The mitochondria targeted antioxidant MitoQ protects against fluoroquinolone induced oxidative stress and mitochondrial membrane damage in human Achilles' tendon cells

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Short title: MitoQ and fluoroquinolone induced oxidative damage in tendon cells

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Abstract

Tendinitis and tendon rupture during treatment with fluoroquinolone antibiotics is thought to be mediated via oxidative stress. We investigated whether ciprofloxacin and moxifloxacin cause oxidative stress and mitochondrial damage in cultured normal human Achilles' tendon cells and whether an antioxidant targeted to mitochondria (MitoQ) would protect against such damage better than a non-mitochondria targeted antioxidant. Human tendon cells from normal Achilles' tendons were exposed to 0-0.3mM antibiotic for 24h and 7d in the presence of 1μM MitoQ or an untargeted form, idebenone. Both moxifloxacin and ciprofloxacin resulted in up to a 3-fold

increase in the release of reactive oxygen species from tenocytes ($p<0.0001$) and loss of mitochondrial membrane permeability ($p<0.001$). In cells treated with MitoQ the rate of reactive oxygen species release was less and mitochondrial membrane potential was maintained. Mitochondrial damage to tenocytes during fluoroquinolone treatment may be involved in tendinitis and tendon rupture.

Introduction

Tendinopathy is a degenerative process thought to be a preliminary phase to tendon rupture. There are several predisposing factors to tendinopathy [1] including excessive exercise, patient age/sex and endocrine factors. However, it has been proposed that the defective repair response predisposing to ineffective tendon healing in overuse tendinopathy is due to dysregulated reactive oxygen species production within the extracellular environment of the tendon [2].

Fluoroquinolone antibiotics are broad spectrum bactericidal agents which inhibit bacterial DNA gyrase and topisomerases and include ciprofloxacin, the first oral fluoroquinolone, introduced in 1987 and moxifloxacin, introduced in 2000. Fluoroquinolone antibiotics are prescribed for a wide range of infections, including respiratory, gastrointestinal, and genito-urinary tract infections and account for around 40% of the antibiotic market in the USA alone.

Many of the older fluoroquinolones have been found to induce oxidative stress and antioxidant depletion in cultured rabbit tendon cells [3,4] and this is thought to contribute to the high incidence of tendon rupture in patients taking fluoroquinolone antibiotics [2,5-8]. Warnings about the risk of tendon rupture and tendonitis have appeared in the package inserts for these drugs for the past 10 years and a call has been made for this to be upgraded to a so-called “black box” warning [9]. There are

over 250 reports of tendon ruptures linked to fluoroquinolone use in the Food and Drug Administration (FDA) adverse event database between 1997 and 2006.

Mitochondria are a major source and target of oxidative stress and in the face of inadequate antioxidant protection, this results in mitochondrial dysfunction, which may contribute to tendinopathy. Oxidative damage to mitochondria in chondrocytes and tenocytes induced by fluoroquinolones, has been reported [10] which may have consequences for cell function and integrity. Ciprofloxacin and moxifloxacin have differential modulatory effects on inflammatory responses [11,12]. However, the effect of moxifloxacin in terms of oxidative stress in human tendon cells has not been reported, although here is evidence of moxifloxacin causing tendinopathy [13].

Coenzyme Q10 (CoQ10) is the predominant form of the electron carrier and antioxidant ubiquinone in man , and has an important role in protecting mitochondria against oxidative stress. Lipophilic cations, such as triphenyl-phosphonium (TPP), accumulate in mitochondria several hundred-fold via the large membrane potential (negative inside) generated by mitochondria during oxidative phosphorylation [14]. This property has been exploited by covalently attaching CoQ10 to the TPP cation, generating mitochondria-targeted CoQ (MitoQ) which is selectively accumulated within mitochondria [15-18].

The majority of the tendon ruptures reported in patients on fluoroquinolone antibiotics involve the Achilles' tendon [1,8,13]. We determined whether moxifloxacin and ciprofloxacin treatment induced oxidative stress and mitochondrial dysfunction in cultured human Achilles' tendon cells *in vitro* and whether MitoQ and an untargeted form of CoQ10, idebenone, were protective.

Materials and methods

Cell culture

Tendon cells were a generous gift from Professor Richard Aspden of the Bone and Musculoskeletal Research Group, University of Aberdeen. Following Ethical Committee approval and written informed consent, tendons were harvested by collagenase digestion from normal human Achilles' tendons obtained from amputated legs as previously described in detail [19]. Following resuscitation from frozen storage in liquid nitrogen, cells were cultured in 75cm^2 flasks containing Ham's F-12 medium supplemented with 10% fetal calf serum, 2 mM glutamine and 0.2μg/ml insulin, at 37°C in a moist atmosphere with 5% CO_2 and were used for experiments at passage 2, to avoid any phenotypic drift [20]. Phenotype was established by analyzing type I collagen, decorin and integrin-1β expression [20].

Ciprofloxacin and moxifloxacin were a kind gift from Bayer Plc, Newbury, UK. Confluent cell monolayers were exposed to 0-0.3mM concentrations of ciprofloxacin or moxifloxacin with either 1μM MitoQ mesylate, 1μM idebenone or 1μM decylTPP bromide salt (dTPP) as control (Antipodean Pharmaceuticals, San Francisco, CA, USA). The concentrations of antibiotics were chosen to mimic the range of intracellular therapeutic levels achieved *in vivo*. Ciprofloxacin and moxifloxacin were prepared in sterile water and MitoQ, dTPP and idebenone were prepared in ethanol. Appropriate solvent controls were used. A sub-lethal concentration of hydrogen peroxide (0.006% v/v) was used as a positive control.

Reactive oxygen species production

For measurement of reactive oxygen species (ROS) production, resuscitated cells were grown in 96 well plates at a density of 5000 cells/well for 24h before experimentation. The cells were loaded with 50μM 5-(6)-carboxy- 2,7'-

dichlorodihydrofluorescein-diacetate for 60min then washed twice in phosphate buffered saline (PBS). Antibiotics plus dTPP, MitoQ or idebenone were then added as described above. The rate of formation of the fluorescent derivative 5-(6)-carboxy-2,7'-dichlorofluorescein, as a result of oxidation by intracellular ROS, was measured with a FLUOstar Optima spectrofluorimeter (BMG Lab Technologies, Germany) at 37°C over 24h at an excitation wavelength of 485nm and emission wavelength of 530nm [21].

Mitochondrial membrane potential

For measurement of mitochondrial membrane potential, cells were again grown in 96 well plates and cultured with antibiotics plus dTPP, MitoQ or idebenone. JC-1 dye (5,5', 6,6'tetrachloro-1,1',3,3'-tetraethyl-benzimidazolcarbocyanine iodide) is a lipophilic cation which accumulates in intact mitochondria and fluoresces when excited at 490nm. After culture periods of up to 7 days, JC-1 (10μg/ml) was added to each well and cells were incubated in the dark at 37^0C for 20min. The cells were then washed twice in PBS and fluorescence was measured at 590nm. Decreased fluorescence is indicative of loss of mitochondrial membrane potential [22,21]. The JC-1 fluorescence was normalized to viable cell number measured using acid phosphatase activity [24].

Statistical analysis

Data are presented as median and interquartile range from 7 treatment replicates from three separate experiments using cells from three different tendon donors for each experiment. No assumptions have been made about the distribution of the data, which were compared using Kruskal Wallis with *post hoc* testing using the Mann Whitney U test. A p value of <0.05 was taken to be significant.

Results

Reactive oxygen species production

To assess whether oxidative stress occurred upon antibiotic treatment we treated tendon cells with a range of concentrations of ciprofloxacin or moxifloxacin. Actual ROS production varied between tendon donor and so results are shown as % change compared to solvent control cells without antibiotic (Figures 1 and 2).

The change in ROS production rate increased with increasing ciprofloxacin or moxifloxacin dose (both $p<0.0001$, Figures 1 and 2) in solvent control cells such that at the highest antibiotic concentration ROS production was one to three-fold greater than in cells without antibiotic (median [IQ range] rate of ROS production 164[128-200]% of control at 0.3mM ciprofloxacin and 208[185-308]% at 0.3mM moxifloxacin, $p=0.05$ and $p=0.007$ respectively, Figures 1 and 2).

When cells were also treated with 1μM MitoQ, the increase in the rate of ROS production was prevented in both ciprofloxacin and moxifloxacin treated cells. The median rate of ROS production with MitoQ plus 0.3mM ciprofloxacin was 97[88-98]% of control, and 95[86-105]% of control with MitoQ plus 0.3mM moxifloxacin (both NS). The untargeted form of CoQ10, idebenone, reduced ROS production rate to some extent but was less effective than MitoQ (Figures 1 and 2). ROS production rate remained high in cation carrier control treated (dTPP) cells ($p<0.05$, Figures 1 and 2).

Mitochondrial membrane potential

As a measure of the effect of ciprofloxacin and moxifloxacin on mitochondrial function, we assessed the effects on mitochondrial membrane potential as determined by JC-1 fluorescence. Increasing concentrations of ciprofloxacin or moxifloxacin caused decreased JC-1 fluorescence i.e. decreased mitochondrial membrane potential

after 7 days, compared to cells without antibiotics (both $p<0.001$, Figures 3 and 4). At the highest concentration of ciprofloxacin (0.3mM) median [IQ range] JC-1 fluorescence was 21[19-26] x 10^3 units compared to 39[37-42] x 10^3 units without antibiotics ($p=0.04$, Figure 3). In cells treated with 0.3mM moxifloxacin JC-1 fluorescence was 13[10-20] x 10^3 units compared to 31[29-34] x 10^3 units without antibiotics ($p=0.02$, Figure 4).

In cells treated with MitoQ, mitochondrial membrane potential was maintained or even augmented. In cells treated with MitoQ and 0.3mM ciprofloxacin, JC-1 fluorescence was 68[63-80] x 10^3 units ($p<0.0001$ compared to without MitoQ) and in cells treated with MitoQ and 0.3mM moxifloxacin, JC-1 fluorescence was 61[58-70] x 10^3 units ($p<0.0001$ compared to without MitoQ, Figures 3 and 4). This large increase in JC-1 fluorescence was not seen when cells were treated with either dTPP or idebenone plus antibiotic. There was no change in membrane potential at 24h (data not shown).

DISCUSSION

We have shown that both moxifloxacin and ciprofloxacin cause oxidative stress resulting in mitochondrial membrane damage in normal human Achilles' tendon cells *in vitro*. We have also shown that the mitochondria targeted antioxidant MitoQ prevents ciprofloxacin and moxifloxacin induced release of reactive oxygen species and loss of mitochondrial membrane potential and an untargeted analog of CoQ10 is less effective.

Previous studies using other cells have also shown a protective effect of MitoQ against oxidative stress. Treatment of bovine aortic endothelial cells with MitoQ protected cells from peroxide induced oxidative stress and maintained mitochondrial function [17]. In studies using fibroblasts from patients with Friedreich's ataxia, MitoQ prevented cell death after *in vitro* glutathione depletion and was several hundred-fold more effective than the untargeted form of CoQ10, idebenone [18]. We have also very recently reported that MitoQ, but not untargeted forms of CoQ, protect human endothelial cells against oxidative stress and modulate cytokine release in a model of sepsis [25].

Fluorinated-4-quinolones, including ciprofloxacin and moxifloxacin, are nalidixic acid analog antibiotics, which exert their bactericidal effect by inhibiting DNA gyrase activity. Tendinitis and tendon rupture have been reported to occur in around 1% of patients treated with fluoroquinolones. Experiments in animals showed that fluorinated quinolones damage cartilage and tendons [10] and it was suggested on the basis of histological evidence that damage may occur as a result of mitochondrial injury [26]. The fluoroquinolones pefloxacin and norfloxacin both caused decreased mitochondrial enzyme activity in rabbit tendon cells [4]. Another study reported increased ROS production coupled with oxidation of important intracellular redox

couples such as glutathione in a rabbit tendon cell line exposed to ciprofloxacin, pefloxacin, ofloxacin or levofloxacin [27]. This damage was less in cells treated with the antioxidant anethole dithiolethione [28]. Others reported that ciprofloxacin-induced oxidative damage was partially prevented by vitamin E in fibroblasts or astrocytes *in vitro* [29-30] and by vitamin E or allopurinol in liver and brain from rats. [33] and in mice, pefloxacin-induced oxidative damage was prevented by administration of N-acetylcysteine [34]. Thus ciprofloxacin induces oxidative stress in various cell types including animal tendon cells. However although moxifloxacin has been implicated as a cause of tendinitis in a case report [13], there have been no reports of moxifloxacin-induced oxidative stress. We also found no reports of fluoroquinolone antibiotics mediating mitochondrial damage in human tendon cells and no information on whether antioxidant protection of mitochondria can prevent such damage. We found that both ciprofloxacin and moxifloxacin caused concentration dependent increases in radical production resulting in loss of mitochondrial membrane potential and that the mitochondria targeted antioxidant Mito Q was protective. Treatment of antibiotic exposed cells with a non-mitochondria targeted analog of coenzyme Q10, idebenone did not protect the cells to the same extent.

These results show that both ciprofloxacin and moxifloxacin cause oxidative stress and mitochondrial membrane damage which may contribute to the development of tendinitis and tendon rupture in some patients. Our data also show that an antioxidant targeted to mitochondria can protect against this damage. Thus it is possible that MitoQ treatment may be of value in patients undergoing antibiotic treatment with fluoroquinolone antibiotics who are at increased risk of tendinitis. Oral MitoQ treatment for up to a year has been tested in two Phase II trials, in patients with

hepatitis C and patients with Parkinson's disease, showing that it was well tolerated, with no serious adverse events.

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Legends to figures

Figure 1

Change in production of reactive oxygen species in human Achilles' tendon cells treated with ciprofloxacin compared to cells without antibiotic and in the presence of either MitoQ, dTPP or idebenone. Values are median and interquartile range of 7 replicate measurements from each of three different tendon donors.

Figure 2

Change in production of reactive oxygen species in human Achilles' tendon cells treated with moxifloxacin compared to cells without antibiotic and in the presence of either MitoQ, dTPP or idebenone. Values are median and interquartile range of 7 replicate measurements from each of three different tendon donors.

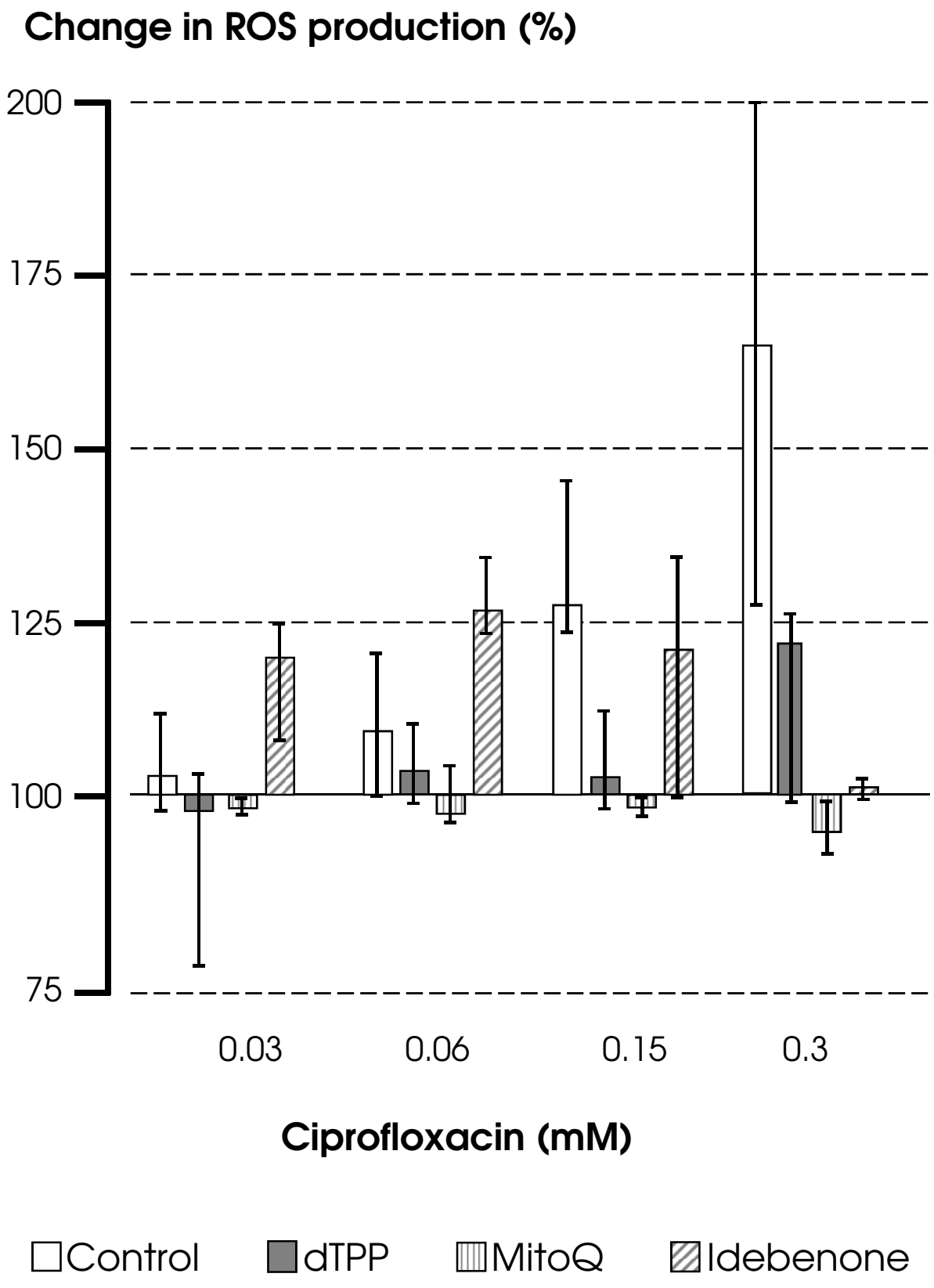
Figure 3

Mitochondrial membrane potential measured as JC-1 fluorescence in human Achilles' tendon cells treated with ciprofloxacin compared to cells without antibiotic and in the presence of either MitoQ, dTPP or idebenone. Values are median and interquartile range of 7 replicate measurements from each of three different tendon donors.

Figure 4

Mitochondrial membrane potential measured as JC-1 fluorescence in human Achilles' tendon cells treated with moxifloxacin compared to cells without antibiotic and in the presence of either MitoQ, dTPP or idebenone. Values are median and interquartile range of 7 replicate measurements from each of three different tendon donors.

Fig 1


Change in ROS production (%)
200
175
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125
100
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0.15
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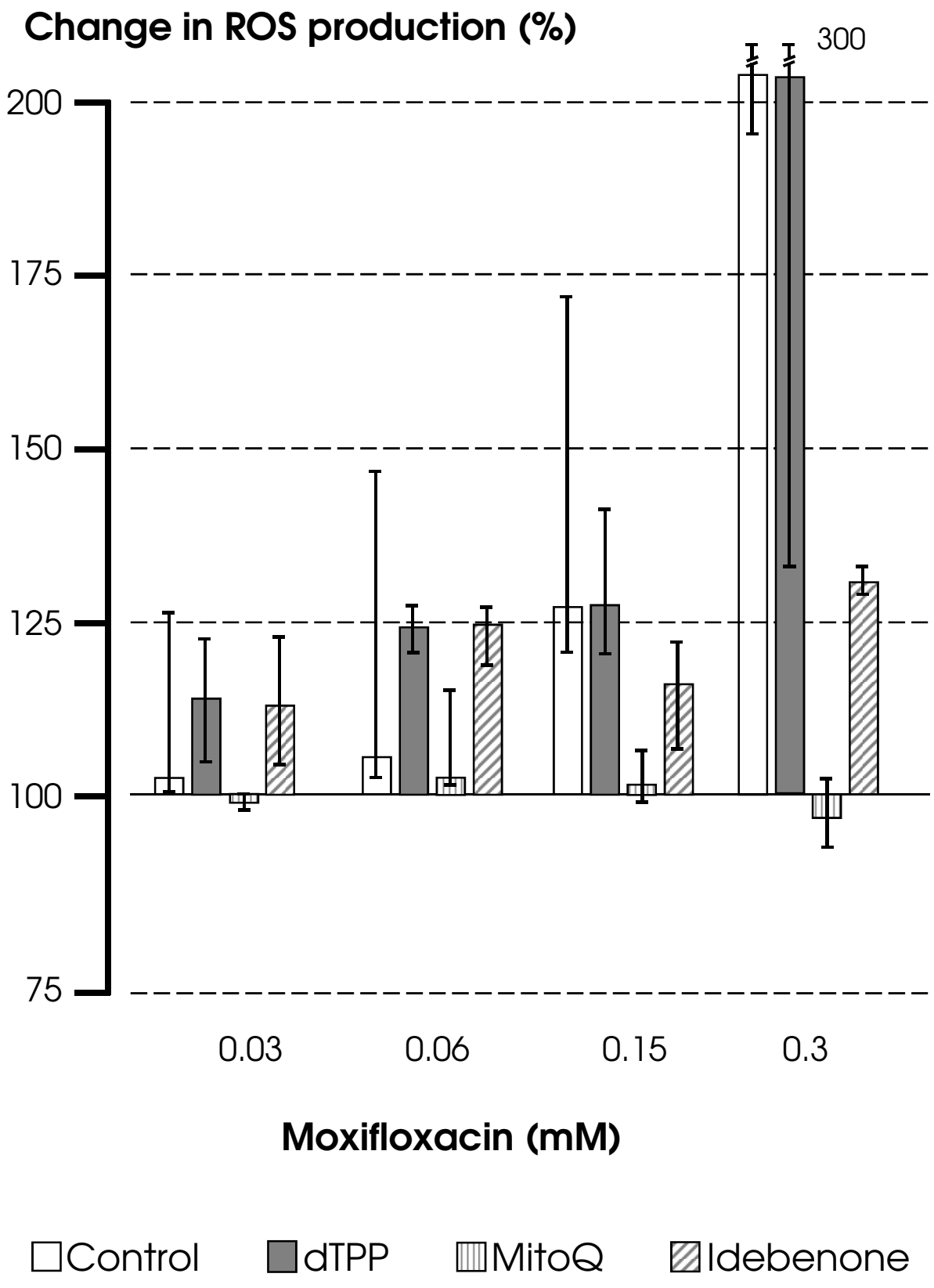
Ciprofloxacin (mM)
Control
dTPP
MitoQ
Idebenone

Fig 2


Change in ROS production (%)
300
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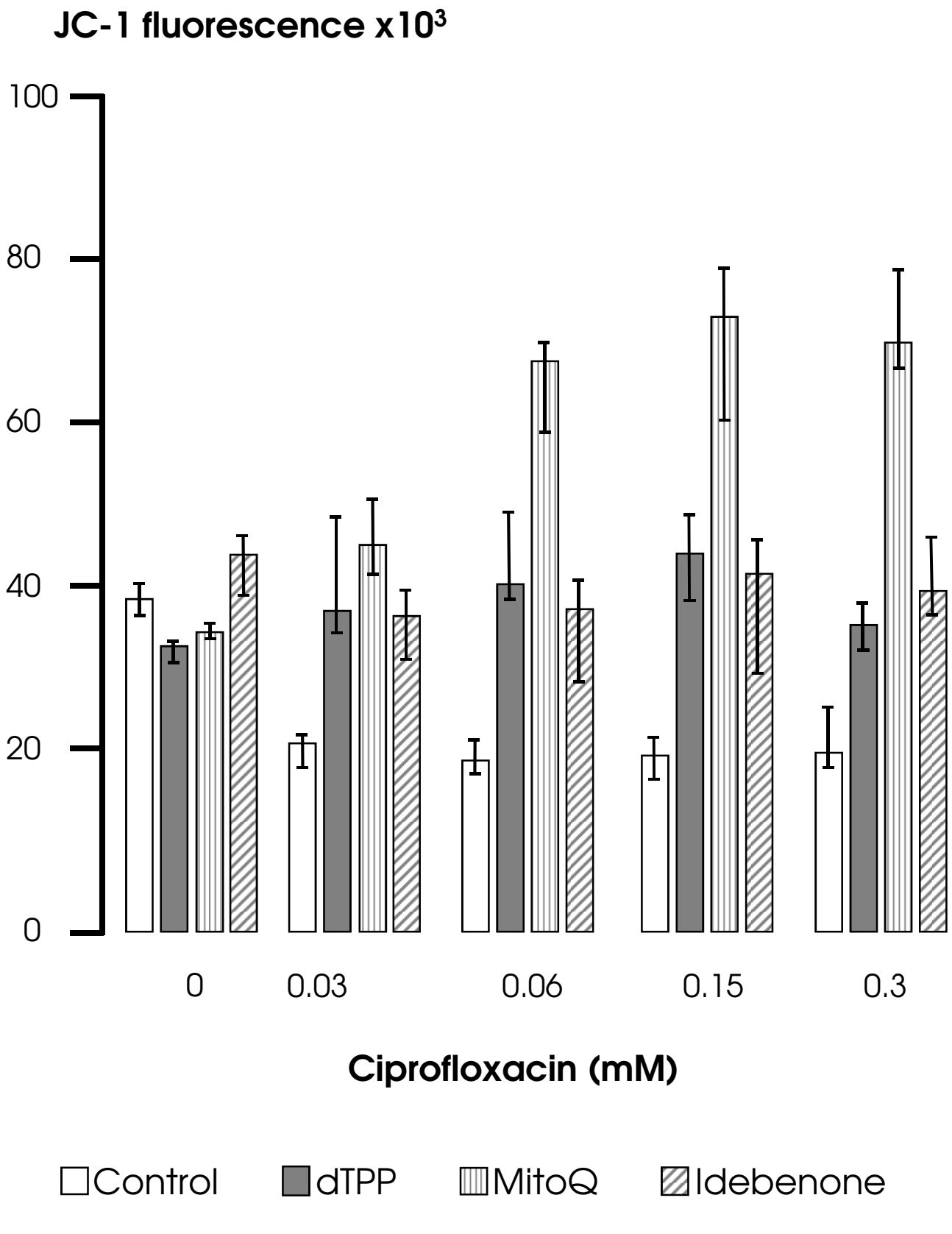
Control
dTPP
MitoQ
Idebenone

Fig 3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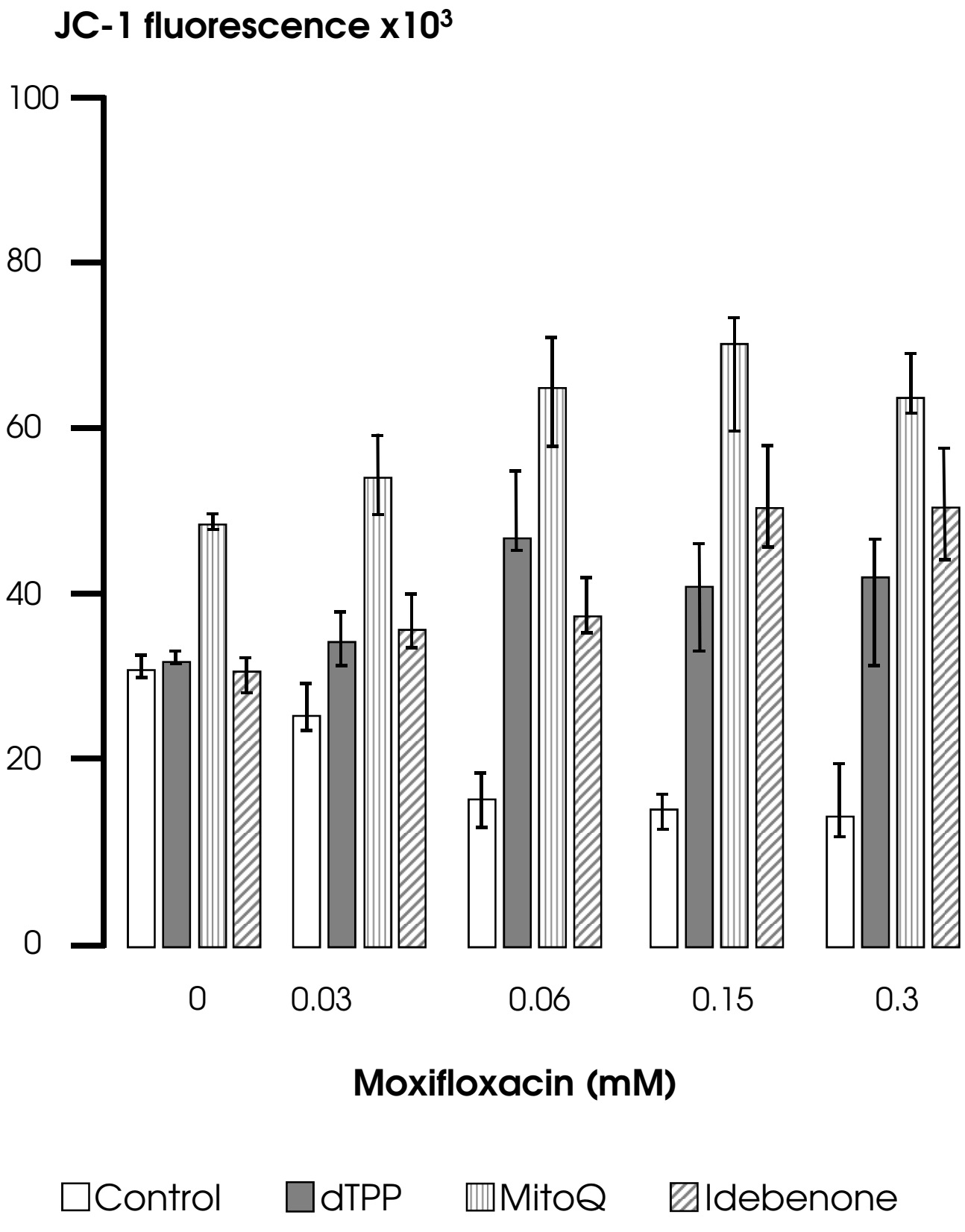
Ciprofloxacin (mM)
Control
dTPP
MitoQ
Idebenone

Fig 4


JC-1 fluorescence x10³
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