Aus dem Institut für Medizinische Immunologie
der Medizinischen Fakultät Charité – Universitätsmedizin Berlin

DISSERTATION

Effect of DHODH inhibition by teriflunomide on axonal mitochondria

Wirkung der DHODH- Inhibition durch Teriflunomide auf axonale Mitochondrien

zur Erlangung des akademischen Grades
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List of abbreviations

aCSF	Artificial cerebrospinal fluid
ATP	Adenosine triphosphate
BMBF	Bundesministerium für Bildung und Forschung
CNS	Central nervous system
DHODH	Dihydroorotate dehydrogenase
DMSG	Deutsche Multiple Sklerose Gesellschaft
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
ETC	Electron transport chain
H_2O_2	Hydrogen peroxide
IMM	Inner-mitochondrial membrane
MS	Multiple sclerosis
NOR	Nodes of Ranvier
PNS	Peripheral nervous system
PPMS	Primary progressive multiple sclerosis
Q	Ubiquinone
QH_2	Ubiquinol
RNS	Reactive Nitrogen species
ROS	Reactive oxygen species
RRMS	Relapsing remitting multiple sclerosis
SPMS	Secondary progressive multiple sclerosis
TFN	Teriflunomide
ZNS	Zentralen Nervensystem

Abstract

Multiple sclerosis (MS) is a chronic inflammatory disease of the central nervous system (CNS), affecting more than 2.5 million people worldwide. MS is characterized by inflammation, demyelination and neurodegeneration. However, its exact pathophysiology is not clearly understood. Many studies have reported that mitochondrial alterations occur already in early stages of MS contributing to neurodegeneration even in absence of demyelination. In MS, the activated infiltrating as well as CNS resident immune cells are potential sources of reactive oxygen and nitrogen species that lead to oxidative stress causing mitochondrial and subsequent neuronal damage. Previously, we demonstrated that oxidative stress alters axonal mitochondrial dynamics and mitochondrial membrane potential in myelinated axons. Thus, we hypothesize that targeted inhibition of mitochondrial damage may prevent neurodegeneration and could represent a potential treatment option in the context of MS.

Teriflunomide (TFN) is an approved drug for MS that inhibits dihydroorotate dehydrogenase (DHODH), a mitochondrial inner membrane associated enzyme. However, the effects of TFN on mitochondria has not been well investigated. Hence, in this project we aimed to investigate the effect of TFN on mitochondria and hypothesized that TFN treatment does not compromise mitochondrial functionality.

To test our hypothesis, we treated murine peripheral spinal root explants and observed their mitochondrial dynamics using confocal microscopy. DMSO, the substance used to dilute TFN, did not affect mitochondrial dynamics. In unmanipulated axons, 50 µM TFN increased mitochondrial elongation and length but decrease their velocity. Then, we induced oxidative stress by incubating the roots with 50 µM hydrogen peroxide (H_2O_2). Additionally, we applied three different concentrations of TFN- 1, 5 and 50 µM together with H_2O_2. During exogenous application of H_2O_2, mitochondria became rounder, shorter and smaller. Additionally, less mitochondria were motile and travelled shorter distance with decreased velocity.

Contrastingly, oxidative stress-induced alterations in mitochondrial morphology was prevented by 1 and 50 µM TFN. Regarding mitochondrial motility, only 1 µM TFN prevented all motility-related alterations. Meanwhile, 50 µM restored the decrease in motile mitochondria and motility distance but not velocity. Moreover, 1 µM TFN reduced the oxidation potential of mitochondria. However, 5 µM TFN could not restore alterations in mitochondrial dynamics as well as its oxidation potential. This non-monotonic dose response of TFN may be attributed to interactions with pathways related to tyrosine kinases and cyclooxygenases.

Thus, our data demonstrates the relationship between DHODH inhibition and mitochondrial dynamics and point to a potential neuroprotective effect of TFN in context of oxidative stress-related mitochondrial damage.

Abstrakt

Die Multiple Sklerose (MS) ist eine chronische entzündliche Erkrankung des zentralen Nervensystems (ZNS) und betrifft weltweit mehr als 2,5 Millionen Menschen. Entzündung, Demyelinisierung und Neurodegeneration gelten als die Kennzeichen der Erkrankung, die genaue Ursache ist jedoch unklar. Viele Studien belegten über mitochondriale Veränderungen in einem frühen Stadium der MS, von denen angenommen wird, dass sie zur Neurodegeneration beitragen, sogar bevor Demyelinisierung vorliegt. Soweit bekannt ist, löst in der MS das Eindringen von Leukozyten aus der Peripherie ins Gehirn eine Entzündung aus. Infolgedessen produzieren die aktivierten Immunzellen im ZNS große Mengen an reaktiven Sauerstoff- und Stickstoffspezies, die zu einer oxidativen Stresssituation führen. Diese schädigt sowohl Neuronen als auch Mitochondrien und führt letztlich zur Neurodegeneration. Wir haben in früheren Studien gezeigt, dass oxidativer Stress die axonale mitochondriale Dynamik sowie das Membranpotenzial in myelinisierten Axonen verändert.

Teriflunomid (TFN) wirkt über die Hemmung eines mitochondrialen, mit der inneren Membran assoziierten Enzyms, der Dihydroorotat-Dehydrogenase (DHODH). Die Wirkung von TFN auf die Mitochondrien ist jedoch unvollständig untersucht. Deswegen stellten wir die Hypothese auf, dass die Behandlung mit TFN die mitochondriale Funktionalität nicht beeinträchtigt.

Um unsere Hypothese zu testen, analysierten wir murine periphere Spinalwurzel-Explantate mittels konfokaler Mikroskopie. Das Vehikel für TFN, DMSO, zeigte keinen Einfluss auf die mitochondriale Dynamik. Wir applizierten 50 µM TFN oder 50 µM Wasserstoffperoxid (H_2O_2), um oxidativen Stress zu induzieren oder drei Konzentrationen von TFN - 1, 5 und 50 µM zusammen mit H_2O_2. In einem unbehandelten Axon erhöhte 50 µM TFN die mitochondriale Schwellung und Länge, verringerte aber die Motilitätsgeschwindigkeit. Unter exogener Applikation von H_2O_2 wurden die Mitochondrien runder, kürzer und kleiner. Außerdem waren weniger Mitochondrien beweglich und legten eine kürzere Strecke mit verringerter Geschwindigkeit zurück.

Im Gegensatz dazu wurde die durch oxidativen Stress induzierte Veränderung der mitochondrialen Morphologie durch 1 und 50 µM TFN verhindert. Hinsichtlich der mitochondrialen Motilität verhinderte nur 1 µM TFN alle motilitätsbezogenen Veränderungen und 50 µM verhinderte die Abnahme der beweglichen Mitochondrien und der Motilitätsdistanz, aber nicht der Geschwindigkeit. Außerdem reduzierte 1 µM TFN das Oxidationspotential der Mitochondrien. 5 µM TFN konnte jedoch die Veränderungen der mitochondrialen Dynamik sowie des Potentials nicht wiederherstellen. Diese nicht-lineare Dosis-Antwort von TFN kann auf Interaktionen mit Signalwegen zurückgeführt werden, die mit Tyrosinkinasen und Cyclooxygenasen in Verbindung stehen.

Somit zeigen unsere Daten den Zusammenhang zwischen DHODH-Inhibition und mitochondrialer Dynamik und deuten auf eine mögliche neuroprotektive Wirkung von TFN im Kontext von oxidativem Stress-bedingten mitochondrialen Schäden hin.

Introduction

1. Introduction

1.1. Multiple sclerosis (MS)

Multiple sclerosis (MS) is an autoimmune neurodegenerative disease of central nervous system (CNS) and is one of the most common cause of non-traumatic neurological disability among young adults. MS affects more than 2.5 million people worldwide (Bargiela and Chinnery 2019). In Germany, incidence of the disease is estimated to be 120,000 to 140,000 according to 2005/2006 nationwide registry conducted by German MS Society (DMSG, Deutsche Multiple Sklerose Gesellschaft Bundesverband e.V.) (Flachenecker, Stuke et al. 2008), and around 200,000 people as of 2014 (Petersen, Wittmann et al. 2014). In MS, the degeneration of neurons forms sclerotic lesions in CNS that manifest into a wide range of symptoms related to vision, muscle weakness and tingling in the extremities depending on the site of lesion and multiple other factors (Filippi, Bar-Or et al. 2018). Depending upon the course of disease, MS is categorized into relapsing-remitting multiple sclerosis (RRMS), secondary progressive multiple sclerosis (SPMS) and primary progressive multiple sclerosis (PPMS). In most cases, patients develop RRMS disease course with periods of new symptoms or relapses that improves partially or completely over several days or weeks. RRMS may eventually progress into SPMS where the remission phase of the disease is absent and there is progressive deterioration of the symptoms. In a subset of patients, the disease develop as PPMS with steady progression of signs and symptoms from the very beginning of the disease course without the period of relapse (Lublin, Reingold et al. 2014, Lassmann 2018).

1.2. Pathophysiology of MS

Although, MS is characterized by inflammation, demyelination and neurodegeneration (Dutta and Trapp 2007, Compston and Coles 2008), the exact cause and mechanisms by which neuroinflammation and myelin damage lead to neurodegeneration have not been fully elucidated. Infiltration of activated leukocyte from periphery into the brain is assumed to initiate inflammatory cascades in CNS (Maurer and Rieckmann 2000, Lublin, Reingold et al. 2014, Barcelos, Troxell et al. 2019). Chronic inflammation in CNS is considered to trigger demyelination and subsequent neurodegeneration leading to loss of functions related to the region of lesion. However, some studies have reported neuroaxonal damage even in absence of demyelination, indicating other contributors to neurodegeneration (Nikic, Merkler et al. 2011, Barcelos, Troxell et al. 2019).

1.3. Mitochondrial hypothesis of neurodegeneration

Mitochondrial dysfunction is considered as one of the contributors of neurodegeneration in MS (Barcelos, Troxell et al. 2019, Bargiela and Chinnery 2019, Campbell, Licht-Mayer et al. 2019,

Wang, Xu et al. 2019). During chronic inflammation, activated immune cells are known to produce high amount of reactive oxygen and nitrogen species (ROS & RNS) that leads to oxidative stress (Mossakowski, Pohlan et al. 2015). In normal situation, excess ROS are neutralized by cell's antioxidant system, however, excessive ROS levels beyond scavenging capacity of the cells' antioxidant system leads to oxidative stress that can cause extensive protein oxidation, lipid peroxidation (Salim 2017) as well as global histone modification and DNA methylation (Niu, DesMarais et al. 2015). Subsequently, oxidative stress may cause both mitochondrial and neuronal damage, further inducing production of ROS and RNS and resulting in a vicious cycle of oxidative insult, mitochondrial damage and neuronal damage (Figure 1).

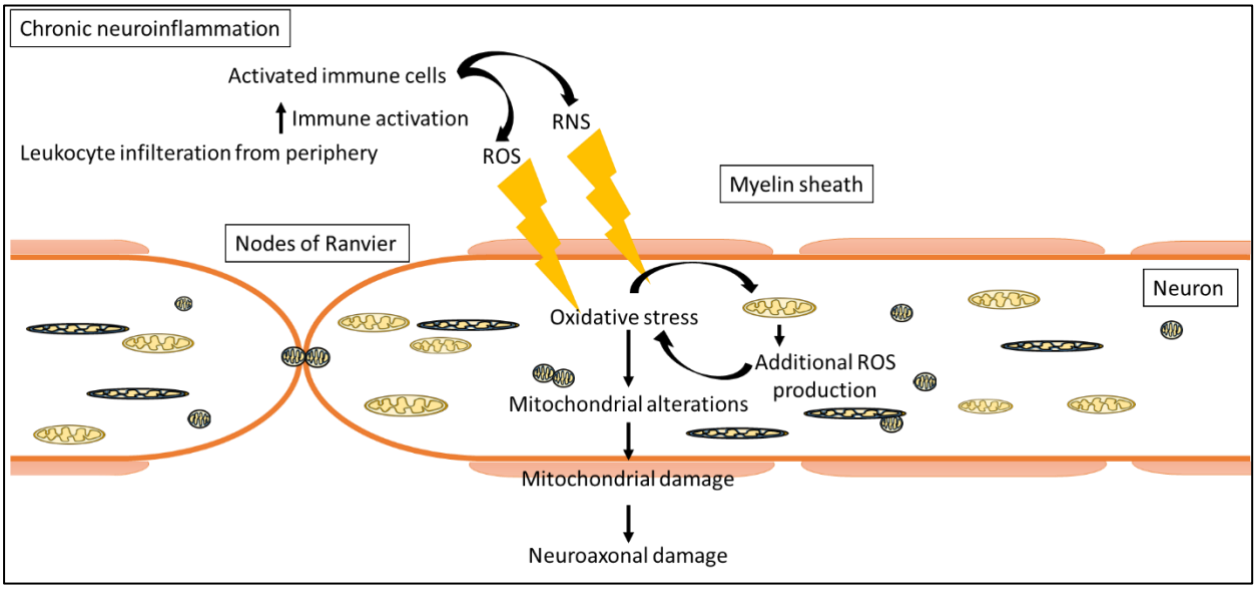


Figure 1: Schematic representation showing consequence of inflammation induced oxidative stress in neurons. Immune activation in CNS by infiltrating immune cells from periphery leads to production of ROS and RNS. During chronic neuroinflammation, sustained ROS and RNS cause oxidative stress that damages both mitochondria and neurons. Damaged mitochondria further produce ROS, contributing to oxidative stress, ultimately leading to mitochondrial alterations, damage and neuroaxonal damage. (Source: Original figure)

Importantly, mitochondrial alterations are reported in earlier stages of MS, even before demyelination (Mahad, Ziabreva et al. 2008, Nikic, Merkler et al. 2011) indicating energy failure that contributes to neuroaxonal damage. Besides MS, oxidative stress and mitochondrial alterations have also been reported in many other neurodegenerative diseases that are not primarily immune related, such as Alzheimer's disease and Huntington's disease among others (Barcelos, Troxell et al. 2019). Hence, understanding of mitochondrial alterations due to oxidative stress may provide a key to the management of neuroaxonal damage in context of neurodegenerative diseases.

1.4. Mitochondrial dynamics in health and in stress

Mitochondria are highly dynamic organelles that change in shape, size and motility behavior

according to the functional need of cells. In normal situation, mitochondria undergo fusion, fission and biogenesis to shuffle, segregate and renew mitochondrial pool allowing mixing of metabolites and mitochondrial DNA, proliferation as well as cellular adaptation to the bioenergetics of the cell (Knott, Perkins et al. 2008). In general, fusion leads to the formation of elongated mitochondria that are considered optimal for ATP generation, whereas fission assists in mitochondrial division as well as mitophagy (Farmer, Naslavsky et al. 2018). These changes in mitochondrial morphology also impacts mitochondrial motility. In general, 20-30% of mitochondria are motile (Lin and Sheng 2015). The purpose of mitochondrial transport again depends on the cell's bioenergetics. Mitochondrial transport are crucial for mitochondrial distribution that are directed in the area of increasing energy demand as well as for the removal of damaged mitochondria (Cheng and Sheng 2021). Interestingly, most of the ATP synthesis occurs in stationary mitochondria (Sun, Qiao et al. 2013, Lin and Sheng 2015, Cheng and Sheng 2021). Hence, it requires a well- coordinated implementation of mitochondrial fusion-fission, biogenesis, mitophagy and motility in a healthy cell.

During mild damage or stress, fusion and fission dynamics assist in distributing functional metabolites and mitochondrial DNA among functional mitochondria as well as get rid of the damaged part to protect mitochondria from further damage (Knott, Perkins et al. 2008). Moreover, mitochondrial fusion is considered important for accelerating ATP production during stress (Farmer, Naslavsky et al. 2018). However, in case of extensive damage or stress, mitochondria may undergo fission in excessive magnitude causing fragmentation of large mitochondrial population, which is detrimental. Consequently, mitochondrial motility pattern alters depending on the amount of damage on mitochondrial pool. While mild change in mitochondrial bioenergetics may trigger motility for mitochondrial distribution; excessive stress may immobilize mitochondria causing accumulation of unremoved dysfunctional mitochondria (Cheng and Sheng 2021).

1.5. Teriflunomide (TFN)

Teriflunomide (TFN) is a disease-modifying drug approved for RRMS (O'Connor, Wolinsky et al. 2011, Miller, Wolinsky et al. 2014). The mechanism of action of TFN is through reversible inhibition of the inner mitochondrial membrane-associated enzyme, dihydroorotate dehydrogenase (DHODH) (Figure 2). DHODH is involved in the fourth enzymatic step of *de novo* pyrimidine biosynthesis by the ubiquinone-mediated oxidation of dihydroorotate to orotate (Bar-Or 2014) (Loffler, Carrey et al. 2020). Inhibition of *de novo* pyrimidine biosynthesis leads to the regulation of rapidly dividing cells, thus reducing B and T cell proliferation subsequently limiting inflammation (Bar-Or 2014, Kretzschmar, Pellkofer et al. 2016, Miller 2017).

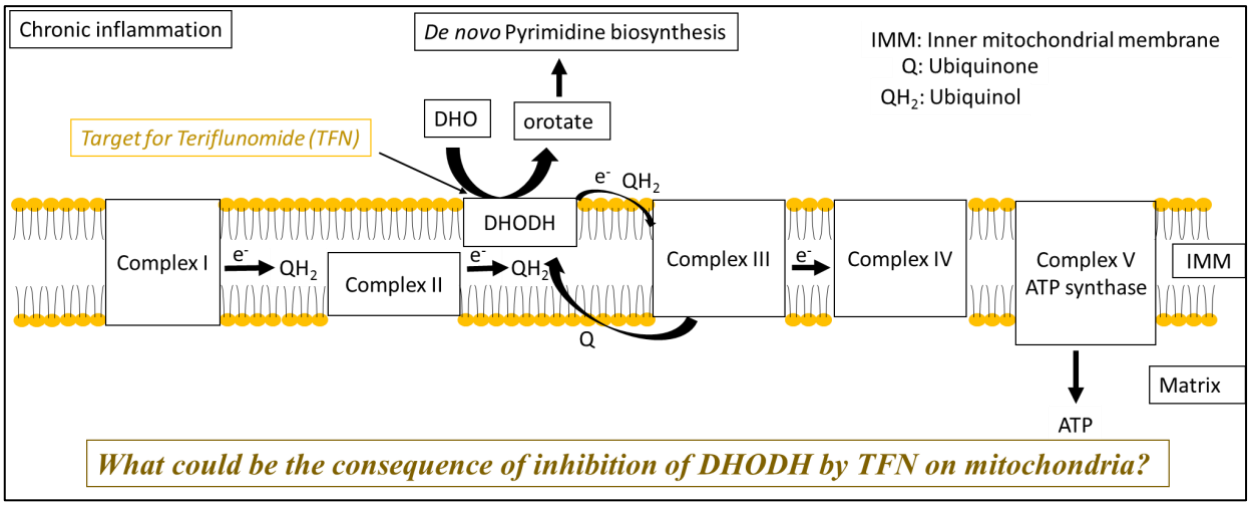


Figure 2: Schema depicting role of dihydroorotate dehydrogenase (DHODH) in de novo pyrimidine biosynthesis and electron transport chain. The complexes associated with electron transport chain, DHODH and the ATP synthase are located in the inner mitochondrial membrane of mitochondria (IMM). DHODH donates electron to ubiquinone (Q) reducing it to ubiquinol (QH_2) during the conversion of dihydroorotate (DHO) to orotate in the fourth step of *de novo* pyrimidine biosynthesis. The reduced QH_2 is reoxidized by complex III transferring electrons to complex IV. Teriflunomide (TFN) acts by reversibly inhibiting DHODH. (Source: Original figure)

The enzyme DHODH is also functionally linked to complex II and complex III of mitochondrial electron transport chain (ETC) (Boukalova, Hubackova et al. 2020). It reduces ubiquinone (Q) to ubiquinol (QH_2) during oxidation of dihydroorotate, which is then oxidized back to Q by complex III (Figure 2). Despite close association with ETC, the effect of DHODH inhibition by TFN on mitochondrial dynamics and functionality remains unclear. As the drug is well established for MS disease management and due to presence of compensatory mechanism related to DHODH reduction of Q in ETC, we hypothesized that TFN does not have detrimental effects on mitochondria.

1.6. TFN dose

TFN has been reported to potently inhibit 50% of DHODH (IC50) at 1 µM concentration (Palmer 2013, Kaplan J. 2015). At higher concentrations ranging from 50-100 µM, TFN has been reported to inhibit protein tyrosine kinase (Oh and O'Connor 2013, Palmer 2013). In patients, 14 mg TFN is recommended per day for the treatment of RRMS, which leads to an average steady-state maximum TFN concentration (Cmax) of 168 µM in blood plasma (Oh and O'Connor 2013). However, the concentration of TFN that ends up into the brain could only be extrapolated from the animal data.

Meanwhile, in rats, the final concentration of TFN in brain was found to be only approximately 2 – 4 % of the blood concentration (~ 2.5 - 4,1 µM) (Kaplan J. 2015). Thus, by extrapolation of rat data, we could approximate around 3-7 µM TFN concentration in CNS in patients. Based on this

information, we investigated three different concentrations of TFN, 1 µM, 5 µM and 50 µM in our mice study using peripheral spinal root explants.

1.7. Rationale

Most of the drugs for MS aim at managing inflammation, including TFN. While MS pathology is multifactorial, management of other factors that contributes to the pathophysiology of MS is crucial. As the mechanism of action of TFN on limiting inflammation is associated with mitochondria, potential harmful or protective effect on mitochondria during oxidative stress may be implicated to avoid any serious side effects or to exploit additional therapeutic benefit that could be used in other diseases associated with mitochondrial dysfunction and oxidative stress.

In this PhD project, we investigated the effect of DHODH inhibition by TFN on mitochondria. Additionally, we explored its implication on mitochondrial protection during oxidative stress.

This thesis is based on the following publication:

Malla B, Cotten S, Ulshoefer R, Paul F, Hauser AE, Niesner R, Bros H, Infante-Duarte C (2020) Teriflunomide preserves peripheral nerve mitochondria from oxidative stress-mediated alterations. Ther Adv Chronic Dis 11:2040622320944773. (IF: 5.091)

(Ranked 36th out of 267 journals in the selected JCR year of 2018 with impact factor of 4.45 and in the category: pharmacology and pharmacy)

Objectives

2. Objectives

2.1. Objectives related to the thesis

The present PhD project primarily aimed at investigating the effects of TFN on mitochondria. The specific aims of the project are given below:

- To investigate the effect of TFN on axonal mitochondrial dynamics
- To investigate the effect of TFN on axonal mitochondrial dynamics during oxidative stress
- To investigate the effect of TFN on mitochondrial oxidation potential during oxidative stress

2.2. Additional Objectives not covered in this thesis

In addition to above mentioned aims, the PhD project also explored mitochondrial dynamics, oxygen consumption and neuronal activity in central nervous system model during oxidative stress in presence and/or absence of TFN. The specific additional aims are as follows,

- To establish a model of murine acute brain slices for investigation of mitochondrial dynamic in terms of mitochondrial morphology and motility
- To investigate the effect of TFN on neuronal mitochondrial dynamics during oxidative stress
- To investigate the effect of TFN on ATP levels during oxidative stress
- To investigate the effect of TFN on oxygen partial pressure during oxidative stress
- To investigate the effect of TFN on neuronal activity during oxidative stress

Papers related to these aims were submitted with long delays in reviewing process.

Methods

3. Methods

3.1. Ethics Statement

All experimental procedures performed in mice were reviewed and approved by the local authority on animal studies, Landesamt für Gesundheit und Soziales Berlin (LaGeSo). We performed the animal studies following strict regulations of the European Communities Council Directives of 22 September 2010 (2010/63/EU). The studies were performed under animal experimentation application (TVA) number T002/10.

3.2. Animals

We used naïve C56Bl6 male and female adult mice that were at least 8 weeks or older. All the animals were kept in animal facilities with access to food, water, appropriate enrichment in the environment as well as maintained with temperature, air-flow and 12 h light-dark cycle.

3.3. Preparation of solutions and drugs

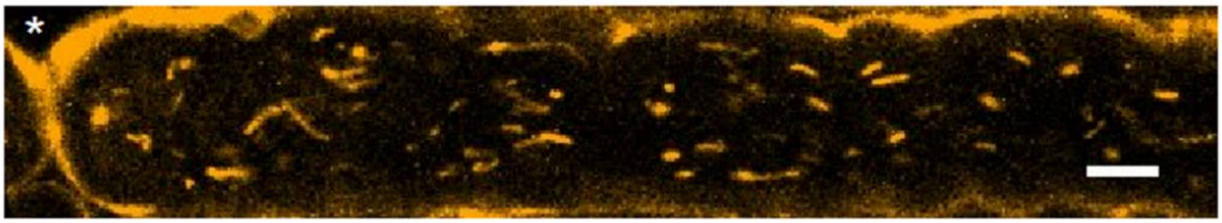
Explanted roots were incubated in artificial cerebrospinal fluid (aCSF) (in mM): 124 NaCl, 1.25 NaH_2PO_4, 10.0 Glucose, 1.80 $MgSO_4$, 1.60 $CaCl_2$, 3.00 KCl, 26.0 $NaHCO_3$. $NaHCO_3$ was added just before using the solution. For induction of oxidative stress, explanted roots were incubated with 50 µM of H_2O_2 (H_2O_2; 30% w/w in H_2O, with a stabilizer) dissolved in aCSF for 30 minutes. TFN in the form of powder was dissolved in dimethyl sulfoxide (DMSO) and stored at -20°C. To investigate the effect of TFN, three different concentrations were tested along with H_2O_2 for 30 minutes.

3.4. Preparation of peripheral spinal root explant

To prepare ventral spinal root explants, C57BL/6 mice were anesthetized using isoflurane and sacrificed by cervical dislocation. Then, the connective tissue was separated and the dorsal side of the spinal cord was exposed. At the thoracic level of the vertebrae, initial sectioning was made in the sides and proceeded in a rostral to caudal direction until the last vertebrae. The spinal cord was gently lifted to expose the ventral roots. The exposed ventral roots were cut before the formation of the peripheral nerves. The spinal cord with attached roots was transferred to aCSF saturated with carbogen (95% O_2; 5% CO_2) and lumbar ventral roots of at least 0.8 cm were selected and separated from the spinal cord using a dissecting microscope. The peripheral roots were then incubated in submerged incubation chamber (Brain Slice Keeper- BSK6-6, Scientific Systems Design Inc., Ontario, Canada) and continuously perfused with the carbogen.

3.5. Labeling of mitochondria and quantification of relative change in intracellular ROS

MitoTracker CMTMRos orange (Life Technologies, Darmstadt, Germany) is a fluorescent dye that fluoresce after entering live cell and then are sequestered into mitochondria depending on the membrane potential. To label axonal mitochondria, ventral spinal roots were incubated in fresh aCSF containing 300 nM, MitoTracker orange for 30 minutes and washed with aCSF (Figure 3). Kweon et al., 2001 described that the fluorescence intensity of the MitoTracker Orange is proportional to the levels of intracellular ROS (Kweon, Kim et al. 2001). Then, the images obtained from confocal microscopy were used for the quantification of mitochondrial fluorescence intensity using the Image J software.



(Adapted and modified from Malla et al., 2020, Ther Adv Chr Dis)

Figure 3: Mitochondrial labelling with MitoTracker Orange. Mitochondria within the orange boundry of the axon are labelled in orange. Asterix shows the nodes of Ranvier. Scale bar: 5µm

3.6. Confocal Microscopy

All the imaging experiments were performed using inverted laser-scanning confocal microscope adapted for live- cell imaging (LSM 710, Carl Zeiss, Jena, Germany). For imaging, explanted ventral roots were placed in a chamber with glass coverslip at the bottom and were immobilized using a custom-built net. The imaging chamber were filled with carbogenated aCSF. MitoTracker Orange was excited with a DPSS laser at 561 nm. Time-lapse video of 30 frame (2 sec/frame) with a resolution of 512x512 pixels were acquired in three separate *regions of interest* (ROI) that justified the following criteria:

- ROI with clearly defined node of Ranvier,
- ROI with visibly labeled mitochondria and
- ROI no closer than 0.2 cm from the ends of the roots

Typically, the first image was captured from the middle of the root, whereas the following two were from the right and left of the middle region (Figure 4).

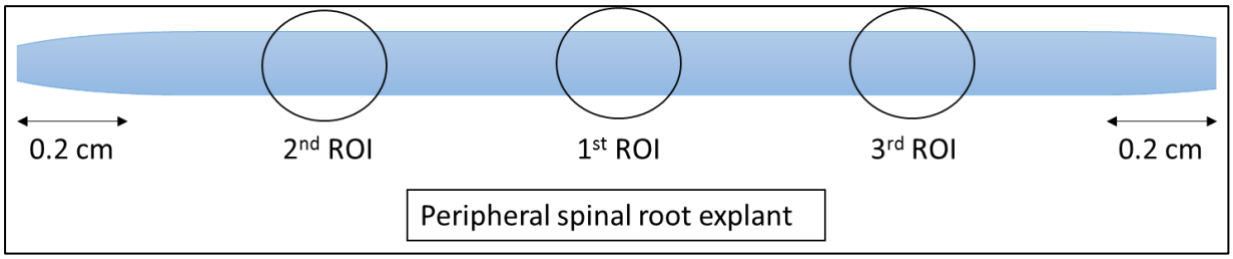


Figure 4: Schematic diagram showing the location of ROIs in peripheral root explants. The ROI should locate at least 0.2 cm away from the ends of the root. Typically, the three ROIs are located in the middle and right and left from the middle. (Source: Original figure)

3.7. Analysis of mitochondrial dynamics

We used an automated analysis function of Volocity®6.3 software (Perkin Elmer, Rodgau, Germany) to analyze time-lapse images for investigating mitochondrial dynamics. For the analysis of mitochondrial morphology, we used the first frame of every video. Mitochondrial shape factor, length and area of individual mitochondria were investigated as morphological parameters for assessing change in mitochondrial morphology, where shape factor is defined as a measure of circularity ranging from 0 and 1 (closer to '0' was a longer mitochondrion, whereas '1' was a perfect circle) (Figure 5).

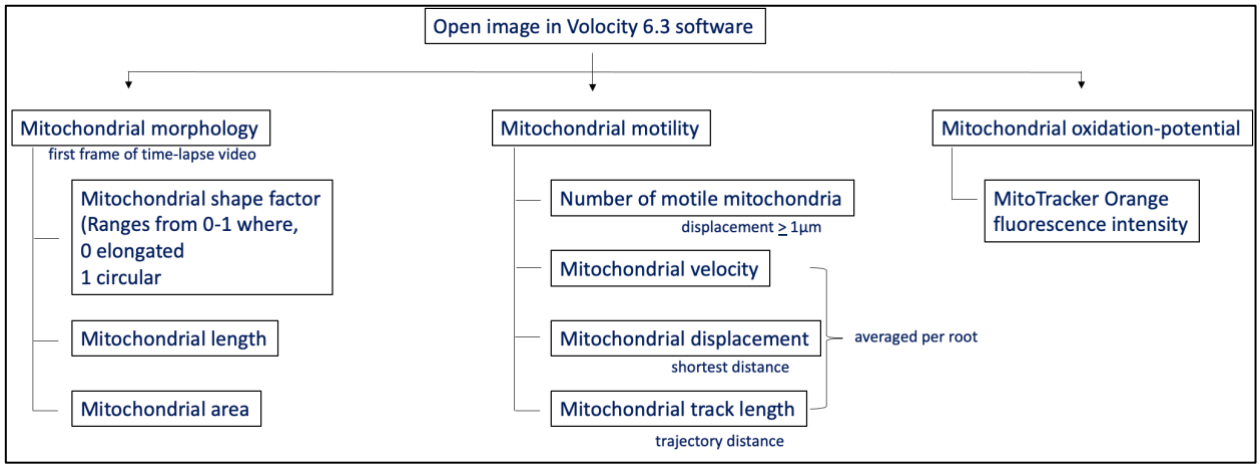


Figure 5: Schematic illustration showing different mitochondrial parameters under investigation. (Source: Original figure)

For the quantification of mitochondrial motility, number of moving mitochondria, their velocity, displacement and track length within each root were analyzed. Mitochondria were tracked manually using Volocity 6.3 software (Perkin Elmer, Rodgau, Germany), where, displacement is the measure of the shortest distance in µm, covered by a mitochondrion. Displacement was measured as a straight line from the starting to the end position during 30 frames. Track length is the measure of real distance path longitude followed by the mitochondrion. Any mitochondrion with a displacement of at least 1 µm was considered 'mobile'. Measurements from three ROI were averaged for each root.

3.8. Statistical Analysis

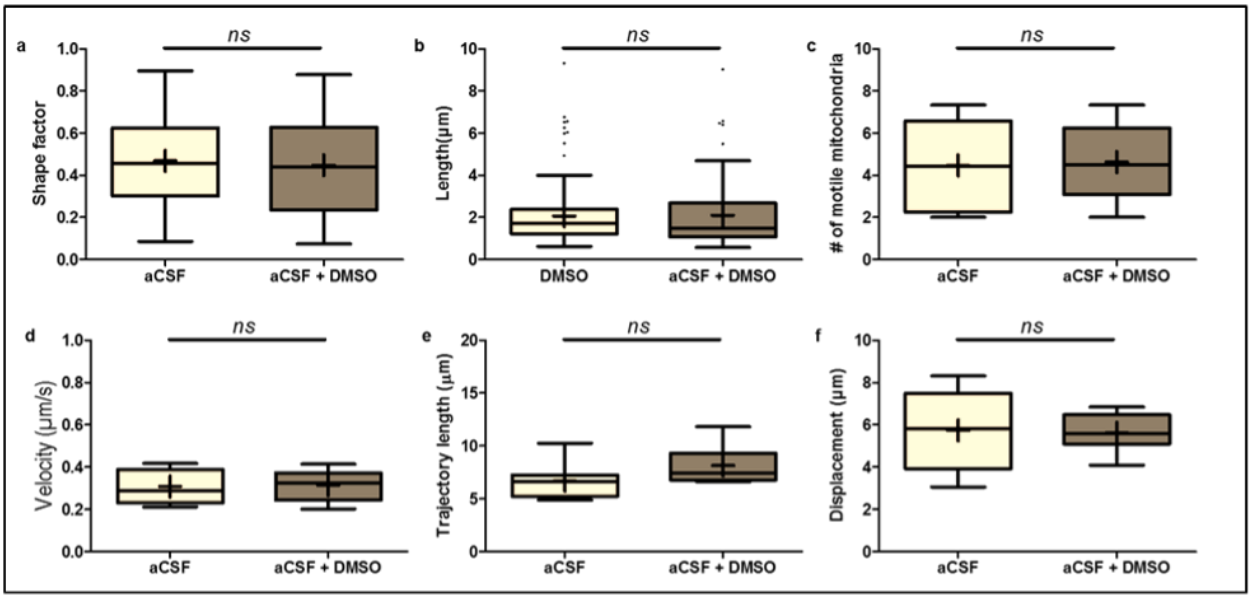
Prism 5.01 Software (GraphPad, CA, USA) was used for the analysis of the data. First, the normality test was carried out using D'Agostino & Pearson omnibus K2 normality test. The two variable comparisons were conducted using Mann Whitney test. All normally distributed data were analyzed using a one-way ANOVA with Bonferroni's post hoc test. The data that were not normally distributed were analyzed using a Kruskal-Wallis test followed by a post hoc Dunn's multiple comparisons test.

Results

4. Results

4.1. DMSO did not influence mitochondrial dynamics in peripheral spinal root explants

As a control of the experimental conditions, we investigated whether dimethyl sulfoxide (DMSO), used to dissolve TFN, would interfere with mitochondrial dynamics in peripheral root explants. For this, six explanted roots were incubated with either aCSF alone or aCSF + DMSO. Then, we compared mitochondrial morphology and motility between roots incubated in aCSF only and aCSF + DMSO. We observed no influence of DMSO on mitochondrial shape, length, no. of motile mitochondria, velocity, trajectory length and displacement (Figure 6a-f, Table 1).



***(Adapted and modified from Supplementary, Malla et al., 2020, Ther Adv Chr Dis*)**

Figure 6: DMSO did not influence mitochondrial dynamics in explanted roots. a) shape factor, b) mitochondrial length, c) no. of motile mitochondria, d) velocity, e) trajectory length and f) displacement. Graphs are shown in Tukey boxplots, where the central line denotes the median; the lower and upper boundaries denote the 1st and 3rd quartile, and the whiskers denote the spread of the data. Inside the box, '+' delineates the mean. *ns*: not significant statistically.

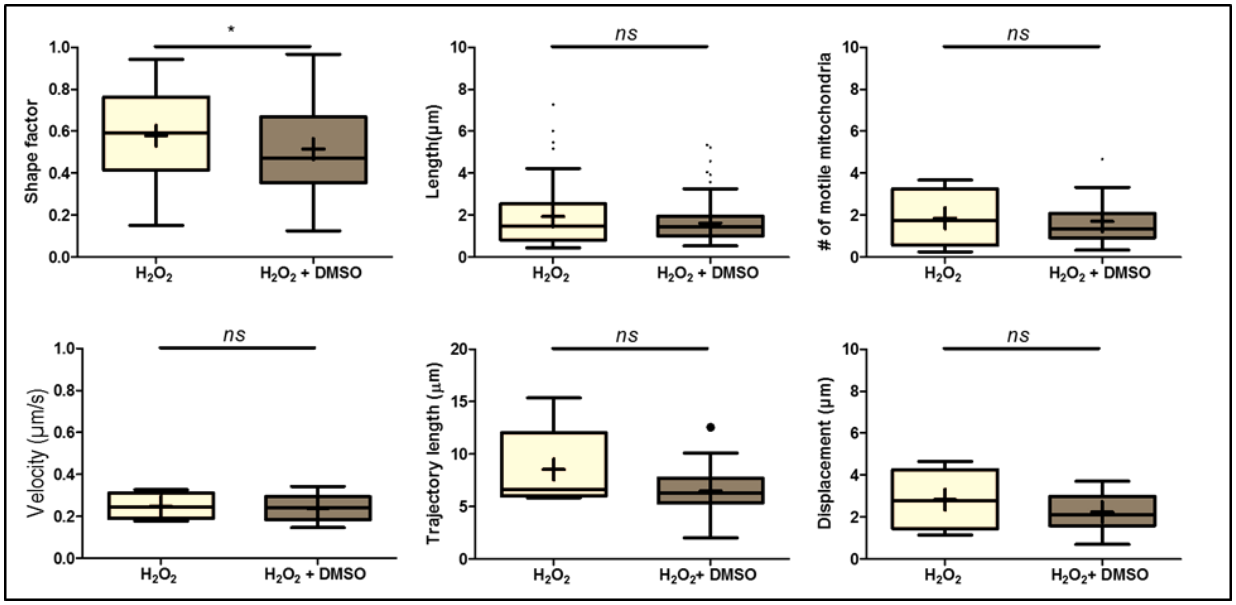
	Shape factor	Length (µm)	# motile mitochondria	Velocity (µm/s)	Trajectory length (µm)	Displacement (µm)
aCSF	0.47 +0.19	2.06 + 1.37	4.47 + 2.26	0.31 + 0.08	6.70 + 1.69	5.74 + 1.92
aCSF + DMSO	0.45 + 0.22	2.11 + 1.59	4.63 + 1.88	0.31 + 0.07	8.14 + 1.78	5.62 + 0.88

(***Adapted and modified from Malla et al., 2020, Ther Adv Chr Dis***)

Table 1: Summary of morphology and motility parameters of mitochondria in peripheral root explants incubated in aCSF and aCSF with DMSO. Values are shown as Mean ± SD. * $p < 0.05$; ** $p < 0.01$

4.2. DMSO did not influence mitochondrial dynamics in peripheral spinal root explants exposed to H_2O_2

Furthermore, we investigated any interference or additional effect of DMSO during oxidative stress. For this, we performed control experiments on 4-10 explanted roots to determine the influence of DMSO on mitochondrial dynamics in presence of H_2O_2. Similar to aforementioned set of experiment, we compared mitochondrial morphology and motility between roots treated with H_2O_2 and H_2O_2 + DMSO in aCSF. We observed slight reduction in mitochondrial shape factor without influencing other morphology or motility related parameters (Figure 7a-f, Table 2).



***(Adapted and modified from Supplementary, Malla et al., 2020, Ther Adv Chr Dis*)**

Figure 7: DMSO did not influence mitochondrial dynamics in explanted roots exposed to H_2O_2. a) shape factor, b) mitochondrial length, c) no. of motile mitochondria, d) velocity, e) trajectory length and f) displacement. Graphs are shown in Tukey boxplots, where the central line denotes the median; the lower and upper boundaries denote the 1st and 3rd quartile, and the whiskers denote the spread of the data. Inside the box, '+' delineates the mean. *p<0.05, *ns*: not significant statistically.

	Shape factor	Length (µm)	# motile mitochondria	Velocity (µm/s)	Trajectory length (µm)	Displacement (µm)
H_2O_2	0.58 ± 0.2	1.93 ± 1.47	1.85 + 1.42	0.25 + 0.06	8.53 + 3.97	2.83 + 1.46
H_2O_2 + DMSO	0.51 ± 0.2	1.62 + 1.47	1.7 + 1.32	0.24 + 0.06	6.47 + 2.27	2.23 +0.94

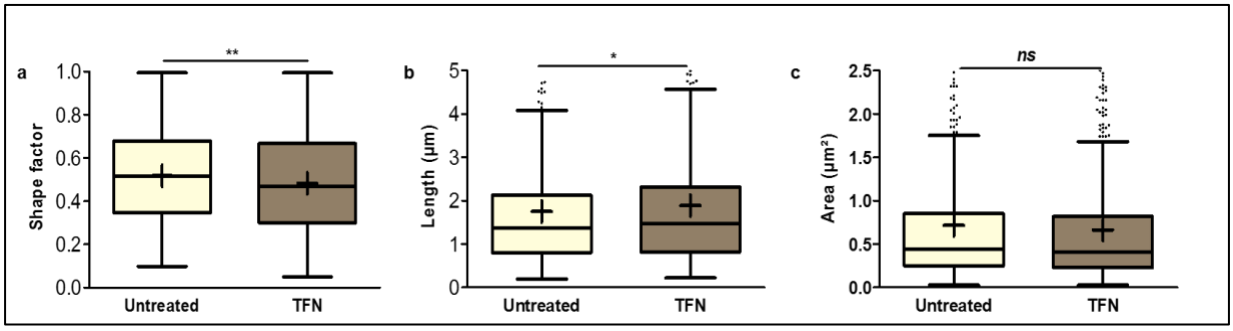
(***Adapted and modified from Malla et al., 2020, Ther Adv Chr Dis*)**

Table 2: Summary of morphology and motility parameters of mitochondria in H_2O_2 and H_2O_2 along with DMSO treated peripheral root explants. Values are shown as Mean ± SD. * p < 0.05; ** p < 0.01

4.3. TFN induced mitochondrial elongation and increase in length in peripheral spinal root explants

To ascertain that TFN does not lead to mitochondrial fragmentation and absolute mitochondrial immobility in absence of H_2O_2-treatment, we incubated the roots with 50 µM TFN. Although, we did not observe any significant difference on mitochondrial morphology and motility with DMSO addition in aCSF and H_2O_2-treatment, we added equivalent amount of DMSO as used in TFN to the untreated group. We performed 4 independent experiments in 12 ROI per condition to compare untreated (aCSF + DMSO) versus TFN-treated nerves. In total, 568 and 672 individual mitochondria in untreated and TFN-treated roots, respectively were analyzed.

To investigate the effect on mitochondrial morphology, we explored three morphological parameters- shape factor, length and area (Figure 8a-c). TFN reduced mitochondrial circularity and increased mitochondrial length without significantly affecting the area (Table 3)



(***Adapted from Malla et al., 2020, Ther Adv Chr Dis***)

Figure 8: Mitochondria became elongated and longer with 50 µM TFN treatment. (a) Mitochondrial shape factor (circularity) was reduced with TFN-treatment. (b) Mitochondrial length was increased with TFN-treatement. (c) Mitochondrial area did not change with TFN-treatment. Graphs are shown in Tukey boxplots, where the central line denotes the median; the lower and upper boundaries denote the 1st and 3rd quartile, and the whiskers denote the spread of the data. Inside the box, '+' delineates the mean. *ns*: not significant statistically. *p<0.05, ** p<0.01.

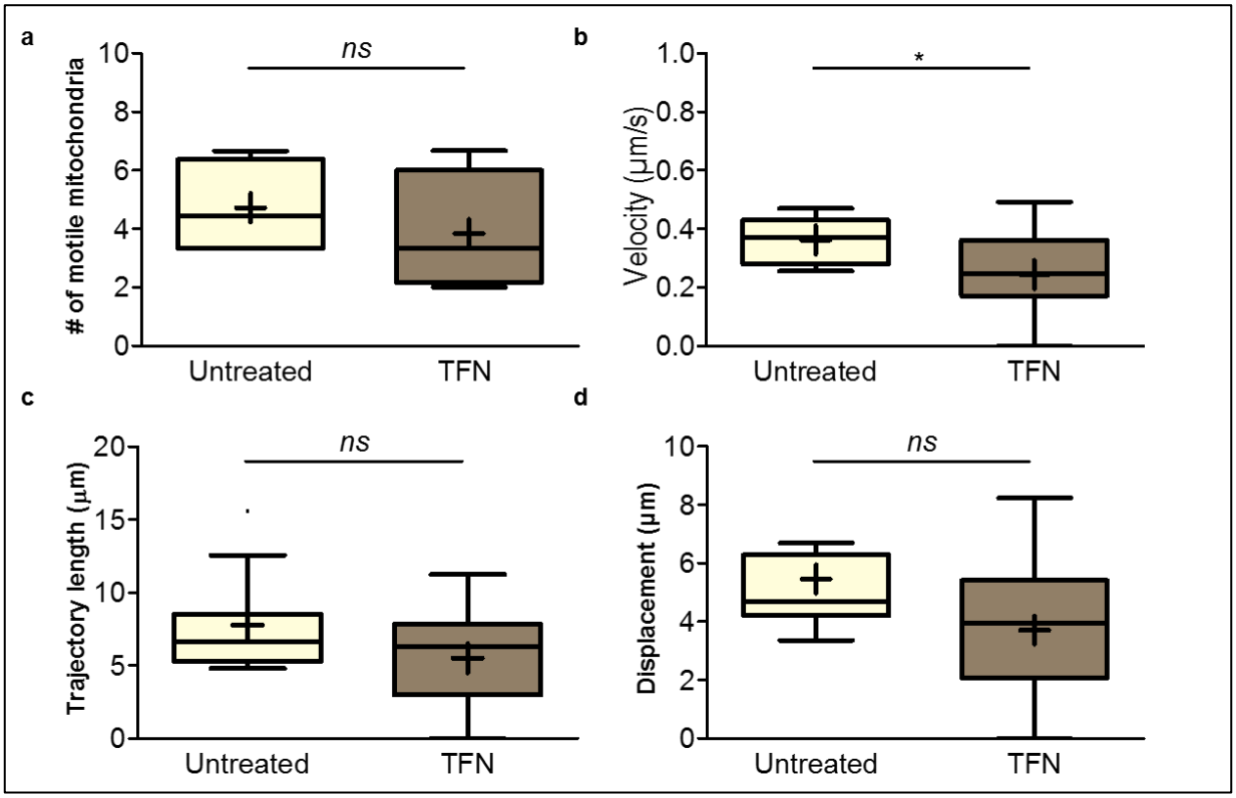
	Shape factor	Length (µm)	Area (µm²)	# motile mitochondria	Velocity (µm/s)	Trajectory length (µm)	Displacement (µm)
Untreated	0.52 ± 0.21	1.75 ± 1.52	0.77 ± 0.86	4.72 ± 1.67	0.36 ± 0.07	7.77 ± 3.26	5.46 ± 2.39
TFN (50 µM)	0.48 ± 0.23	1.89 ± 1.67	0.66 ± 0.82	3.83 ± 2.06	0.24 ± 0.15	5.51 ± 3.49	3.71 ± 2.43
Mann Whitney test	**	*	>0.1	>0.1	*	>0.1	>0.1

(***Adapted and modified from Malla et al., 2020, Ther Adv Chr Dis***)

Table 3: Summary of morphology and motility parameters of mitochondria in untreated and teriflunomide treated peripheral root explants. Values are shown as Mean ± SD. * p < 0.05; ** p < 0.01

4.4. TFN reduced mitochondrial transport velocity

Similarly, we investigated the effect of TFN on mitochondrial motility in untreated (aCSF + DMSO) versus TFN-treated spinal root explants, basing on four-motility parameters- no. of motile mitochondria, velocity, trajectory length and displacement. We observed reduction of mitochondrial transport velocity compared to the untreated roots (Figure 9b). There was no effect on other motility parameters (Figure 9a, c-d, Table 3).



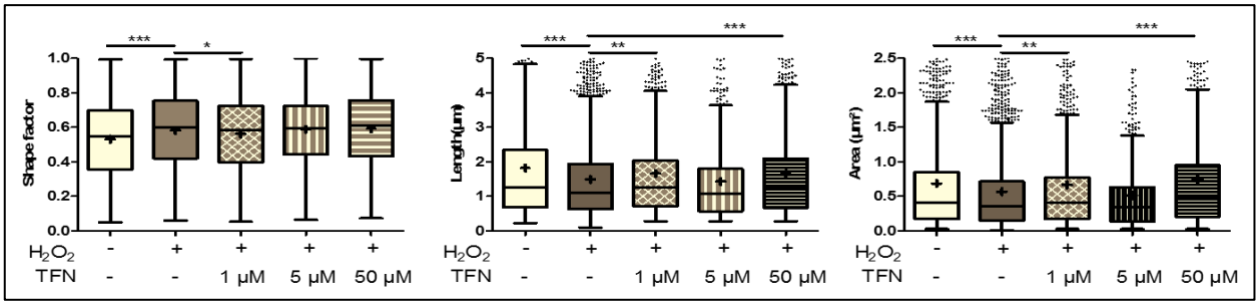
***(Adapted from Malla et al., 2020, Ther Adv Chr Dis*)**

Figure 9: Mitochondrial transport velocity decreased with TFN treatment. (a) Number of moving mitochondria per root in untreated vs. teriflunomide-treated roots. (b) Velocity of mitochondrial transport. (c) Length of the mitochondrial trajectories and (d) Displacement (final position minus initial position) of mitochondria. Graphs are shown in Tukey boxplots, where the central line denotes the median; the lower and upper boundaries denote the 1st and 3rd quartile, and the whiskers denote the spread of the data. Inside the box, '+' delineates the mean. *ns*: not significant statistically. * $p<0.05$.

4.5. TFN prevented oxidative stress-mediated reduction in mitochondrial elongation, length and area

To investigate the effect of TFN on oxidative stress-induced mitochondrial alterations, the explanted roots were either untreated (aCSF + DMSO) or treated with 50 µM hydrogen peroxide (H_2O_2+DMSO) and 3 different concentrations of TFN: 1, 5 and 50 µM in aCSF for 30 minutes at room temperature. We included 19 independent experiments carried out in 19 different mice. Each treatment includes at least 5 independent roots and 15 ROI per culturing condition. For morphological investigations, number of mitochondria in the selected ROI were 2586, 2860, 1306, 875 and 1550 for untreated (aCSF + DMSO), H_2O_2-treated (+DMSO), H_2O_2 + TFN (1 µM), H2O2 + TFN (5 µM) and H_2O_2 + TFN (50 µM), respectively.

During oxidative stress, mitochondrial circularity increased and mitochondrial length and area decreased substantially while, TFN prevented these alterations (Figure 10a-c). Among the three concentrations of TFN, 1 µM prevented alterations of all the three parameters, 50 µM induced increase in mitochondrial length and area only but 5 µM TFN did not have any statistically significant effect on H_2O_2-induced morphological alterations. (Table 4)



***(Adapted from Malla et al., 2020, Ther Adv Chr Dis*)**

Figure 10: Mitochondrial morphology preserved by TFN treatment during oxidative stress. (a) Change in mitochondrial shape factor, (b) length, and (c) area of mitochondria in the presence of H_2O_2 with or without teriflunomide. Graphs are shown in Tukey boxplots, where the central line denotes the median; the lower and upper boundaries denote the 1st and 3rd quartile, and the whiskers denote the spread of the data. Inside the box, '+' delineates the mean. * p<0.05, ** p<0.01, ***p<0.001.

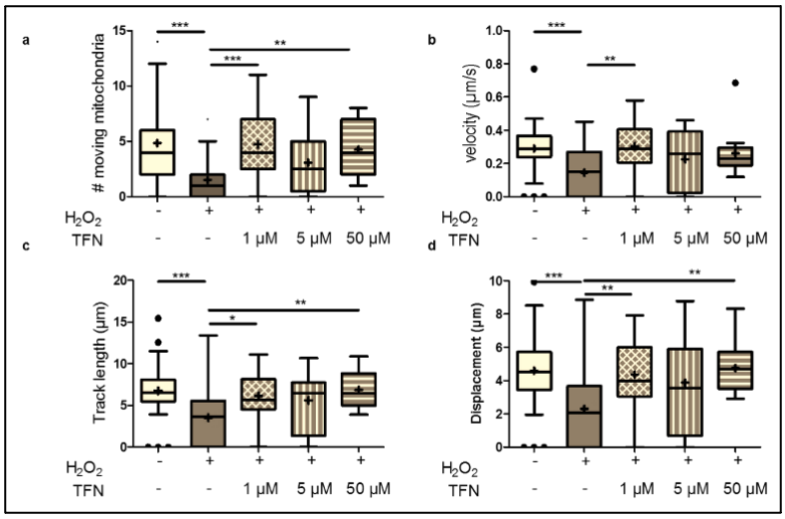
	Shape factor	KW test	Length (µm)	KW test	Area (µm²)	KW test
Untreated	0.53± 0.22	***	1.82 ± 1.79	***	0.69 ± 0.92	***
H_2O_2-treated	0.58 ± 0.21		1.49 ± 1.29		0.57 ± 0.65	
H_2O_2+ TFN (1µM)	0.57 ± 0.21	*	1.66 ± 1.50	**	0.67 + 0.85	**
H_2O_2+ TFN (5µM)	0.59 ± 0.19	>0.1	1.43 ± 1.29	>0.1	0.51 + 0.69	>0.1
H_2O_2+ TFN (50µM)	0.59 ± 0.21	>0.1	1.66 ± 1.42	***	0.75 + 0.93	***

(***Adapted and modified from Malla et al., 2020, Ther Adv Chr Dis*)**

Table 4: Summary of mitochondrial morphology under H_2O_2- treatment alone, and with 50 µM H_2O_2 in presence of 1 µM, 5µM and 50 µM teriflunomide. Values are shown as Mean ± SD. * p < 0.05; ** p < 0.01; *** p < 0.001

4.6. TFN prevented oxidative stress-mediated reduction in no. of motile mitochondria, transport velocity, trajectory length and displacement

As described above, to investigate the effect of TFN on oxidative stress-induced mitochondrial motility, the explanted roots were either untreated (aCSF + DMSO) or treated with 50 µM hydrogen peroxide (H_2O_2+DMSO) and 3 different concentrations of TFN: 1, 5 and 50 µM in aCSF for 30 minutes. For motility investigation, average number of motile mitochondria per root were analyzed. In total, 201, 70, 83, 52 and 64 motile mitochondria for untreated (aCSF + DMSO), H_2O_2-treated (+DMSO), H_2O_2 + TFN (1 µM), H2O2 + TFN (5 µM) and H_2O_2 + TFN (50 µM), respectively were analyzed. We observed that H_2O_2-treatment lead to decrease in the number of motile mitochondria, velocity, trajectory length as well as displacement. TFN at concentrations 1 µM and 50 µM prevented the decrease in motile mitochondria, velocity, trajectory length as well as displacement (Figure 11a-d). However, 5 µM TFN had no effect. (Table 5).



***(Adapted and modified from Malla et al., 2020, Ther Adv Chr Dis*)**

Figure 11: Mitochondrial motility preserved by TFN treatment during oxidative stress. (a) Number, (b) velocity, (c) trajectory, and (d) displacement, of mitochondria. Graphs are shown in Tukey boxplots, where the central line denotes the median; the lower and upper boundaries denote the 1st and 3rd quartile and the whiskers denote the spread of the data. Inside the box, '+' delineates the mean. * p<0.05, ** p<0.01, ***p<0.001.

	# motile mito (axon)	KW test	Velocity (µm/s)	KW test	Trajectory length (µm)	KW test	Displacement (µm)	KW test
Untreated	4.85 ± 3.72	***	0.29± 0.14	***	6.77 ± 3.13	***	4.59 ± 2.16	***
H_2O_2-treated	1.52 ± 1.71		0.15 ± 0.14		3.53 ± 3.67		2.30 ± 2.60	
H_2O_2+ TFN (1µM)	4.71 ± 3.08	***	0.30 ± 0.13	**	6.13 ± 2.59	*	4.37 ± 1.97	**
H_2O_2+ TFN (5µM)	3.08 ± 2.75	>0.1	0.23 ± 0.17	>0.1	5.61 ± 3.69	>0.1	3.87 ± 2.96	>0.1
H_2O_2+ TFN (50µM)	4.27 ± 2.34	**	0.26 ± 0.13	>0.1	6.84 ± 2.15	**	4.75 ± 1.45	**

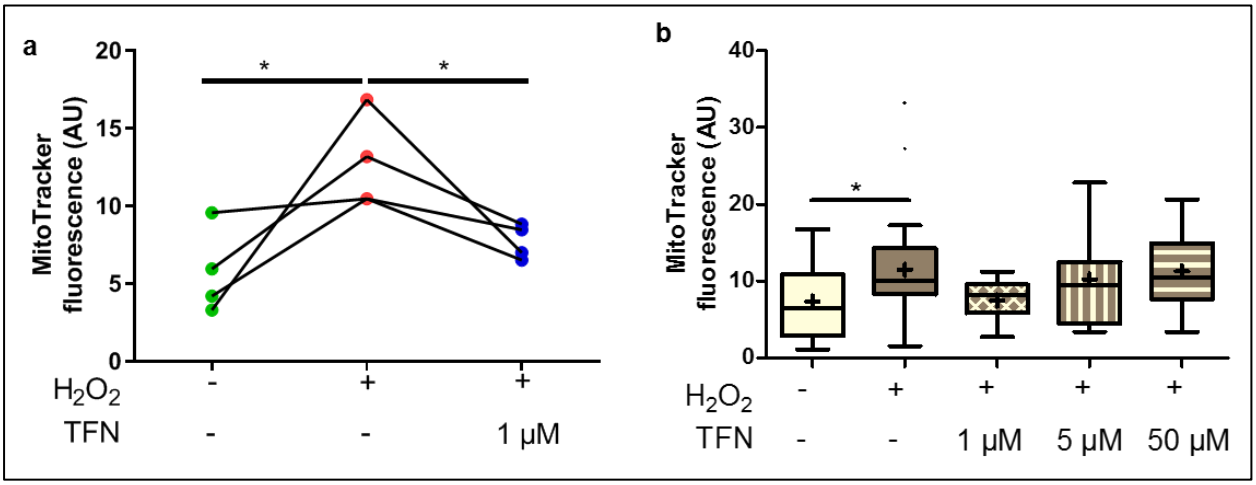
KW test: Kruskal-Wallis test

(***Adapted and modified from Malla et al., 2020, Ther Adv Chr Dis*)**

Table 5: Summary of mitochondrial motility under H_2O_2- treatment alone, and with 50 µM H_2O_2 in presence of 1µM, 5µM and 50 µM teriflunomide. Values are shown as Mean ± SD. * p < 0.05; ** p < 0.01; *** p < 0.001

4.7. TFN prevented change in mitochondrial oxidation potential in peripheral nerve explants during oxidative stress

As MitoTracker CMTMRos Orange fluoresces only upon oxidation, mitochondria in H_2O_2-treated roots with high oxidation potential acquired higher fluorescence relative to untreated. In contrast, 1 µM TFN reduced the fluorescence intensity in the mitochondria as low as in untreated axons (Figure 12a). Pair-wise comparison between untreated vs H_2O_2- treated and H_2O_2- treated vs 1 µM TFN-treated were significant (Table 6). No effect was observed at 5 or 50 µM TFN (Figure 12b).



***(Adapted from Malla et al., 2020, Ther Adv Chr Dis*)**

Figure 12: Mitochondrial oxidation potential preserved by 1 µM teriflunomide during oxidative stress. (a) Fluorescence intensity of MitoTracker Orange staining depicting significant reduction of oxidation potential of 1 µM TFN. (b) MitoTracker Orange fluorescence intensity at 1, 5, and 50 µM TFN treatment during oxidative stress in comparison to untreated and H_2O_2-treated roots. * depicts statistical significance with p value < 0.05

	MitoTracker Fluorescence (AU)	Mann Whitney test
Untreated	**7.35 ± 4.76**	*
H_2O_2-treated	**11.49 ± 6.44**	
H_2O_2+ TFN (1µM)	**7.48 ± 2.75**	*
H_2O_2+ TFN (5µM)	**10.22 ± 6.45**	**>0.1**
H_2O_2+ TFN (50µM)	**11.27 ± 4.79**	**>0.1**

(***Adapted and modified from Malla et al., 2020, Ther Adv Chr Dis*)**

Table 6: Summary of mean fluorescence intensity of mitochondria. (AU: arbitrary units). Values are shown as Mean ± SD. * p < 0.05

Discussion

5. Discussion

Mitochondria is one of the major contributors in MS pathogenesis. In MS, inflammation- induced oxidative stress causes mitochondrial alterations that leads to neuroaxonal damage (Moreira, Zhu et al. 2010, Friese, Schattling et al. 2014, Barcelos, Troxell et al. 2019, Bargiela and Chinnery 2019, Boukalova, Hubackova et al. 2020). Although, most of the current treatments for MS focuses on mitigating neuroinflammation via immune modulation (Rudick and Trapp 2009, Claussen and Korn 2012), preventing neurodegeneration seems to be crucial to slow the progress of disability.

In this context, TFN is the drug for our investigation because this drug has been widely used for managing RRMS. The mechanism of action of TFN is via limiting lymphocytic proliferation by targeting mitochondrial enzyme, DHODH (Klotz, Eschborn et al. 2019). However, inhibition of DHODH affects mitochondria is not well studied. Due to its close association with mitochondrial ETC, we hypothesized that TFN influences mitochondrial stability without compromising mitochondrial functionality.

To test this hypothesis, we used our previously established model of murine spinal root explants (Bros, Millward et al. 2014, Bros, Niesner et al. 2015). Although, the impact of mitochondrial morphology on mitochondrial motility is known to be interrelated and well reported in cell culture models, there are no study that investigated motility changes during oxidative stress in live tissue model as per our knowledge. In this paper, we showed the alterations in mitochondrial velocity, track length and displacement during oxidative stress for the first time using murine peripheral spinal root explants.

As opposed to 300 µM H_2O_2 concentration that we used in our previous study, here we investigated the effect of 50 µM H_2O_2 on axonal mitochondria (Bros et al., 2014). A very high concentration of H_2O_2 damages mitochondria and neurons rapidly to the extent where repair and protection is not possible. In this study, H_2O_2 concentration induced alterations in both mitochondrial morphology and motility and TFN ensured mitochondrial protection.

After excluding any relevant effect of the dilution vehicle for teriflunomide, DMSO, in both untreated (Figure 6) and H_2O_2 – treated mitochondria (Figure 7), we could show that the application of 50 µM TFN in otherwise untreated roots led to significantly longer mitochondria (Figure 8b-d) with reduced velocity (Figure 9b). TFN seem to promote mitochondrial fusion as depicted by elongated and longer mitochondria without affecting number of motile mitochondria. This observation supports other studies that reported increased expression of mitofusin 1 and 2 gene that are involved in mitochondrial fusion during DHODH inhibition (Miret-Casals, Sebastian et al. 2018, Boukalova, Hubackova et al. 2020).

Additionally, we observed that 1 and 50 µM teriflunomide was able to prevent the decrease in shape factor, mitochondrial length and area during exogenous application of H_2O_2 (Figure 10f-h). Due to huge amount of oxidative damage, mitochondria tend to get rid of the damage parts. Functional mitochondria after shedding off the damaged parts may divide itself in order to replenish the loss number of mitochondria resulting in rounder, shorter and smaller mitochondria, indicative of fragmentation, which is in consistent with previous reports (Bros, Millward et al. 2014, Sadeghian, Mastrolia et al. 2016). 1 and 50 µM TFN prevented fragmentation of mitochondria (Figure 10a-c, e). Importantly, mitochondrial fragmentation does not necessarily suggest mitochondrial dysfunction but it is part of mitochondrial biogenesis undergoing fusion and fission depending on the energy demand of the cell (Oliver and Reddy 2019). However, excessive fragmentation could be indicative of extensive mitochondrial damage.

Subsequently, high energetic demand of the cell and loss to huge number of mitochondria during oxidative stress might lead to immediate need of ATP production (Kaasik, Safiulina et al. 2007, Safiulina and Kaasik 2013). Since, mitochondrial immobility is one of the prerequisite for ATP synthesis (Lin and Sheng 2015), fragmented mitochondria might need to stabilize and synthesize ATP during oxidative stress. This might be the reason that H_2O_2- treatment caused reduction in number of motile mitochondria and reduction in velocity, displacement and trajectory lengths (Figure 11a-d). 1 and 50 µM TFN treatment prevented the reduction in the number of motile mitochondria, displacement and trajectory length in H_2O_2- treated roots.

Moreover, oxidation potential of mitochondria was increased during H_2O_2- treatment. 1 µM TFN effectively reduced the increase in mitochondria oxidation potential indicating lower level of ROS in TFN treated roots even during H_2O_2- treatment (Figure 12). During excessive ROS generation, there is over reduction of ubiquinone. Over reduction of ubiquinone leads to reverse electron transport (Scialo, Fernandez-Ayala et al. 2017) resulting in further ROS generation, ATP depletion and subsequent inhibition of ATP synthase (Figure 13a). However, when TFN inhibits DHODH during H_2O_2- treatment, it might limit the pool of reduced ubiquinone, preventing reverse electron transport, thus limiting further ROS generation and mitochondrial dysfunction (Figure 13b).

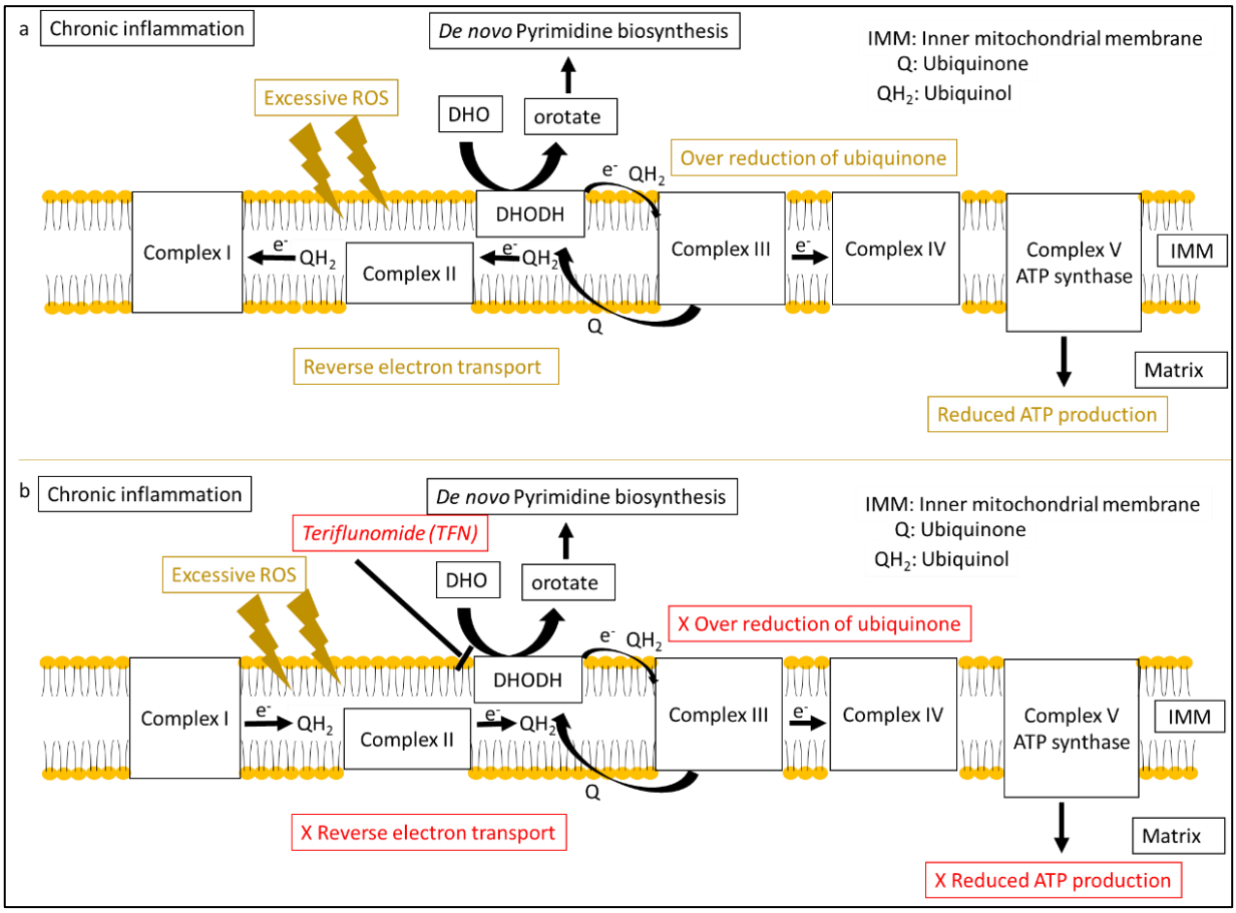


Figure 13: Simplified schematic diagram depicting DHODH- complex III- ubiquinone cycle and the impact of ROS and TFN on ubiquinone, electron transport chain and ATP production. (a) Excessive ROS production leads to over reduction of ubiquinone, reverse electron transport and reduced ATP production. (b) TFN inhibition blocks DHODH that limits reduction of ubiquinone that prevents reverse electron transport and reduced ATP production. (Source: Original figure)

Regarding the doses, TFN potently inhibits DHODH with half-maximum concentration of 1µM in human and ~86 nM in rats (Palmer 2013). At higher concentrations (50-200 µM), TFN is capable of inhibiting other proteins like tyrosine kinase or cyclooxygenase-2 (Oh and O'Connor 2013). However, not much is known about the intermediate doses. In our study, 1µM TFN concentration was proven protective and 50 µM TFN restored most of the alterations in mitochondrial dynamics. Contrastingly, 5µM TFN concentration had no effects. While the given half-maximum concentrations for rats are much lower than for humans, it is difficult to ascertain what concentration possess beneficial effect of DHODH inhibition and what concentration lead to more detrimental consequences of inhibition of other proteins like tyrosine kinases. In our study, inhibition of DHODH by1µM TFN restored mitochondria dynamics and oxidation potential during oxidative stress.

Conclusions and Limitations

6. Conclusion

In conclusion, mitochondria are dynamic organelles that undergo fusion- fission and motile-stationary cycles according to the demand of the cells. In situation of oxidative stress, mitochondria tend to fragment and, the remaining few that are motile travels shorter distance and slowly in comparison to non-stressed mitochondria. By inhibiting DHODH, TFN prevents ROS induced ROS production, restores oxidative stress-induced alterations in mitochondrial morphology and motility as well as reduces oxidation potential in peripheral roots. As, DHODH inhibition possess therapeutic capability for mitochondrial protection, TFN drug could be further investigated for its efficacy in other diseases, in which oxidative stress and mitochondrial alterations are confounding components.

7. Limitations and perspectives

This thesis is based on investigation of mitochondrial morphology and motility during oxidative stress in peripheral spinal root explants. Although, experimental setting in peripheral spinal root explants is close to the biological system and easy to extract and manipulate, the duration of experimentation is limited to 3-4 hrs only. After 3-4 hrs of explantation, the axons undergo irreparable damage due to lack of physiological environment. Hence, longer experimentations with treatments could not be carried out in this model.

Apart from the model, another important aspect that needs consideration is the concentration of TFN. The kinetics of delivery of TFN in murine peripheral root explant and in brain would be different. Hence, although we used TFN within the recommended concentration, exact amount absorbed in the roots is not known. To answer this question, further investigation in brain tissue is needed.

Furthermore, using peripheral spinal root explants, we intended to mimic oxidative stress situation induced by inflammation in MS by applying hydrogen peroxide. As much as one can argue about using a live tissue model, this model still lacks the complexity of CNS. Unlike parallel arranged axons in peripheral root explants, CNS axons are directed in all directions and hence difficult to follow in three-dimensions. Moreover, MS being a CNS disease, a brain model could provide better insight on how mitochondria are affected during oxidative stress and if TFN is protective towards neuronal mitochondria. In the second part of my PhD project, we established the CNS model and investigated the effect of oxidative stress and TFN on neuronal mitochondria.

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Statutory Declaration

I, Bimala Malla, by personally signing this document in lieu of an oath, hereby affirm that I prepared the submitted dissertation on the topic 'Effect of DHODH inhibition by teriflunomide on axonal mitochondria', in German, 'Wirkung der DHODH- inhibition durch teriflunomide auf axonale Mitochondrien', independently and without the support of third parties, and that I used no other sources and aids than those stated.

All parts, which are based on the publications or presentations of other authors, either in letter or in spirit, are specified as such in accordance with the citing guidelines. The sections on methodology (in particular regarding practical work, laboratory regulations, statistical processing) and results (in particular regarding figures, charts and tables) are exclusively my responsibility.

Furthermore, I declare that I have correctly marked all of the data, the analyses, and the conclusions generated from data obtained in collaboration with other persons, and that I have correctly marked my own contribution and the contributions of other persons (cf. declaration of contribution). I have correctly marked all texts or parts of texts that were generated in collaboration with other persons.

My contributions to any publications to this dissertation correspond to those stated in the below joint declaration made together with the supervisor. All publications created within the scope of the dissertation comply with the guidelines of the ICMJE (International Committee of Medical Journal Editors; www.icmje.org) on authorship. In addition, I declare that I shall comply with the regulations of Charite - Universitätsmedizin Berlin on ensuring good scientific practice.

I declare that I have not yet submitted this dissertation in identical or similar form to another Faculty.

The significance of this statutory declaration and the consequences of a false statutory declaration under criminal law (Sections 156, 161 of the German Criminal Code) are known to me.

Date	Bimala Malla, MSc. Medical Neuroscience

Declaration of contribution to the top-journal publication for a PhD degree

Bimala Malla contributed the following to the below listed publication:

Publication 1: ***Malla B***, Cotten S, Ulshoefer R, Paul F, Hauser AE, Niesner R, Bros H, Infante-Duarte C, ***Teriflunomide preserves peripheral nerve mitochondria from oxidative stress-mediated alterations***. Ther Adv Chronic Dis, 2020. (Impact Factor: 5.091)

Contribution: Bimala Malla contributed to the study design, performed the experiments, image analysis and statistical analysis under the supervision of HB and CID. Specifically, she created figures 6-12 and related tables from her experiments in which some data points were included from the experiments contributed by SC. Additionally, she created figures 1, 2, 4, 5 and 13. Then, she interpreted the data, wrote the manuscript, and responded to the reviewers' questions during revision in agreement with all other co-authors.

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Prof. Dr. Carmen Infante-Duarte

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Bimala Malla, MSc. Medical Neuroscience

Excerpt from Journal Summary List

Journal Data Filtered By: **Selected JCR Year: 2018** Selected Editions: SCIE,SSCI Selected Categories: **"PHARMACOLOGY and PHARMACY"** Selected Category Scheme: WoS

Gesamtanzahl: 267 Journale

Rank	Full Journal Title	Total Cites	Journal Impact Factor	Eigenfactor Score
1	NATURE REVIEWS DRUG DISCOVERY	32,266	57.618	0.054890
2	PHARMACOLOGICAL REVIEWS	12,653	18.886	0.011950
3	ADVANCED DRUG DELIVERY REVIEWS	36,350	15.519	0.037430
4	Annual Review of Pharmacology and Toxicology	7,820	12.103	0.009900
5	DRUG RESISTANCE UPDATES	2,856	11.708	0.003590
6	TRENDS IN PHARMACOLOGICAL SCIENCES	12,317	11.523	0.018180
7	MEDICINAL RESEARCH REVIEWS	4,560	9.791	0.004920
8	PHARMACOLOGY & THERAPEUTICS	15,434	9.396	0.022540
9	JOURNAL OF CONTROLLED RELEASE	47,630	7.901	0.052240
10	ALIMENTARY PHARMACOLOGY & THERAPEUTICS	20,998	7.731	0.033430
11	NEUROPSYCHOPHARMACOLOGY	25,672	7.160	0.039090
12	DRUG DISCOVERY TODAY	14,244	6.880	0.021560
13	European Heart Journal-Cardiovascular Pharmacotherapy	442	6.723	0.001430
14	BRITISH JOURNAL OF PHARMACOLOGY	34,006	6.583	0.033440
15	CLINICAL PHARMACOLOGY & THERAPEUTICS	16,170	6.336	0.016950
16	Reviews of Physiology Biochemistry and Pharmacology	738	6.214	0.000540
17	Acta Pharmaceutica Sinica B	2,418	5.808	0.004930
18	PHARMACOLOGICAL RESEARCH	11,583	5.574	0.017940
19	Neurotherapeutics	4,475	5.552	0.009060
20	Expert Opinion on Drug Delivery	6,666	5.400	0.008980
21	CURRENT OPINION IN PHARMACOLOGY	6,511	5.203	0.010500

Rank	Full Journal Title	Total Cites	Journal Impact Factor	Eigenfactor Score
22	JOURNAL OF ANTIMICROBIAL CHEMOTHERAPY	30,927	5.113	0.048620
23	DRUGS	10,520	4.993	0.013240
24	BIODRUGS	1,685	4.903	0.003370
25	BIOCHEMICAL PHARMACOLOGY	27,370	4.825	0.021340
26	Pharmaceutics	1,482	4.773	0.002320
27	ANTIMICROBIAL AGENTS AND CHEMOTHERAPY	65,138	4.715	0.086660
28	EUROPEAN JOURNAL OF PHARMACEUTICS AND BIOPHARMACEUTICS	16,651	4.708	0.016480
29	DRUG METABOLISM REVIEWS	2,590	4.702	0.002050
30	CLINICAL PHARMACOKINETICS	8,333	4.680	0.009020
31	EXPERT OPINION ON THERAPEUTIC TARGETS	4,703	4.621	0.008050
32	INTERNATIONAL JOURNAL OF ANTIMICROBIAL AGENTS	11,529	4.615	0.017010
33	Current Neuropharmacology	3,508	4.568	0.005650
34	International Journal of Nanomedicine	20,118	4.471	0.027780
35	EUROPEAN NEUROPSYCHOPHARMACOLOGY	7,488	4.468	0.015500
36	Therapeutic Advances in Chronic Disease	655	4.455	0.001750
37	Journal of Pharmaceutical Analysis	1,084	4.440	0.002050
38	Expert Opinion on Drug Discovery	3,097	4.421	0.006350
39	MOLECULAR PHARMACEUTICS	16,792	4.396	0.028020
40	NEUROPHARMACOLOGY	20,604	4.367	0.034460
41	PROGRESS IN NEURO-PSYCHOPHARMACOLOGY & BIOLOGICAL PSYCHIATRY	10,674	4.315	0.012400
42	JOURNAL OF NATURAL PRODUCTS	25,908	4.257	0.021150
43	JOURNAL OF PSYCHOPHARMACOLOGY	6,460	4.221	0.010120

Publication

Therapeutic Advances in Chronic Disease *Original Research*

Teriflunomide preserves peripheral nerve mitochondria from oxidative stress-mediated alterations

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Abstract: Mitochondrial dysfunction is a common pathological hallmark in various inflammatory and degenerative diseases of the central nervous system, including multiple sclerosis [MS]. We previously showed that oxidative stress alters axonal mitochondria, limiting their transport and inducing conformational changes that lead to axonal damage. Teriflunomide [TFN]. an oral immunomodulatory drug approved for the treatment of relapsing forms of MS, reversibly inhibits dihydroorotate dehydrogenase [DHODH]. DHODH is crucial for *de nova* pyrimidine biosynthesis and is the only mitochondrial enzyme in this pathway, thus conferring a link between inflammation, mitochondrial activity and axonal integrity.
Here, we investigated how DHOOH inhibition may affect mitochondrial behavior in the context of oxidative stress. We employed a model of transected murine spinal roots, previously developed in our laboratory. Using confocal live imaging of axonal mitochondria, we showed that in unmanipulated axons, TFN increased significantly the mitochondria length without altering their transport features. In mitochondria challenged with 50 µM hydrogen peroxide [H_20_2] to induce oxidative stress, the presence of TFN at 1 µM concentration was able to restore mitochondrial shape, motility, as well as mitochondrial oxidation potential to control levels. No effects were observed at 5 µM TFN, while some shape and motility parameters were restored to control levels at 50 µM TFN.
Thus, our data demonstrate an undescribed link between OHODH and mitochondrial dynamics and point to a potential neuroprotective effect of DHODH inhibition in the context of oxidative stress-induced damage of axonal mitochondria.

Keywords: dihydroorotate dehydrogenase [DHODHI. mitochondria, mitochondrial dynamics, neurodegeneration, oxidative stress, teriflunomide [TFN]

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Introduction

Multiple sclerosis (MS) is a chronic inflammatory disease of central nervous system (CNS) that affects more than 2.5 million people worldwide.[1] In MS, inflammation, demyelination and neurodegeneration are considered to contribute to disease development.[2,3] It is assumed that in MS, a misguided immune response against the CNS is initiated and orchestrated by autoreactive T cells, leading to progressive demyelination, oligodendrocyte injury and axonal loss,[4,5] that affect not only the white but also the grey matter.[6]

The mechanisms by which neuroinflammation and myelin damage lead to neurodegeneration have not been fully elucidated; however, the sustained release of reactive oxygen species (**ROS**) and nitrogen species (**NOS**) by macrophages and activated microglia during inflammation appears to contribute to the damaging cascade.[7-9] Also in cortical lesions, demyelination appears to be associated with excessive oxidative damage.[10] Mitochondrial pathology and subsequent focal axonal injury appears also to be triggered by inflammation-associated ROS and NOS and to

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be independent of demyelinating processes.[11] Axonal mitochondrial damage is an early sign of neurodegeneration that precedes and contributes to focal and reversible alterations in axon morphology. The alterations of the mitochondrial function within axons have been proposed to occur in the early stages of the disease, even before demyelination,[11,12] and to precede neuronal death.[13–16] In autopsied tissue from chronic progressive MS, respiratory deficient neurons were detected both in white and grey matter. Respiration deficits were shown to be caused by multiple deletions of mitochondrial DNA, probably subsequent to inflammation and oxidative stress, that contributed to an enhanced susceptibility of axons and neurons to additional damaging insults.[17] In this line, we and others have shown that oxidative stress disrupts the transport of mitochondria in the axon.[14,18] Hence, mitochondrial dysfunction is considered as one of the major contributors of neuroaxonal damage in MS.

With regard to MS management and treatment, teriflunomide (TFN) (Aubagio; Genzyme, Cambridge, MA, USA) is a once-daily oral immunomodulatory drug for the treatment of patients with relapsing forms of MS.[19] TFN has been shown to reduce relapse events and increase the periods of remission.[20,21]

TFN seems to exert its therapeutic effect by non-competitively and reversibly inhibiting the mitochondrial respiratory chain-associated enzyme dihydroorotate dehydrogenase (DHODH).[22–25] DHODH is involved in *de novo* pyrimidine biosynthesis, thus limiting lymphocytic proliferation and inflammation. However, whether the inhibition of DHODH alone may affect neuronal mitochondria remains uncertain. Importantly, a very recent retrospective, single-center, observational study indicated that the effect of TFN in reducing cortical grey matter atrophy is superior to the effect of the anti-oxidative and anti-inflammatory dimethyl fumarate.[26] Moreover, it has been reported that TFN penetrates into the CNS and exerts its effect directly within the brain.[27] Thus, TFN may indeed have the potential to affect axonal mitochondria directly.

To explore the possible effects of TFN on the nervous system, we have used in this study a previously established model of explanted spinal roots, in which we had shown that mitochondria undergo a series of alterations in response to oxidative stress.[18,28]

In patients treated daily with 14 mg TFN, average steady-state maximum TFN concentration (C_{max}) in plasma is 168 μM.[29] The half maximum concentration (IC_{50}) for interaction of TFN with human DHODH is 1 μM[30,31] and 50–100 μM is considered sufficient to inhibit protein tyrosine kinase *in vitro*.[29,31] Moreover, a study assessing the effect of TFN on eryptosis indicated that concentrations ranging from 3.7 to 37 μM TFN might compensate oxidative stress-mediated erythrocyte changes *in vitro*.[32] In rats, it has been shown that after one single injection of 10 μg/g TFN, approximately 2–4% of the blood concentration was found in the brain (~2.5–4.1 μM).[30] Although an extrapolation to the human reality is not exact, we could suppose in treated patients a TFN concentration within the nervous system of about 3–7 μM. Therefore, in our study, we investigated the effect of TFN on oxidative stress-induced mitochondrial alterations in murine root explants using three different TFN concentrations, 1 μM, 5 μM and 50 μM.

We show that TFN is able to prevent mitochondrial alterations induced by hydrogen peroxide (H_2O_2), suggesting that TFN has additional therapeutically relevant properties related to mitochondrial protection in axons.[31]

Materials and methods

Ethics

All experimental procedures were approved by the local authority on animal studies in Berlin (Landesamt für Gesundheit und Soziales Berlin; ID: T0002/10). Animal studies were performed in strict accordance with the European Communities Council Directive of 22 September 2010 (2010/63/EU).

Solutions and drugs

Explanted roots were bathed in artificial cerebrospinal fluid (aCSF) containing the following: solution I: 124 mM sodium chloride (NaCl), 1.25 mM sodium dihydrogen phosphate (NaH_2PO_4), 10.0 mM glucose, 1.80 mM magnesium sulphate ($MgSO_4$), 1.60 mM calcium chloride ($CaCl_2$), 3.00 mM potassium chloride (KCl);

solution II: 26.0 mM sodium bicarbonate ($NaHCO_3$). Solutions I and II were mixed immediately before use. Hydrogen peroxide (H_2O_2; 30% w/w in H_2O, with a stabilizer) and dimethyl sulfoxide (DMSO) were purchased from Sigma-Aldrich. To induce oxidative stress, explanted roots were incubated with 50 µM of H_2O_2 dissolved in aCSF for 30 min. TFN was applied at different concentrations along with H_2O_2 for 30 min. TFN was provided in powder form by the manufacturer (Sanofi Genzyme), which was dissolved in DMSO and stored at −20°C.

Preparation of ventral spinal roots

Ventral spinal roots were prepared as described in our previous work.[18,28,33] Briefly, C57BL/6 mice at least 3 weeks of age were anesthetized with isoflurane prior to cervical dislocation. After separating the connective tissue and exposing the dorsal side of the spinal cord, an initial sectioning was made at the thoracic level, which proceeded in a rostral to caudal direction until the last vertebrae. The spinal cord was lifted gently to expose the ventral roots, which were cut distal to the spinal cord but before the formation of the peripheral nerves. The explanted spinal cord with attached roots was transferred to aCSF saturated with carbogen (95% oxygen [O_2]; 5% carbon dioxide [CO_2]). Under a dissecting microscope, lumbar ventral roots of at least 0.8 cm were selected and separated from the spinal cord.

Labeling of mitochondria

All experimental incubations were conducted in a submerged incubation chamber (Brain Slice Keeper- BSK6-6; Scientific Systems Design Inc., Ontario, Canada), which allows for multiple treatment conditions and continuous carbogen perfusion of each submersion well. Transected ventral spinal roots were transferred to fresh aCSF containing 300 nM, MitoTracker CMTMRos orange (Life Technologies, Darmstadt, Germany) for 30 min and washed with aCSF.

Confocal microscopy

Explanted ventral roots were placed onto a glass coverslip and transferred to an imaging chamber filled with carbogenated aCSF. To prevent movement of the roots during imaging, a custom-built net was placed on the top of the roots.[28] For all imaging experiments, we used an inverted laser-scanning confocal microscope adapted for live cell imaging (LSM 710; Carl Zeiss, Jena, Germany). MitoTracker Orange was excited with a diode-pumped solid state (DPSS) laser at 561 nm. After finding the middle of the root, 3 × 60-sec videos (2 sec/frame) with a resolution of 512 × 512 pixels were acquired in three separate regions of interest (ROI) according to the following criteria: (a) there was a clearly defined node of Ranvier; (b) there were visibly labeled mitochondria; and (c) the areas were no closer than 0.2 cm from the ends of the roots. Typically, the first ROI was located at the middle of the root, whereas the following two were to the right and left of the middle region.

Analysis of mitochondrial dynamics

Mitochondrial morphology was assessed using an automated analysis function of the Volocity 6.3 software (Perkin Elmer, Rodgau, Germany). The first frame of every video was used for analysis. Shape factor, a measure of circularity ranging from 0 and 1 (closer to '0' was a longer mitochondrion, whereas '1' was a perfect circle), length (µm) and area of individual mitochondria were quantified for assessing change in mitochondrial morphology.

Mitochondrial transport was quantified in terms of the number of moving mitochondria, velocity, displacement, and track length of moving mitochondria. Displacement is the measure of shortest distance in µm, covered by a mitochondrion; it was measured as a straight line from the start to the end position during the 30 frames. Track length is the measure of real distance path longitude followed by the mitochondrion. Mitochondria were tracked manually using Volocity 6.3 software (Perkin Elmer, Rodgau, Germany). Any mitochondrion with a displacement of at least 1 µm was considered 'mobile'. Measurements from three ROI were averaged for each root.

In total, 15 different mice in 15 independent experiments were investigated. Depending on the quality of the explants, at least five independent roots and 15 ROI per culturing condition were included into the analysis (usually up to three different ROI per root). Specifically, 39 and 44 ROI were analyzed for the untreated group and H_2O_2 treated groups, respectively; 15–21 ROI were used to investigate treatments with H_2O_2 + TFN.

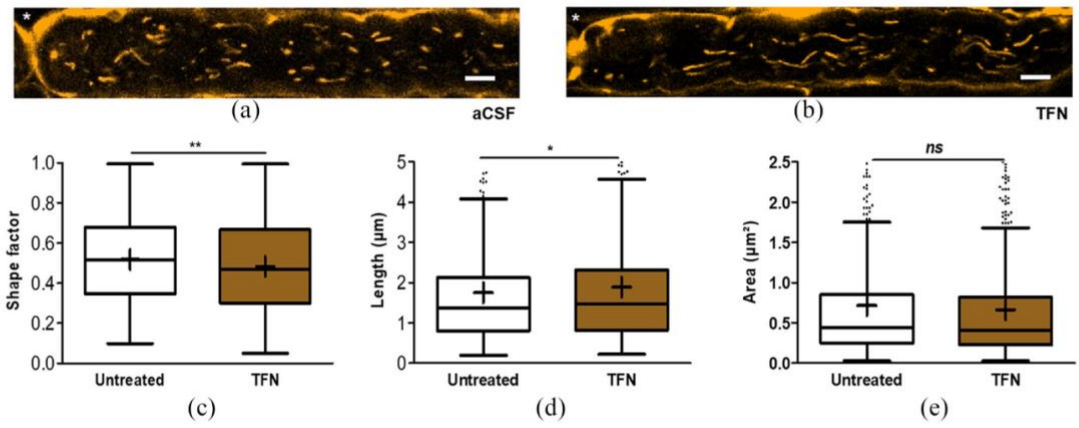

Figure 1. Teriflunomide (TFN) affected mitochondrial shape and length in untreated root explants.
(a) Representative confocal picture of the mitochondria within single axons in an untreated peripheral root explant and (b) treated with TFN. The node of Ranvier is located on the left side shown with an asterix (*). Scale bar: 5 µm. (c) Shape factor (circularity), (d) length and (e) area of mitochondria. The mitochondrial shape and length is significantly less round and longer after treating the axons with TFN (50 µM).
Graphs are shown in Tukey boxplots, where the central line denotes the median; the lower and upper boundaries denote the first and third quartile, and the whiskers denote the spread of the data.
Inside the box, '+' delineates the mean.
ns, not significant statistically.
$^{*}p < 0.05$, $^{**}p < 0.01$.

For the morphological investigations, the number of mitochondria in the selected ROI were 2586, 2860, 1306, 875 and 1550 for untreated, H_2O_2-treated, H_2O_2 + TFN (1 µM), H_2O_2 + TFN (5 µM) and H_2O_2 + TFN (50 µM), respectively. Analyses of motility included 201, 70, 83, 52 and 64 motile mitochondria for each of the above-mentioned groups.

For the comparison of untreated *versus* TFN-treated nerves, four independent experiments were performed. Analyses include 12 ROI per condition. In total, 568 and 672 individual mitochondria were analyzed, respectively.

Quantification of relative change in intracellular ROS

The fluorescence intensity of the MitoTracker Orange was quantified as a measure of intracellular ROS as described by Kweon *et al.*,[34] The images obtained from confocal microscopy were used for the quantification of mitochondrial fluorescence intensity using Image J software.

Statistical analysis and data representation

The data were analyzed with Prism 5.01 software (GraphPad, CA, USA). All datasets were subjected first to D'Agostino and Pearson omnibus K2 normality test for Gaussian distribution. All data fitting the criteria for a normal distribution were subsequently analyzed using a one-way analysis of variance (ANOVA) with Bonferroni's *post hoc* test. All data following a non-parametric distribution were analyzed using a Kruskal–Wallis test followed by a *post hoc* Dunn's multiple comparisons test. All data are given in mean ± SD.

Data are shown in Tukey box and whisker plots. The box and whisker plot shows simultaneously the minimum, first quartile, mean (+), median (dissecting line inside the box), third quartile, and maximum of the data set. Whiskers indicate variability outside the upper and lower quartiles. Outliers are plotted as individual dots that are in line with whiskers. The mean + SD values are given in the corresponding tables.

Results

TFN altered mitochondrial dynamics in peripheral root explants

We labelled peripheral root mitochondria with MitoTracker Orange and the explants were imaged for morphological investigation (Figure 1a, b). Then, the effects of TFN on mitochondrial morphology and transport in unmanipulated explanted roots were investigated. Explanted roots were

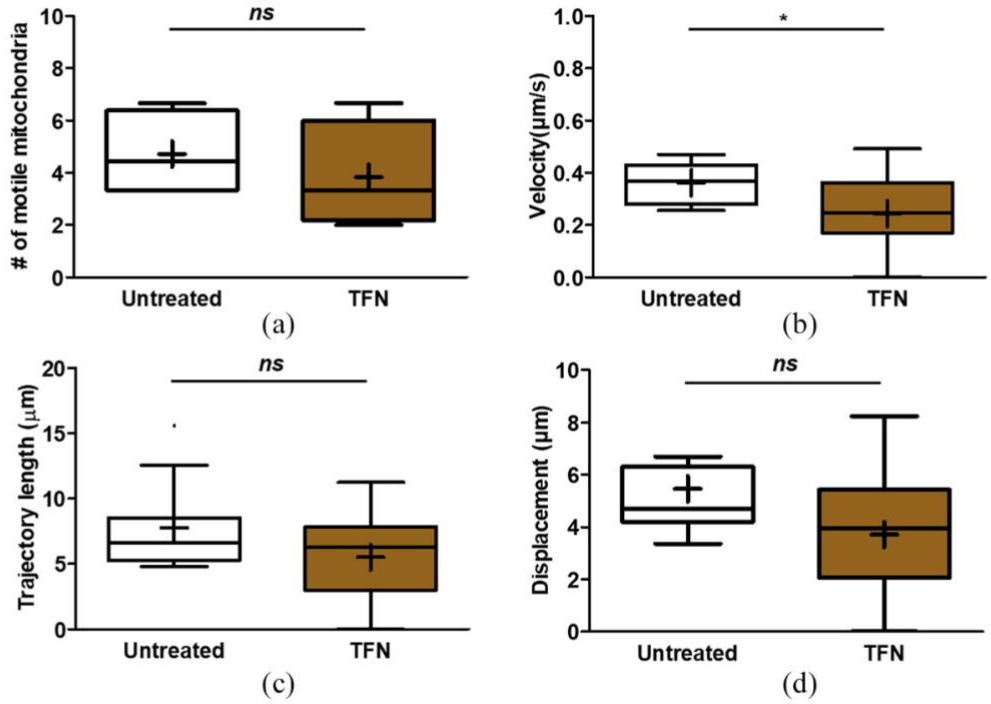

Figure 2. Teriflunomide (TFN) reduced mitochondrial velocity without influencing motile number, trajectory length and displacement of mitochondria in untreated root explants.
(a) Number of moving mitochondria per root during 1 min in untreated *versus* TFN-treated roots, (b) velocity of mitochondrial transport, (c) length of the mitochondrial trajectories and (d) displacement (final position minus initial position) of mitochondria.
Graphs are shown in Tukey boxplots, where the central line denotes the median; the lower and upper boundaries denote the first and third quartile, and the whiskers denote the spread of the data. Inside the box, '+' delineates the mean.
ns, not significant statistically.
*$p < 0.05$.

incubated in the presence or absence of 50 µM TFN. TFN treatment resulted in a statistically significant decrease in mitochondrial circularity (Figure 1c) and an increase in mitochondrial length (Figure 1d). There were no significant changes in mitochondrial area (Figure 1e) after TFN treatment compared with the untreated controls.

For mitochondrial motility, TFN did not significantly change the number of motile mitochondria (Figure 2a) as well as the distance covered by the mitochondria (Figure 2c, d). However, it induced a significant reduction of the mean velocity of mitochondrial transport (Figure 2b). Corresponding statistical information is summarized in Table 1.

TFN prevented oxidative stress-induced morphological changes in mitochondria

We previously reported that oxidative stress leads to substantial changes to both morphology and transport of axonal mitochondria.[18] Here, we investigated whether TFN, applied together with H_2O_2, would be able to prevent these effects. We treated the roots with 50 µM H_2O_2 (both groups containing the vehicle DMSO), and 3 different concentrations of TFN: 1, 5 and 50 µM in aCSF (Figure 3a–e). We analyzed a total of 39 untreated ROI, 44 ROI treated with H_2O_2, and 18, 15 and 21 ROI treated with 1 µM, 5 µM and 50 µM TFN in the presence of 50 µM H_2O_2, respectively, from 15 independent experiments. Consistent with our previous findings, we observed that treatment with 50 µM H_2O_2 induced an overall increase of mitochondrial circularity and a corresponding decrease in mitochondrial length and area. In particular, mitochondria were significantly more circular (Figure 3f), shorter (Figure 3g) and smaller (Figure 3h) than their untreated counterparts.

In the presence of 1 µM TFN, the shape factor of the mitochondria was reduced, that is,

Table 1. Summary of morphology and motility parameters of mitochondria in untreated and teriflunomide-treated peripheral root explants.

	n=	Shape factor	Length (μm)	Area (μm²)	No. of motile mitochondria	Velocity (μm/s)	Trajectory length (μm)	Displacement (μm)
Untreated	39	0.52 ± 0.21	1.75 ± 1.52	0.77 ± 0.86	4.72 ± 1.67	0.36 ± 0.07	7.77 ± 3.26	5.46 ± 2.39
TFN (50 μM)	12	0.48 ± 0.23	1.89 ± 1.67	0.66 ± 0.82	3.83 ± 2.06	0.24 ± 0.15	5.51 ± 3.49	3.71 ± 2.43
Mann–Whitney test		**	*	>0.1	>0.1	*	>0.1	>0.1

Values are shown as mean ± SD.
*$p < 0.05$; **$p < 0.01$

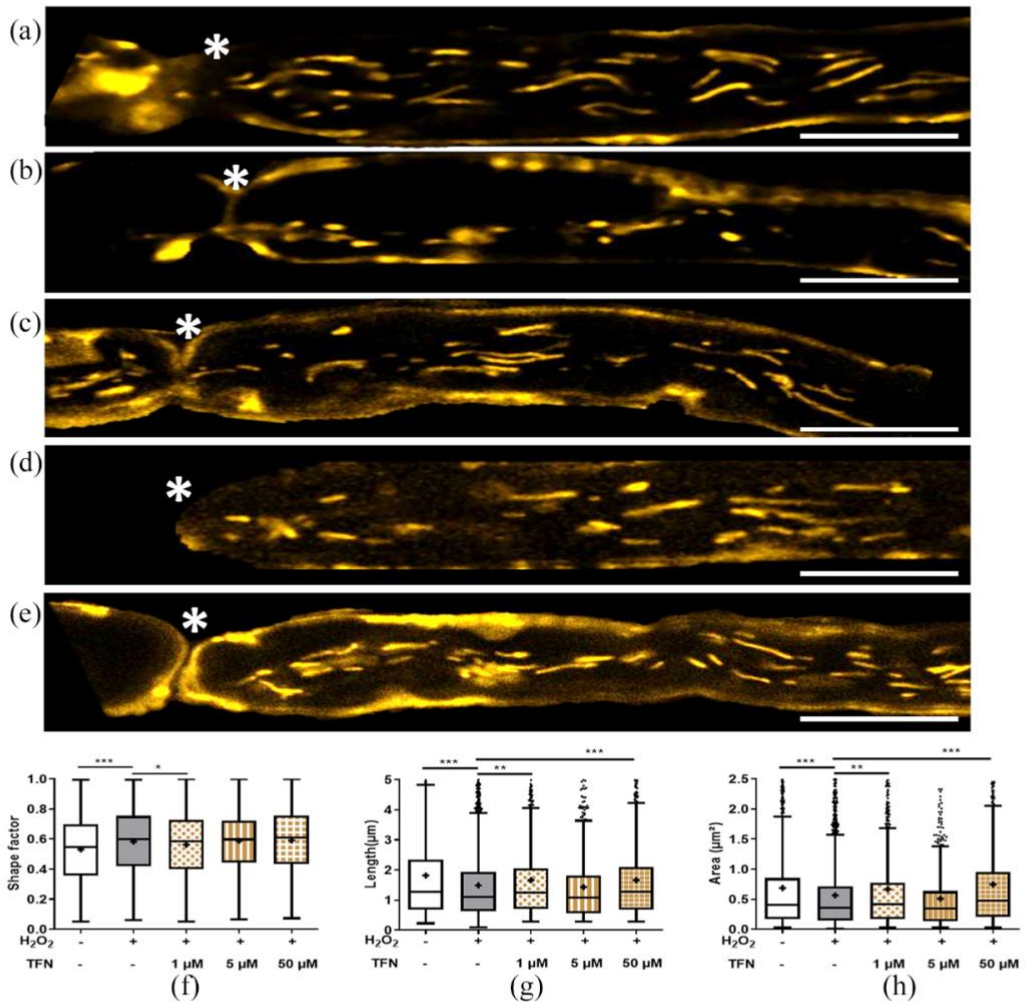

Figure 3. Mitochondrial morphology altered during oxidative stress with/out teriflunomide (TFN) treatment.
Representative image of mitochondria in (a) untreated, (b) hydrogen peroxide (H_2O_2)-treated, and (c, d, and e) H_2O_2-TFN-treated, where TFN was 1, 5 and 50 μM, respectively, in murine peripheral root explants. Scale bar: 10 μm. (f) Change in mitochondrial shape factor, (g) length, and (h) area of mitochondria in the presence of H_2O_2 with or without TFN.
Graphs are shown in Tukey boxplots, where the central line denotes the median; the lower and upper boundaries denote the 1st and 3rd quartile, and the whiskers denote the spread of the data. Inside the box, '+' delineates the mean.
*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

Table 2. Summary of shape factor, length and area of mitochondria under H_2O_2 treatment alone, and with 50 µM H_2O_2 in the presence of 1 µM, 5 µM and 50 µM teriflunomide.

	n=	Shape factor	KW test	Length (µm)	KW test	Area (µm²)	KW test
Untreated	39	0.53 ± 0.22	***	1.82 ± 1.79	***	0.69 ± 0.92	***
H_2O_2-treated	44	0.58 ± 0.21		1.49 ± 1.29		0.57 ± 0.65	
H_2O_2 + TFN (1 µM)	18	0.57 ± 0.21	*	1.66 ± 1.50	**	0.67 + 0.85	**
H_2O_2 + TFN (5 µM)	15	0.59 ± 0.19	>0.1	1.43 ± 1.29	>0.1	0.51 + 0.69	>0.1
H_2O_2 + TFN (50 µM)	21	0.59 ± 0.21	>0.1	1.66 ± 1.42	***	0.75 + 0.93	***

KW, Kruskal–Wallis; H_2O_2, hydrogen peroxide.
Values are shown as mean ± SD.
*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

mitochondria became elongated or rod-shaped (Figure 3f). In contrast, no effects were observed at higher concentration of TFN. Moreover, the lowest and highest TFN concentrations (1 µM and 50 µM) induced a significant increase in mitochondrial length (Figure 3g) and area (Figure 3h), in comparison with the mitochondria exposed to H_2O_2 alone. Paradoxically, treatment with 5 µM TFN with 50 µM H_2O_2, showed no statistically significant effect on H_2O_2-induced morphological alterations (Figure 3f–h) (0.59 ± 0.19 shape factor, 1.43 ± 1.29 µm length, and 0.51 ± 0.69 µm² area; Kruskal-Wallis test followed by Dunn's *post hoc* test $p > 0.1$ in all cases).

Corresponding statistical information is summarized in Table 2.

TFN prevented oxidative stress-induced changes in mitochondrial motility

To investigate TFN effects on mitochondrial motility, roots were treated either with aCSF, 50 µM H_2O_2 (both groups containing DMSO) or 50 µM H_2O_2 in the presence of three different concentrations of TFN: 1, 5 and 50 µM (Figure 4a–e). We observed that H_2O_2 treatment led to an overall decrease in the number of motile mitochondria (Figure 4f). In addition, the moving mitochondria had lower mean velocity (Figure 4g), trajectory length (Figure 4h), and displacement (Figure 4i) than the untreated mitochondria.

Again, the lowest and highest TFN concentration (1 µM and 50 µM) restored the motility-related parameters to control levels, except for the mitochondrial velocity with 50 µM TFN, when compared with the mitochondria exposed to H_2O_2 alone; for the number of moving mitochondria (Figure 4f), velocity (Figure 4g), trajectory (Figure 4h), and displacement (Figure 4i). In contrast, 5 µM TFN had no effect. Corresponding statistical information is summarized in Table 3.

TFN prevented change in mitochondrial oxidation potential in peripheral nerve explants during oxidative stress

MitoTracker Orange CMTMRos is a reduced, non-fluorescent dye that fluoresces on oxidation. Thus, in conditions of high oxidative stress, mitochondria acquire higher fluorescence intensity.[34] We observed that fluorescence intensity was higher in H_2O_2-treated roots compared with untreated controls (Figure 5). In the presence of 1 µM TFN, the fluorescence intensity in the mitochondria was reduced, approaching the values of the untreated axons (Table 4), suggesting that the H_2O_2-mediated increase in the oxidation potential could be prevented by TFN. In contrast, no effect was observed at 5 or 50 µM TFN. Corresponding statistical information is summarized in Table 4.

Discussion

While current treatments for MS focus on reducing inflammation *via* modulation of the

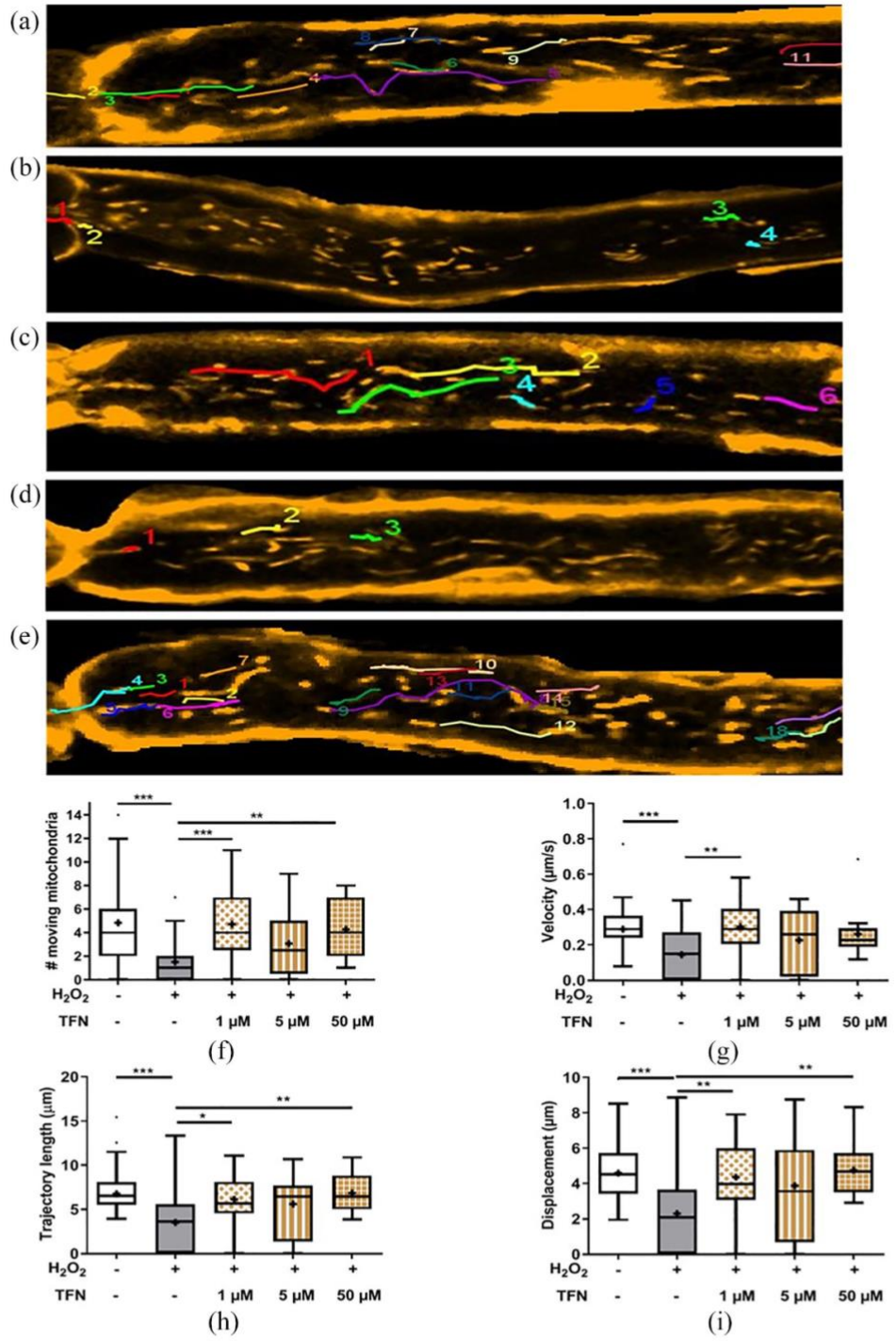

Figure 4. Mitochondrial motility altered during oxidative stress with or without teriflunomide (TFN) treatment. A representative image of mitochondrial tracking in (a) untreated, (b) hydrogen peroxide (H_2O_2)-treated, and (c, d, and e) H_2O_2-TFN treated where TFN was 1, 5 and 50 μM, respectively, in murine peripheral root explants. (f) Number, (g) velocity, (h) trajectory, and (i) displacement of mitochondria.
Graphs are shown in Tukey boxplots, where the central line denotes the median; the lower and upper boundaries denote the first and third quartile and the whiskers denote the spread of the data. Inside the box, '+' delineates the mean.
*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

Table 3. Summary of mitochondrial motility, velocity, displacement and trajectory length under H_2O_2 treatment alone, and along with different concentrations of teriflunomide.

	n=	# motile mitochondria (axon)	KW test	Velocity (μm/s)	KW test	Trajectory length (μm)	KW test	Displacement (μm)	KW test
Untreated	39	4.85 ± 3.72 (39)	***	0.29 ± 0.14	***	6.77 ± 3.13	***	4.59 ± 2.16	***
H_2O_2-treated	44	1.52 ± 1.71 (39)		0.15 ± 0.14		3.53 ± 3.67		2.30 ± 2.60	
H_2O_2 + TFN (1 μM)	18	4.71 ± 3.08 (21)	***	0.30 ± 0.13	**	6.13 ± 2.59	*	4.37 ± 1.97	**
H_2O_2 + TFN (5 μM)	15	3.08 ± 2.75 (12)	>0.1	0.23 ± 0.17	>0.1	5.61 ± 3.69	>0.1	3.87 ± 2.96	>0.1
H_2O_2 + TFN (50 μM)	21	4.27 ± 2.34 (15)	**	0.26 ± 0.13	>0.1	6.84 ± 2.15	**	4.75 ± 1.45	**

KW, Kruskal–Wallis; H_2O_2, hydrogen peroxide.
Values are shown as mean ± SD.
*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

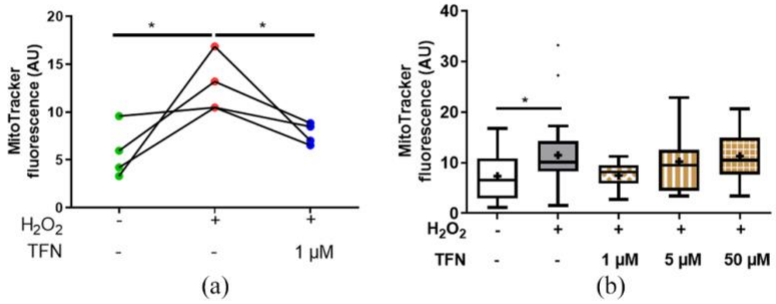

Figure 5. Teriflunomide (TFN) at 1 μM altered the oxidation potential of mitochondria, reducing oxidative MitoTracker Orange fluorescence intensity Fluorescence intensity of MitoTracker Orange staining depicting significant reduction of oxidation potential of 1 μM TFN. (b) MitoTracker Orange fluorescence intensity at 1, 5 and 50 μM TFN treatment during oxidative stress in comparison with untreated and H_2O_2-treated roots.
*Statistical significance with *p* value < 0.05.

Table 4. Summary of mean fluorescence intensities of mitochondria.

	n=	MitoTracker fluorescence (AU)	Mann–Whitney test
Untreated	39	7.35 ± 4.76	*
H_2O_2-treated	44	11.49 ± 6.44	
H_2O_2 + TFN (1 μM)	18	7.48 ± 2.75	*
H_2O_2 + TFN (5 μM)	15	10.22 ± 6.45	>0.1
H_2O_2 + TFN (50 μM)	21	11.27 ± 4.79	>0.1

AU, arbitrary units.
Values are shown as mean ± SD.
*$p < 0.05$.

immune system,[25,35] there is a general lack of treatment targeting inflammation-promoted neurodegeneration,[36,37] which is an integral component of disability progression.[37,38] As early mitochondrial alterations are reported in inflammatory neurodegenerative diseases,[8,11–16] the maintenance of mitochondrial integrity could be a key goal to achieve neuronal protection during neuroinflammation.[39] Here, we hypothesized that TFN, due to its ability to inhibit DHODH,[40] an enzyme functionally linked with complex III activity of the mitochondrial respiratory chain,[41] may influence mitochondrial stability in the context of oxidative stress. To test our hypothesis, we used our previously established model of murine explanted ventral roots, in which the morphology and the transport of mitochondria can be analyzed within peripheral axons.[18,28]

After excluding any relevant effect of DMSO (the dilution vehicle), in both untreated and H_2O_2-treated mitochondria (Supplemental Figures 1–2), we investigated the effect of TFN treatment in unmanipulated or oxidative stress-exposed spinal root explants.

Interestingly, in non-stressed roots, TFN seems to promote mitochondrial fusion, induce mitochondrial elongation (Figure 1d, e), and reduce mitochondrial velocity (Figure 2b). Mitochondrial fusion is important for the formation of mitochondrial networking that assists in reshuffling and redistributing the mitochondrial content.[42,43] Thus, the inhibition of DHODH and subsequent effects on complex III of the electron transport chain (ETC) and respiration[41] may promote mitochondrial fusion as an attempt to redistribute the electron transport complexes that are still capable of maintaining the proton gradient and synthesizing adenosine triphosphate (ATP).

In contrast, during oxidative stress, we observed a reduction in mitochondrial length and size, which is indicative of fragmentation/fission of mitochondria that might undergo mitophagy. Mitochondrial fission has also been proposed to increase the number of mitochondria and their cellular distribution in order to meet the increasing energy demands of the cell.[44,45] Although mitochondrial fission is extensively discussed in terms of mitophagy as well as apoptosis,[43–46] intensive mitochondrial fission may translate into mitochondrial failure and the strategy to optimize mitochondrial functionality before undergoing apoptosis. It also serves to get rid of damaged, irreparable mitochondrial parts.[45,47] Importantly, in the presence of 1 μM and 50 μM TFN, reduction in mitochondrial length and area due to oxidative insult could be prevented (Figure 3g, h). Intensive fragmentation during oxidative stress could not be prevented with 5 μM TFN.

Further, consistent with previous findings, we observed a reduced mitochondrial motility during oxidative stress,[14,18] that is, reduced trajectories and transport velocity. The impairment of mitochondrial transport was preserved with TFN treatment. In axons, around 10–30% of mitochondria are motile, while more than 70% remain stationary.[48] This motile and stationary pool of mitochondria is dependent on the current energy demands of the cell.[49,50] In addition, disrupted motility could lead to impairment of mitochondrial fusion.[46,49,51] Thus, TFN may promote fusion by influencing the motility. On the other hand, it has been proposed that inhibition of DHODH by TFN may reduce the total amount of ROS in the cell.[52] Thus, TFN-mediated ROS reduction may also lead indirectly to an increased motility of stressed mitochondria.

Along this line, to assess the effects of TFN on ROS in our system, we monitored the fluorescence intensity of MitoTracker Orange CMTMRos (see Methods).[34] As expected, fluorescence intensity of CMTMRos significantly increased with H_2O_2 treatment, while inhibition of DHODH with 1 μM TFN reduced the ROS level (Figure 5). As complex III of the ETC is considered one of the major contributors to ROS formation, its compromised activity in the presence of TFN might reduce ROS production in mitochondria in peripheral spinal root explants. 5 μM and 50 μM TFN could not effectively reduce ROS, which might be attributed to the inhibition at higher concentrations of additional signaling pathways including tyrosine kinases.[53]

On the other hand, the intermediate dose of 5 μM TFN showed no effects on H_2O_2-induced shape or motility changes. Why, in our experimental setup a dose effect is missing, remains uncertain. The high variability of our data, which is intrinsic to the nature of mitochondrial dynamics and reflects the heterogeneity of the mitochondrial population in both physiological and diseased conditions, could have contributed to mask a true dose effect. To minimize this problem, several experiments

with large amounts of mitochondria were analyzed (see Methods section). Moreover, depending on its concentration, TFN may function by a different mode of action. While low TFN concentrations are effective in inhibiting DHODH (1–1.5 μM), concentrations needed to achieve DHODH-independent effects such as inhibition of protein tyrosine kinase or cyclooxygenase-2 are much higher (50–200 μM).[29] However, little is known about the mode of action of intermediate concentrations. One could speculate that in our model, TFN at 5 μM may achieve partially known or yet undefined DHODH-independent effects that rather counterbalance the beneficial effects observed at 1 μM, while at 50 μM DHODH-dependent and independent mechanisms may synergize against dysfunctions observed under oxidative stress. Future experiments using long-living explants are needed to evaluate to what extent TFN effects at different concentration are DHODH-dependent and thus reversible.

Our previous data on root explants demonstrated that mitochondrial alterations caused by oxidative stress precede axonal damage.[18] Now, we show that these alterations could be pharmacologically reversed *in vitro* by TFN. Targeting dysfunction of axonal mitochondria should become one of the key goals in drug development not only for MS but also for other classic neurodegenerative disorders such as Parkinson's or Alzheimer diseases.[54] In this line, we showed in the animal model of MS a protective effect of epigallocatechin-3-gallate (EGCG),[55,56] a polyphenol, that among others, inhibits the formation of ROS and protects neurons.[57,58] Also dimethyl fumarate, used to treat MS, prevents oxidative stress-related mitochondrial dysfunction, apoptosis and autophagy in murine oligodendrocytes *in vitro*.[59] Importantly, endogenous substances currently being investigated in MS may be exploited as therapeutics due their mitoprotective capacities, such as high dose biotin,[60] vitamin D[61] or octadecaneuropeptide, a neurotrophic peptide produced principally by astrocytes, which is able to counteract oxidative stress-induced alterations.[62]

In summary, our present findings suggest a protective effect of TFN on axonal mitochondria exposed to oxidative stress. Investigations expanding on these findings are needed to determine whether mitochondrial protection at the axonal level can be translated into protection of axons and neurons.

Conflict of interest

The authors declare that there is no conflict of interest.

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Supplemental material

Supplemental material for this article is available online.

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Curriculum Vitae

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‚Persevere calmly and sincerely ‘

To my fellow PhD candidates and all the readers of this thesis, persevere through your passion for science. Renouncing it would be easy but genuinely following it is what makes you deserve this degree. This thesis is dedicated to all the PhD fellows who are sincerely working towards their goal, who are stumbling but have not given up and living this process of metamorphosis.

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