Reviewer comments and authors' responses

Cytotoxicity of mitochondrial Complex I inhibitor rotenone: a complex interplay of cell death pathways

From MitoFit Preprint version 1 to version 2

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Manuscript submitted 2022-04-14, 2022-08-08 (revision)

Manuscript accepted 2022-08-16

Reviewer 1

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Manuscript reviewed 2022-05-02

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*Only major points from review and responses included.

Reviewer 1

While the results are quite interesting, they are not essentially new since the consequences of rotenone treatment on cell viability, metabolism, and redox balance were known for years (https://pubmed.ncbi.nlm.nih.gov/10347173/; https://www.jbc.org/article/S0021-9258(18)81269-8/pdf). Importantly, seminal literature on the cytotoxic effects of rotenone and imbalanced cellular iron homeostasis are missing and some key references include the following: https://pubmed.ncbi.nlm.nih.gov/26385697/;

https://pubmed.ncbi.nlm.nih.gov/28502703). Indeed, ferrostatin was even shown to exhibit cytoprotective effects against rotenone treatment in this very same cell line (https://pubmed.ncbi.nlm.nih.gov/26385697/), which emphasizes the need of authors to properly address the available literature not only to support their hypothesis and conclusions but also to provide a broader background for the readers.

Authors

We thank the reviewer for constructive criticisms. Indeed, the Introduction was short and incomplete. In the revised version we have elaborated it sufficiently to provide a broader perspective and also to point out the knowledge-gaps. We have cited and discussed not only the references recommended by the reviewer, but also additional relevant references. Most importantly, we have pointed out that despite substantial data cited in the literature on rotenone-induced cell death, mitochondrial metabolism and redox imbalance, the mechanisms of rotenone induced cytotoxicity remains debatable which justifies further work on it.

Reviewer 1

In the same line, the results shown in figure 1 are similar to a previously reported work (https://pubmed.ncbi.nlm.nih.gov/26385697/) which shows that ferrostatin reverts the cytotoxic effects of rotenone on SH-SY5Y cells, underscoring the need to properly address the available literature. In this regard, considering the available literature, and the data provided in their manuscript, the authors should propose a mechanistic explanation for the cellular and metabolic consequences of rotenone treatment in order to provide the readers a broader picture of the observed events.

Authors

We thank the reviewer for the meticulous review. We have cited and discussed the paper (Kabiraj et al., 2015 DOI 10.1007/s10930-015-9629-7) as recommended by the reviewer. Further, we have provided a mechanistic interpretation of rotenone toxicity in the Discussion section. Regarding the Fig 1 of our paper and the study of Kabiraj et al., 2015 doi.10.1007/s10930-015-9629-7, we would like to point out that Kabiraj et al., 2015 first checked the cytotoxicity of ferrostatin-1 on SH-SY5Y cells, and then showed how it prevented rotenone-induced morphological changes, ROS production, activation of a mediator (PARP-1) of apoptosis, α-synuclein aggregation etc. in SH-SY5Y cells. The authors did not show by a typical cell death assay that ferrostatin-1 prevented rotenone induced cell death as shown in Fig.1 of our manuscript. Moreover, other measurement parameters of our current study were different.

Reviewer 1

Although malondialdehyde and DCFDA are extensively used elsewhere as proxies of redox imbalance, they are not reliable measures for this purpose. Particularly, there are many technical issues related to the use of fluorescent probes that must be considered. For example, it is known that DCFDA is a probe that is not specific for "ROS" and therefore this does not mean that increased DCFDA fluorescence means increased "ROS" levels but, instead, increased reactive (oxygen, nitrogen, and others) species or even iron/heme levels (please refer to https://pubmed.ncbi.nlm.nih.gov/20331437/ and https://pubmed.ncbi.nlm.nih.gov/29739855/) to properly balance their conclusions.

Also, the authors might consider improve the detection of redox imbalance by using alternative approaches including the assessment of HPLC-specific detection of superoxide by DHE fluorescence (https://www.ncbi.nlm.nih.gov/pubmed/16971501),MitoB (https://pubmed.ncbi.nlm.nih.gov/23726990/), and other methods. Despite the assessment of GSH content is welcomed, the authors should balance that observed changes in GSH could also reflects altered biosynthesis of glutathione in a way that both reduced and oxidized pools could be affected if only one of the redox pair is measured. In addition, I strongly suggest the authors to consider a thorough revision of the manuscript in order to properly balance their conclusions based on the assessment of "oxidative stress" by the use of these tools. Finally, I suggest the authors to use the general term "oxidants" instead of ROS throughout the manuscript considering the issue pointed out above.

Authors

We thank the reviewer for raising these interesting issues, and we would like to address them. We admit that H_2DCFDA is not a specific probe for superoxide radical, hydrogen peroxide or hydroxyl radical (the typical ROS members). It does react with lipid hydroperoxides, peroxy radicals, peroxynitrite radicals and others. However, 'ferroptosis' is defined by the accumulation of many different radicals like typical ROS, lipid hydroperoxides, lipid derived peroxy radicals as well as end products of lipid peroxidation. Thus, it is not important to identify a specific oxyradical like the superoxide radical to establish the occurrence of 'ferroptosis' in rotenone-toxicity; in fact H_2DCFDA assay will be a good assay in this case because it reacts with many types of oxyradicals. The suggestion of the reviewer for using Mito B or other probes could be used in future work when exploring the details of the initiation of ferroptosis by rotenone. However, we agree with the other comment that H_2DCFDA assay is dependent on intracellular heme, cytochrome c etc, which is a limitation of the assay. We have discussed all these issues adequately in the revised version in the methodology with citations including some citations recommended by the reviewer. Regarding replacing the term 'ROS' by 'oxidants', I feel this is not necessary, though technically it is sound. In fact, the term ROS is not used in a 'restrictive sense' now-a-days, and instead it is used as an 'umbrella term' to include superoxide radicals, H_2O_2, hydroxyl radical, lipid hydroperoxides, peroxy radicals, protein derived radicals, peroxynitrite radicals and many others. In fact, one of the references that the reviewer has recommended for citation has defined ROS precisely in that way [https://pubmed.ncbi.nlm.nih.gov/29739855]. We have also used the term ROS in that sense and explicitly stated this in the revised version while mentioning about H_2DCFDA assay.

We agree that MDA is a 'proxy' for redox imbalance, but again in 'ferroptosis' the accumulation of MDA, one of the end-products of lipid peroxidation, has been documented in multiple studies. MDA being a very active compound can react with proteins, and signalling effects of MDA are being reported.

We agree with the suggestion that both GSH and GSSG should have been measured, and this will be done in our future studies on rotenone toxicity. However, analyzing with other parameters like increased ROS and MDA levels, GSH depletion has been taken as indicative of oxidative stress; GSH depletion in our study is also recovered by Fer-1 and Lip-1 which are known to prevent oxidative stress.

Reviewer 1

The authors should improve the description of the methodologies used in their work as some details were not provided. For example, quantification of ATP content was carried out by using the "luciferase-based assays" but it is not known whether this applied only for the mitochondrial synthesized ATP by OXPHOS or by other pathways (glycolysis, Creatine Kinase). In the same line, assessment of mitochondrial membrane potential by TMRE fluorescence was not properly detailed including probe concentration, excitation/emission as well as the inclusion of critical controls such as proton ionophore (FCCP, DNP) to ascertain that TMRE fluorescence is truly derived from mitochondria.

Authors

Agreed. Necessary changes have been made in the revised version.

Reviewer 1

The discussion section does not provide a clear explanation for the effects observed in the manuscript. For example, the failure of ferrostatin-1 and liproxstatin-1 to revert Complex I-III activity was not properly discussed and balanced. In this regard, the authors should consider respirometry experiments in permeabilized cells to address the possibility that viability and redox balance markers are preserved as a consequence of a metabolic rewiring induced by ferrostatin-1 and liproxstatin-1 to compensate for reduced Complex I activity. This could imply that respiratory rates maintained by Complex I-substrates would be reduced upon rotenone and ferrostatin-1/liproxstatin-1 treatment, but not by using alternative substrates, for example succinate, glycerol phosphate, palmitoylcarnitine and so on, which should be increased. Indeed, this could nicely explain the maintenance of mitochondrial membrane potential and ATP levels under rotenone+ferrostatin-1/liproxstatin-1 treatment (considering the technical limitations associated to these measures as I pointed out above). However, even in the absence of these experiments, the authors might consider substantially improving the discussion section to properly address this and other key points raised in the manuscript.

Authors

We are grateful for this constructive suggestion which helped us to reanalyze our data with the help of the existing literature as well as with a just-published paper from our research group on rotenone-toxicity in a different context. We have made substantial changes in the Discussion, suggesting the involvement of two different mechanisms in rotenone toxicity: a simple bioenergetic failure of Complex I inhibition in some situations and a predominantly oxidative death with involvement of mitochondrial permeability transition pore (mPTP) in other cases (doi.org/10.1016/j.ejphar.2022.175129). The possible effects of dose and duration of rotenone exposure and the nature of the cells on rotenone-toxicity have been mentioned. Also we have suggested why Fer-1 and Lip-1 could prevent rotenone mediated cell death without recovering Complex I activity.

Regarding the suggestions of the reviewer about metabolic re-programming caused by Fer-1 and Lip-1 in rotenone treated cells, we believe that this would be an interesting study; as of now, however, we do not have data to either prove or disprove it. However, we have used this interesting idea in a somewhat different context in the Discussion section.

Reviewer 2

Trypan Blue assay: What was the minimum number of cells counted per analysis?

Authors

The automated counter provides the number of cells / µL and the percentages of live and dead cells. However, we routinely cross-checked the Trypan blue assay by manual counting by two independent observers; each person counted 250 to 300 cells separately for each assay. This is now mentioned in the revised version.

Reviewer 2

The discussion needs more detail on:

- Comment on the use of undifferentiated SHSY5Y versus differentiated cells. Is there any data on ferroptosis on differentiated cells that can be considered in the discussion?

- Is ferroptosis potentially linked to the duration/concentration of treatment? Please, discuss the potential role of the length of incubation (48h) versus shorter treatments (24 hours for example) and higher concentration of Rotenone in inducing cell death processes. Would a higher dose and shorter treatment, leading to the similar % of cell death, be inducing ferroptosis and/or other cell death mechanisms?

- There is evidence that Rotenone may have Complex I independent toxicity potential. Please discuss the potential role of this characteristic in the induction of ferroptosis.

Authors

We thank the reviewer for very constructive and useful suggestions which have made our manuscript stronger. The possible effects of dose and duration of rotenone treatment, the nature of the cells including the state of differentiation on the mechanisms of cytotoxicity of rotenone have been mentioned in the Discussion from a survey of the existing literature. However, more elaborate studies are necessary to establish these effects; we will explore this aspect in our future work. Further, we have discussed some apparently Complex I – independent toxicity of rotenone such as disruption of microtubules, activation of JNK / p38 etc.

Reviewer 3

You showed that 48-h rotenone treatment inhibits Complex I-III activity by 50 %. Is there any information available in the literature how much the Complex I-III is inhibited in Parkinson's disease (or in animal models of Parkinson's disease)?

Authors

In the original paper first describing Complex I deficiency in post-mortem PD brain, 31% inhibition of Complex I and 39% of Complex I-III were reported. In rotenone-induced PD animal models, reported values are variable; some did not find any inhibition, others reported 30 to 40% inhibition and still others even more (around 80%). These variations are because of different strains of rats used, different doses, duration and administration procedures of rotenone (oral, intravenous or intraperitoneal or intracerebroventricular) and different assay procedures. Since cell culture based results

cannot be extrapolated directly at the quantitative level with either post-mortem or animal model studies, we did not mention all these in the manuscript.

Reviewer 3

How ferrostatin-1 and liproxstatin-1 exert their ferroptosis-specific antioxidant effect? What is the mechanism of action? Why it is considered that these antioxidants are specific to ferroptosis? Please add it to the introduction.

Authors

Ferroptosis apparently depends on the accumulation lipid hydroperoxides, lipid derived oxyradicals and other lipid peroxidation intermediates. Thus, lipophilic antioxidants like ferrostatin-1 and liprotaxin-1 are very good inhibitors of ferroptosis. However, other lipophilic antioxidants like alpha-tocopherol and butylated hydroxytoluene are also potent inhibitors of ferroptosis. Thus, ferrostatin-1 and liproxstatin-1 are not specific inhibitors of ferroptosis; these are novel inhibitors of ferroptosis identified by high throughput screening procedures. We have, therefore, rephrased some sentences and added these statements in the Introduction as suggested by the reviewer.

Reviewer 3

Do you know any publication describing the positive effect of the antioxidants ferrostatin-1 and liproxstatin-1 in animal models of Parkinson's disease (or any other disease models accompanied with ferroptosis) given as food supplement?

Authors

Ferrostatin-1 and liproxstatin-1 are being explored as neuroprotective compounds in animal models of traumatic brain injury and ischemic stroke. However, we have not discussed this in our manuscript.