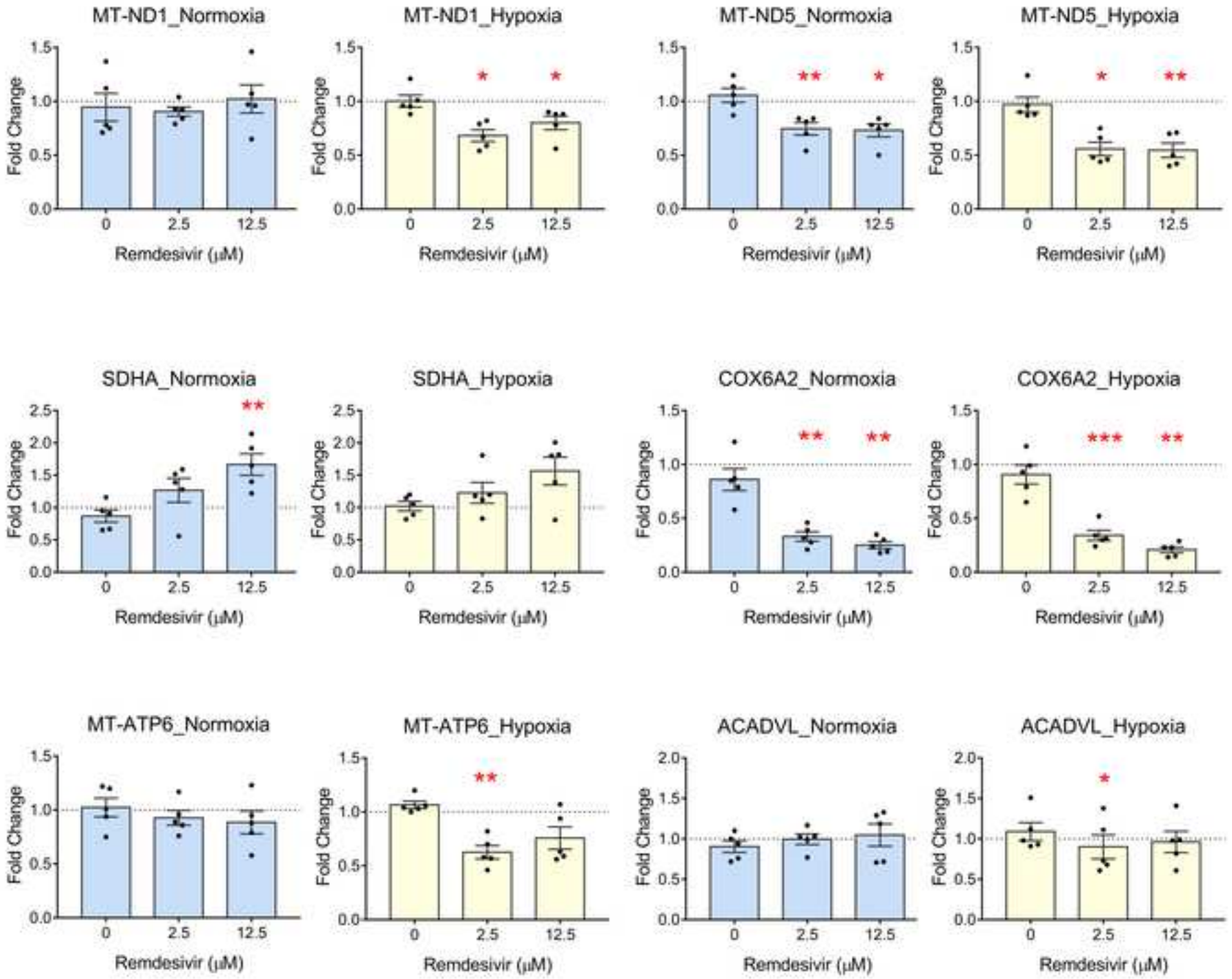


Figure 2



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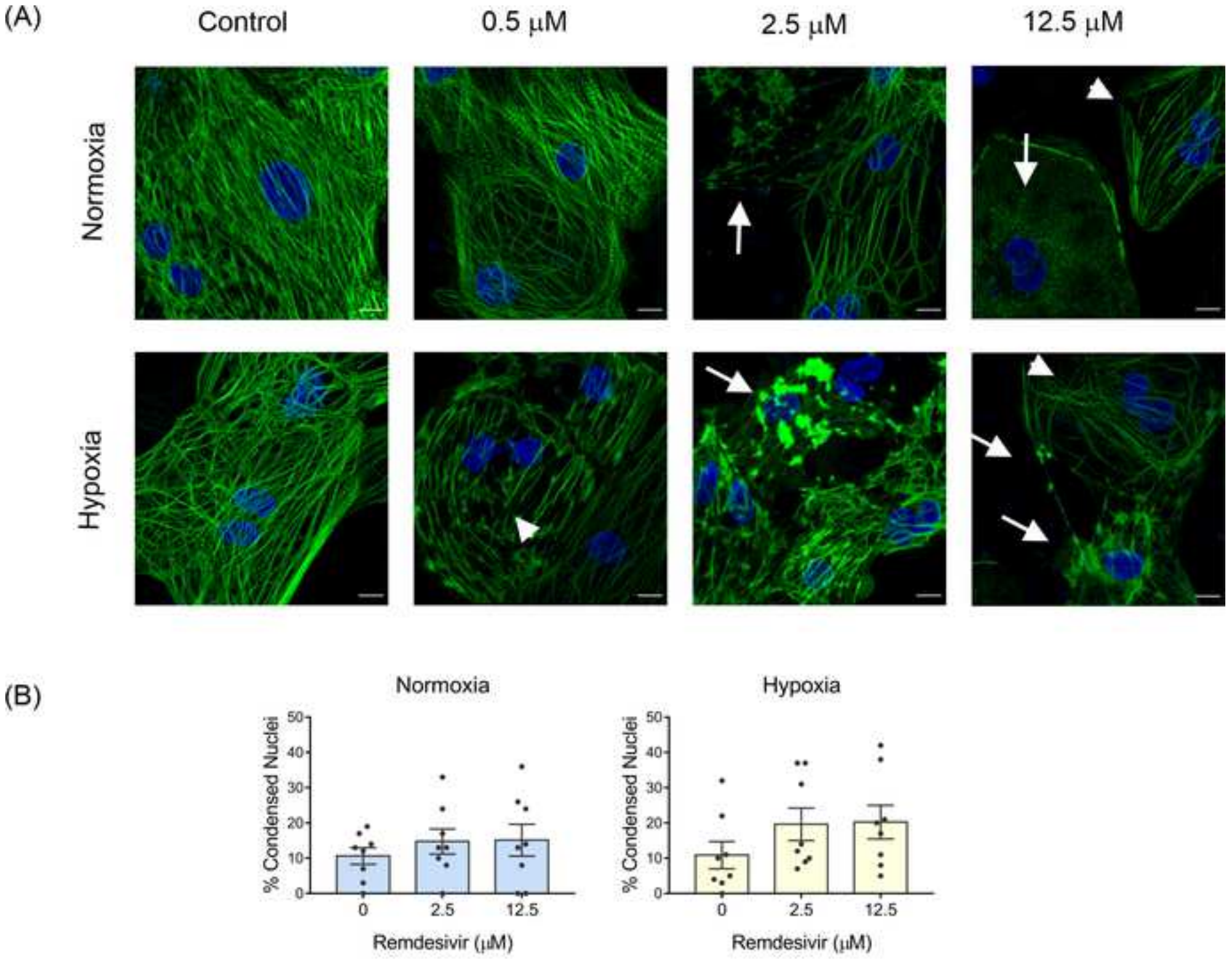

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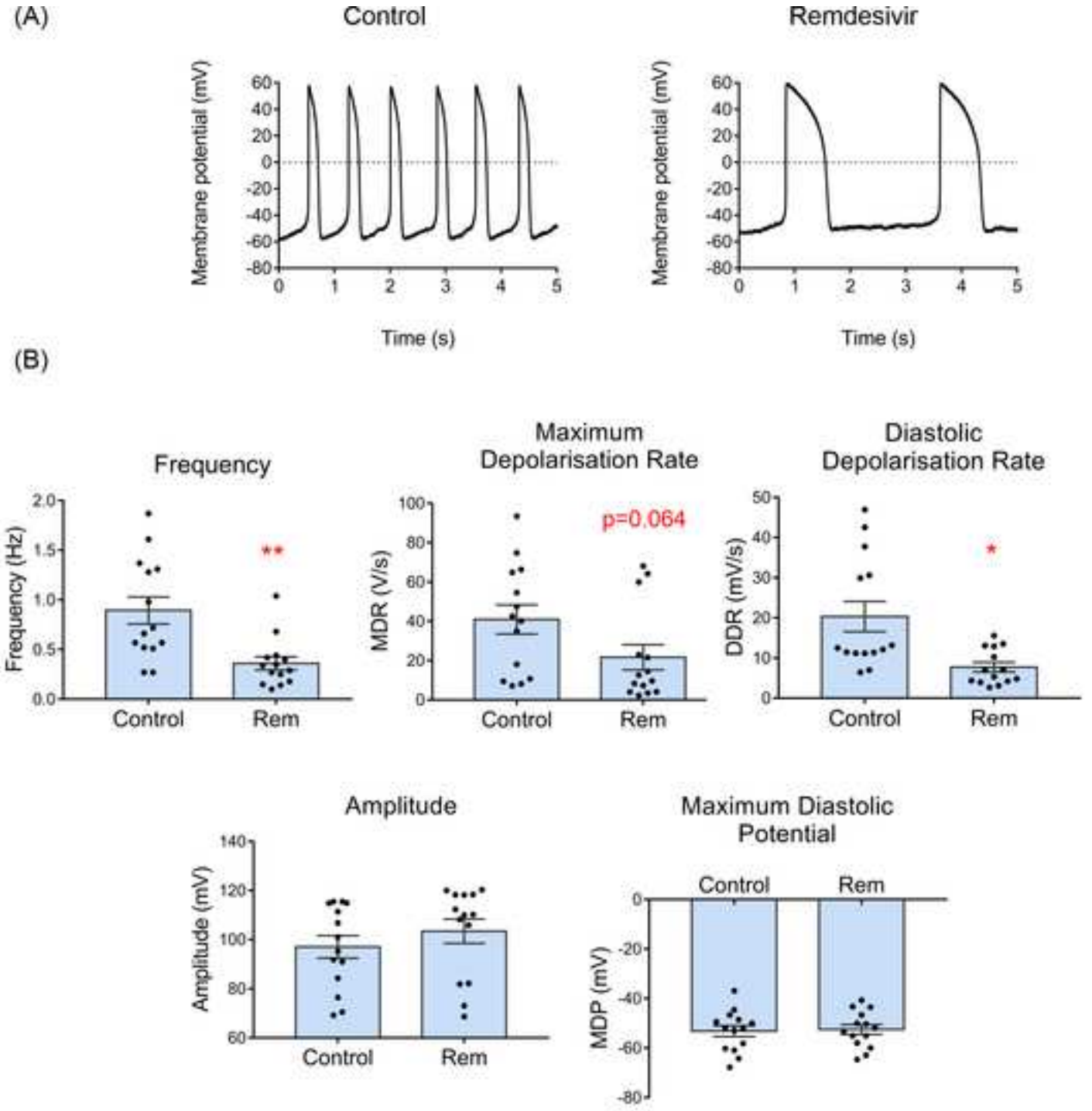

Figure 5

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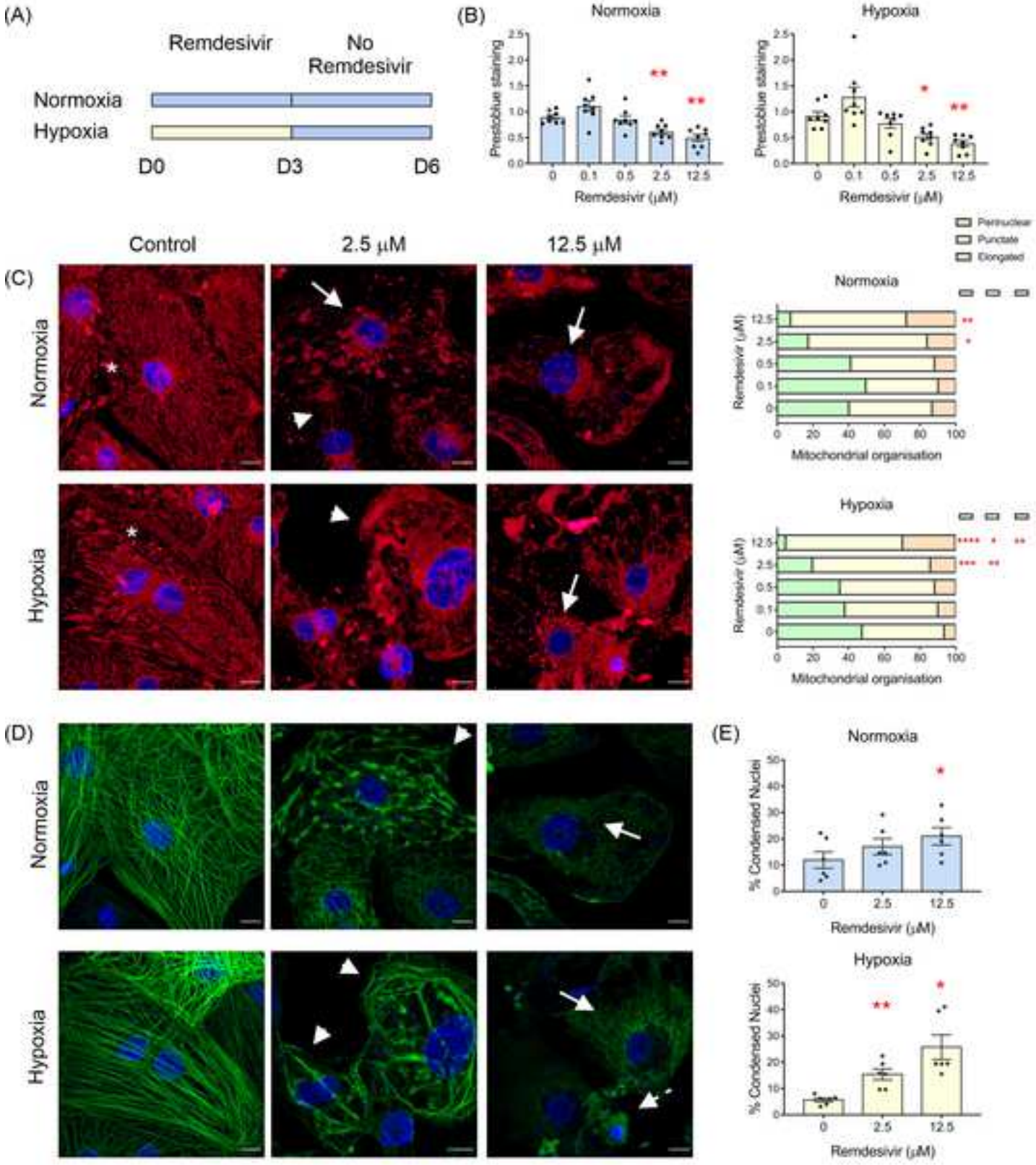

Figure 6

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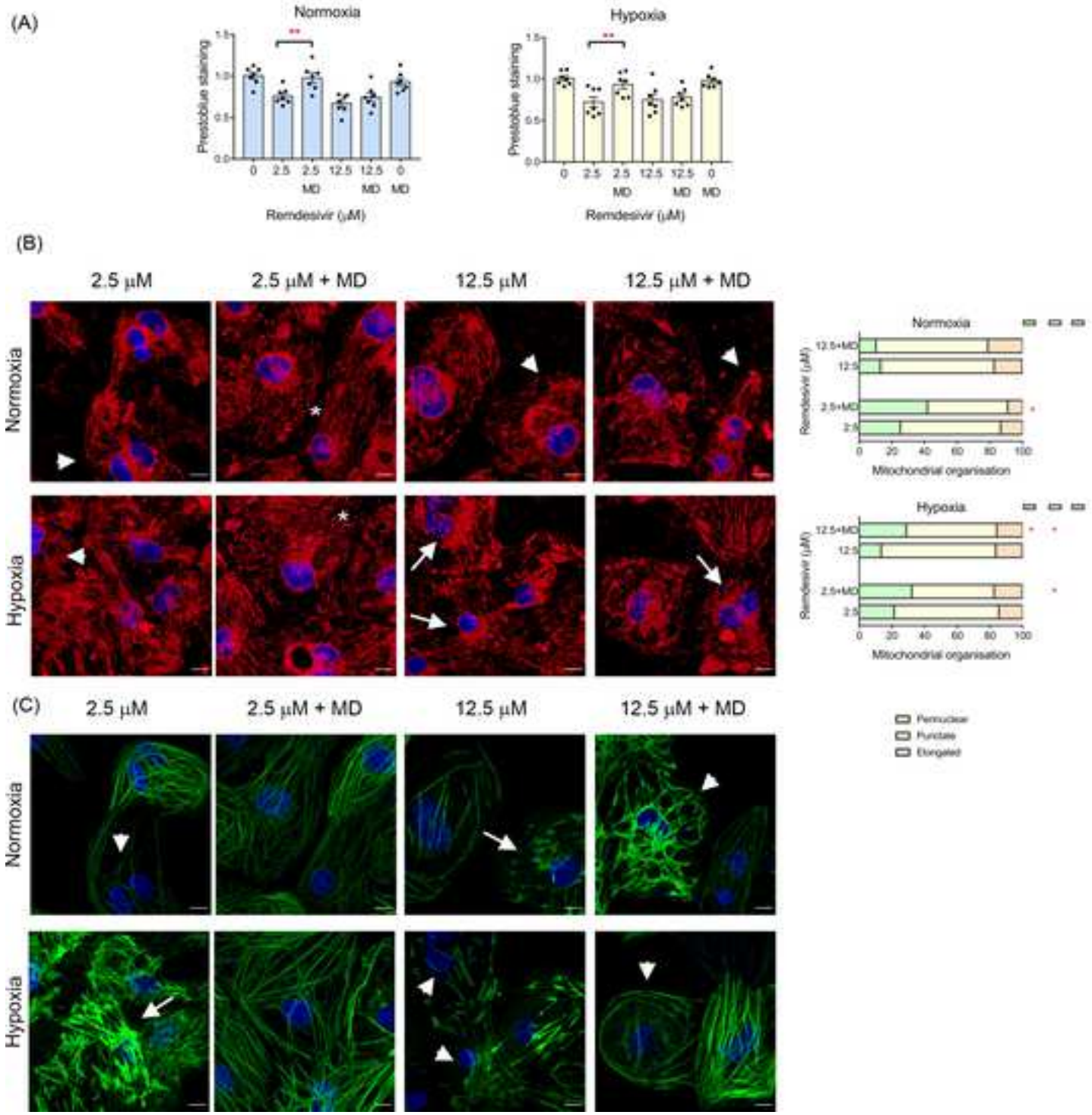

Graphical Abstract