Heliyon 7 (2021) e06028

Contents lists available at ScienceDirect

Heliyon

journal homepage: www.cell.com/heliyon

Research article

Increase of complex I and reduction of complex II mitochondrial activity are possible adaptive effects provoked by fluoride exposure

Heloisa Aparecida Barbosa Silva Pereira [a], Tamara Teodoro Araújo [a], Aline Dionizio [a], Juliana Sanches Trevizol [a], Fabrício Soares Pereira [a], Flávia Godoy Iano [a], Valdecir Faria Ximenes [b], Marília Afonso Rabelo Buzalaf [a,*]

[a] Department of Biological Sciences, Bauru Dental School, University of São Paulo, Al. Octávio Pinheiro Brisolla, 9-75, 17012-901, Bauru, São Paulo, Brazil
[b] Department of Chemistry, Faculty of Sciences, São Paulo State University (UNESP), P. O. Box 473, 17033-36, Bauru, São Paulo, Brazil

ARTICLE INFO

Keywords:
Liver
Fluoride
Oxidative stress
Mitochondria
Mitochondrial activity
Chronic exposure

ABSTRACT

Fluoride (F) can induce changes in the expression of several liver proteins, most of them localized in the mitochondria and its effect is dose- and time-dependent. This study analyzed the effect of distinct F concentrations and exposure periods on the mitochondrial activity of complex I-III and II-III in the liver. Thirty-six 21-day-old male Wistar rats were divided into 2 groups (n = 18) according to the duration of the treatment (20 or 60 days). They were subdivided into 3 subgroups (n = 6) according to the concentration of F (0 mg/L, 15 mg/L or 50 mg/L). After the experimental periods, the animals were anesthetized, liver mitochondria were isolated and stored for activity analyses. The determination of complexes II-III and I-III was based on the reduction of cytochrome c^{3+} to cytochrome c^{2+} performed spectrophotometrically. Bioinformatics analyses were performed using data from a previous study (Pereira et al., 2018). The mitochondrial complex I-III was significantly activated in the groups treated with 50 mgF/L for 20 days and 15 mgF/L for 60 days. The complex II-III was significantly reduced in the group treated with the higher F dose for 60 days. The networks indicated more changes in mitochondrial proteins in the group treated with the higher dose for 20 days; the reduction is probably linked to the activation of the complex I-III. The reduction in the complex II-III upon exposure to the higher F dose in the long term might be part of an adaptative mechanism of the body to counteract the deleterious effects of this ion on the energy metabolism.

1. Introduction

Fluoride (F) is important in caries prevention when added to the drinking water (McDonagh et al., 2000) and dentifrice (Bratthall et al., 1996). However, excessive F ingestion can provoke deleterious effects in soft tissues, such as liver, muscle, kidney and heart, which has been extensively documented in animal studies (Kobayashi et al., 2009; Pereira et al., 2013; Lima Leite et al., 2014; Lobo et al., 2015; Pereira et al., 2018; Araujo et al., 2019).

High levels of F can also impair the antioxidant defense of rodents (He and Chen 2006; Iano et al., 2014). Some studies suggest that these effects depend on the dose of F administered and period of exposure (Dabrowska, Balunowska et al., 2006; Dabrowska, Letko et al., 2006; Pereira et al. 2016, 2018; Araujo et al., 2019). Mitochondria are one of the major producers of intracellular reactive oxygen species (ROS). Overproduction can indicate several changes, in addition to causing irreversible metabolic damage, such as the activation of intrinsic or extrinsic apoptotic pathways (Elmore 2007; Starkov 2008; Ni et al., 2020). The consumption of F seems to be related to alterations in mitochondrial function (Pereira et al., 2013; Lima Leite et al., 2014; Lobo et al., 2015; Sun et al., 2016; Pereira et al., 2018; Araujo et al., 2019). The respiratory components present in the inner membrane of mitochondria have critical role in oxidative stress, apoptosis and cell proliferation (Brenner and Kroemer 2000; Wang 2001; Rustin 2002). In addition, mitochondria can modulate calcium signaling (Babcock and Hille 1998; Rizzuto et al., 2000), which is a universal secondary messenger. Assessing mitochondrial activity is an extremely important tool for verifying changes in the mitochondrial pattern. Reduction on mitochondrial activity has been reported in osteoblastic cells upon chronic administration of F (Fina et al., 2014). Since liver plays a key role in detoxification of oxidative damage (Li et al., 2015) and disorders in this organ compromise all biological systems, isolation of mitochondria from liver is an important strategy in toxicology studies (Palmeira et al., 1994). Such disorders comprise changes in metabolic pathways, especially involving energy metabolism.

* Corresponding author.
E-mail address: mbuzalaf@fob.usp.br (M.A.R. Buzalaf).

https://doi.org/10.1016/j.heliyon.2021.e06028
Received 18 August 2020; Received in revised form 30 November 2020; Accepted 14 January 2021

We showed previously that upon exposure to F the proteins most affected in liver are the ones involved in metabolism and energy (Pereira et al., 2013; Lobo et al., 2015; Pereira et al., 2018). Alterations in energy metabolism are linked to mitochondrial dysfunction that in turn is associated to oxidative stress, calcium homeostasis and apoptosis (Araujo et al., 2019). Recent studies of our group have shown that these alterations induced by F are dose- and time-dependent (Pereira et al., 2018; Araujo et al., 2019). Thus, the present study investigated the effect of F in the mitochondrial activity in the liver of rats chronically exposed to water containing different F concentrations, for two experimental periods. To better characterize the underlying mechanisms, bioinformatics analyses involving alterations in mitochondrial proteins were also conducted, employing proteomic data obtained in a previous study (Pereira et al., 2018).

2. Material and methods

2.1. Animals and treatment

All experimental protocols were approved by the Ethics Committee for Animal Experiments of Bauru School of Dentistry, University of São Paulo (#037/2011).

Male 3-week-old weanling *Wistar* rats were randomly allocated into two groups (n = 18/group), in accordance with the periods of treatment (20 or 60 days). Each group was then divided into 3 subgroups (n = 6/subgroup), according to the F concentrations (as sodium fluoride) administered in the drinking water: 0 (control), 15 or 50 mg/L. The reason for selecting these concentrations is because they produce plasma F levels in rodents that are similar to those observed in humans who drink water containing 3 and 10 mgF/L, respectively (Dunipace et al., 1995). These F levels are naturally found in the water in endemic areas of dental and skeletal fluorosis, respectively. All rats were kept in pairs in standard cages with *ad libitum* water and chow. The humidity and temperature in the climate-controlled room, which had a 12 h light/dark cycle, were 40%–80% and 23 ± 1 °C, respectively. At the end of the experimental period, the rats were anesthetized (ketamine chlorhydrate and xylazine chlorydrate). The liver was collected, mitochondria were immediately isolated as described below and stored at -80 °C for further activation analysis.

2.2. Isolation of mitochondria

Two grams of liver tissue were homogenized in 2 mL of buffer MSTE consisting of 0.23 M mannitol, 0.07 M sucrose, 10 mM Tris-HCl, 1 mM EDTA, pH 7, sonicated for 45 s and centrifuged at 700 g for 10 min at 4 °C for elimination of the cell membranes. The supernatant was collected and centrifuged at 8,000 g for 10 min at 4 °C, leading to the formation of a heart-shaped pellet, which was suspended in 1 mL of MSTE buffer and stored at -80 °C for analysis (Fina et al., 2014).

2.3. Protein quantification

Mitochondrial proteins were quantified using the Bradford Biorad Protein Assay Biorad kit based on the method of Bradford (1976), in triplicate. For each reaction, we used 5.0 μL of sample and 250 μL of the staining reagent. After homogenization, the sample was transferred to the spectrophotometer and read at 595 nm. The obtained value was interpolated in a previously established curve using the same methodology for known concentrations of BSA (bovine serum albumin) protein standard.

2.4. Isolation of the mitochondrial membrane

The mitochondria were suspended in MSTE buffer and thawed three times in order to disrupt the mitochondrial membranes. These membranes were used for the measurement of the activity of the mitochondrial complexes.

2.5. Activity of respiratory complexes

The determination of complex II-III (succinate-c-cytochrome) and I-III (NADH-cytochrome c reductase) is based on the reduction of cytochrome c^{3+} to cytochrome c^{2+} and was performed spectrophotometrically at 550 nm for 30 and 120 min at 30 °C. The reaction media was composed of 100 mM H_2KPO_4/HK_2PO_4 pH 7.4 buffer, mitochondrial membranes (for final concentration of 0.02 mg protein/mL), 25 mM KCN (for inhibition of activated complex IV), 4 mM NADH, 0.7 M Succinate and 0.5 mM Cytochrome C^{3+}. The analysis was done in spectrophotometer at 550 nm. The activities were calculated as μmol. min^{-1}. mg $protein^{-1}$ (Navarro et al., 2010), using the formula $Activity = \frac{\Delta Abs/_{min} \; x \; Vfinal_{\mu l}}{19.1 \; x \; sample_{\mu l} \; x \; mg/ml \; proteins}$ (Fina et al., 2014). For the analysis of complex I-III, NADH was added but succinate was not, while for the analysis of complex II-III, succinate was added but NADH was not.

2.6. Bioinformatics analysis

We used data generated in our previous study where we performed proteomic analysis of the liver of rats exposed to water containing 15 and 50 mgF/L for 20 or 60 days (Pereira et al., 2018). The groups of proteins used for analysis of Gene Ontology annotation of Broad Cellular Component were those presenting significant difference in expression between the groups (both up and downregulation), as indicated by the Protein Lynx Global Server (PLGS, Waters Corporation) software and expressed as $p < 0.05$ for downregulated proteins and $1 - p > 0.95$ for upregulated proteins. For this, gene entries for each pair-wise comparison (control group vs. treated groups 15 mgF/L and 50 mgF/L, both for 20 or 60 days) were obtained from the Uniprot protein ID accession numbers. Gene Ontology annotation of Broad Cellular Component was done using Cluego v2.0.7 + Clupedia v1.0.8 (Bindea et al., 2009; Bindea et al., 2013), a Cytoscape (Bauer-Mehren 2013; Millan 2013) plugin. For this, Uniprot IDs were analyzed with default parameters: Enrichment (Right-sided hypergeometric test) correction method using Bonferroni step down, analysis mode "Function" and load gene cluster list for *Rattus norvegicus*, Evidence Codes "All", set networking specificity "medium" (GO levels 3 to 8), and Kappa Score Threshold 0.03. For this analysis, only proteins present in cell compartment mitochondria were included. Protein-protein interaction networks were downloaded from PSICQUIC (Orchard 2012), built in Cytoscape version 3.7 as previously reported (Millan 2013).

2.7. Statistical analysis

Data of activity of respiratory complexes passed normality (Kolmogorov-Smirnov test) and homogeneity (Bartlett test) and were analyzed by ANOVA and Tukey's test, using the software Statistica version 7.0. The significance level was set at 5%. In all figures, data are shown as means ± SD.

For proteomic data, the difference in expression between the groups (biological triplicates) described in Tables S1–S4 was obtained using the Protein Lynx Global Server (PLGS) software (Waters Co., UK) and expressed as $p < 0.05$ for down-regulated proteins and $1- p > 0.95$ for up-regulated proteins (Pereira et al., 2018).

3. Results

3.1. Activity of respiratory complexes

The activity of the mitochondrial complex I-III was significantly increased upon treatment with 50 mgF/L for 20 days and with 15 mgL/F

group for 60 days in respect to control. The experimental groups did not significantly differ from each other, regardless the period of treatment (Figure 1).

As for the complex II-III, the activity was significantly reduced upon treatment with 50 mgF/L for 60 days in respect to control (Figure 2).

3.1.1. Functional classification

In the functional classification according with the cellular component (CC), the genes belonging to mitochondrial compartment were selected and the percentages of gene association were calculated. For the period of 20 days, the terms with the highest percentage of associated genes related to mitochondria were mitochondrial part (86%) for the group treated with 15 mgF/L (Figure 3A) and mitochondrion (85%) for the group treated with 50 mgF/L (Figure 3B). As for the 60-day period, the term with the highest percentage of associated genes related to mitochondria was mitochondrial matrix (73%), regardless the dose of F (Figure 3C and D).

3.1.2. Interaction networks

The associated genes with the terms indicated in the functional classification related to mitochondria were used to build the interaction networks. The ActiveModules 1.8 plug-in to Cytoscape was used to build subnetworks within the molecular interaction network whose genes had significant alterations in fold changes and p-values, as displayed in the original proteomic analysis (Pereira et al., 2018). A subnetwork was built for each comparison described above (totaling 4 networks). They give a broad global view of potentially relevant interacting partners of proteins whose abundances change.

The related proteins are displayed in the Tables S1, S2, S3 and S4.

As can be seen in Table S1, the animals exposed to the lower F concentration (15 mgF/L) for 20 days in comparison to control had in the interaction subnetwork four proteins with reduced expression: 40S ribosomal protein S3 (Rps3; P62909), Clusterin (P05371), Cytochrome b-c1 complex subunit 6, mitochondrial bellows to complex III (Uqcrh; Q5M9I5), Cytochrome c oxidase subunit 4A, mitochondrial bellows to complex IV (Cox5a; P11240) and two proteins with increased expression: Nucleoside diphosphate kinase A (Nme1; Q05982) and Dihydrolipoyllysine-residue acetyltransferase component of pyruvate dehydrogenase complex, mitochondrial (Dlat; P08461).

As for the exposure to the highest F concentration for 20 days (50 mgF/L, Table S2), the interaction subnetwork revealed reduction in the expression of Clathrin heavy chain 1 (Cltc; P11442) and Rps3, while the other proteins were increased: Heterogeneous nuclear ribonucleoprotein K (Hnrpk; P61980), Fructose-bisphosphate aldolase A (Aldoa; P05065), Fumarate hydratase, mitochondrial (Fh; P14408), Aconitate hydratase, mitochondrial (Aco2; Q9ER34), Stomatin-like protein 2, mitochondrial (stoml2; Q4FZT0), Cytochrome b-c1 complex subunit Rieske (Uqcrfs1; P20788), MICOS complex subunit Mic60 (Immt; Q3KR86) (Figure 4B).

Regarding the longer exposure period (60 days), considering the comparison 15 mg F/L *vs.* control (Table S3, Figure 4C), the proteins identified with increased expression were: ATP synthase subunit beta, mitochondrial (Atp5f1b-P10719), 3-ketoacyl-CoA thiolase, mitochondrial (Acaa2-P13437), Methylcrotonoyl-CoA carboxylase beta chain, mitochondrial (Mccc2-Q5XIT9), Glutamate dehydrogenase 1, mitochondrial (Glud1-P10860). Three proteins were decreased: 10 kDa heat shock protein, mitochondrial (Hspe1-P26772), Dihydrolipoyl dehydrogenase, mitochondrial (Dld-Q6P6R2) and Leucine-rich repeat-containing protein 59 (Lrrc59-Q5RJR8).

As for the comparison 50 mg F/L *vs.* control (Table S4, Figure 4D), Malate dehydrogenase, mitochondrial (Mdh2-P04636) and Protein/nucleic acid deglycase DJ-1 (Park7-O88767) were increased, while Dihydrolipoyl dehydrogenase, mitochondrial (Dld-Q6P6R2), 10 kDa heat shock protein, mitochondrial (Hspe1-P26772) and Lrrc59 were decreased.

In all the networks, most of the proteins with change in expression interacted with Solute carrier family 2, facilitated glucose transporter member 4 (Slc2a4-P19357), known as GLUT-4 (Figure 4A-D).

4. Discussion

The present study was developed to provide additional insights into the effects of F in the liver, with special focus on the oxidative stress provoked by distinct doses and periods of exposure to F on mitochondria. F is known for its ability to inhibit enolase (Warburg and Christian 1942), which is associated with the marked alterations seen in proteins related to energy metabolism upon exposure to F (Pereira et al., 2013; Lobo et al., 2015; Pereira et al., 2018). This is consistent with the fact that, in all interaction networks (Figure 4A-D), most of the proteins with altered expression interacted with GLUT-4, a glucose transporter that plays a key role in removal of glucose from circulation. Remarkably, in all interaction networks, glycolytic and TCA cycle enzymes were increased in the F-treated groups (Figure 4A-D). This increase might be an attempt of the organism to keep the energy flow due to the ability of F to inhibit enolase (Warburg and Christian 1942). Increase in these enzymes, especially in those involved in TCA cycle, increases NADH and $FADH_2$ that are directed to oxidative phosphorylation (OXPHOS). This is in-line with the increase in the mitochondrial activity of the complex I-III upon exposure to the higher dose of F in the shorter period and upon exposure to the lower dose of F in the longer period. The complex I (NADH: ubiquinone oxidoreductase) is the first enzyme of the respiratory complex chain that is responsible for oxidizing NADH generated through the Krebs cycle in the mitochondrial matrix (Sharma et al., 2009). This complex is the main point of entry of electrons into the

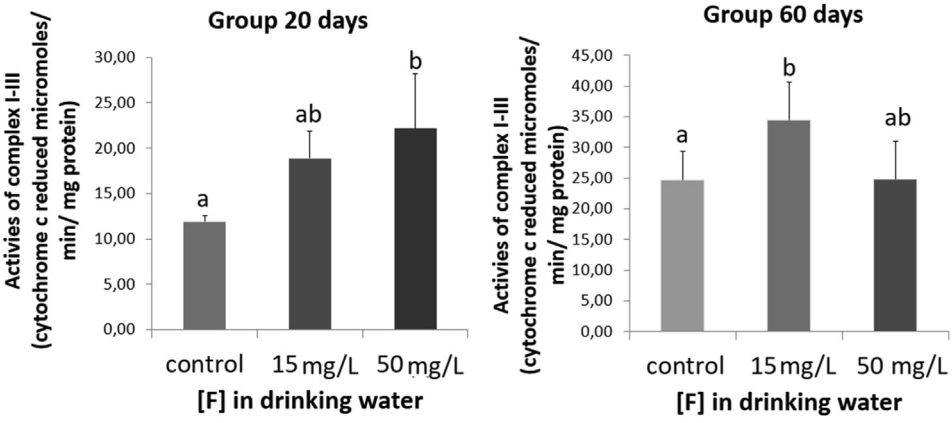

Figure 1. Activities of complex I-III of isolated mitochondria of liver rats. The rats received water containing different fluoride concentrations (0-control, 15 and 50 mgF/L) for 20 days or 60 days. Distinct superscript letters indicate significant differences among the groups (ANOVA and Tukey's test, $p < 0.05$) $n = 6$. Mean ± SD.

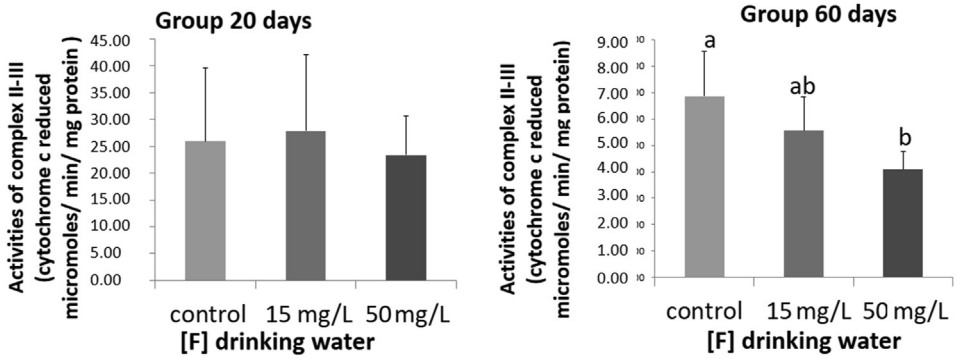

Figure 2. Activities of complex II-III of isolated mitochondria of liver rats. The rats received water containing different fluoride concentrations (0-control, 15 and 50 mgF/L) for 20 days or 60 days. Distinct superscript letters indicate significant differences among the groups (ANOVA and Tukey's test, $p < 0.05$) $n = 6$. Mean ± SD.

respiratory chain and plays an important role in energy metabolism due to its implication in the regulation of ROS. The increase in ROS production can be extremely harmful and consequently damage macromolecules within the mitochondria, including lipids, proteins and mitochondrial DNA (Van Houten et al., 2006; Sharma et al., 2008; Guo et al., 2013). The protein Rps53 was reduced in both doses (15 and 50 mgF/L) at 20 days. This protein is a major component of the 40S subunit of the ribosome and assists protein translation, DNA repair (Hegde et al., 2004; Kim et al., 2009), cell signaling (Kim and Kim 2006) and apoptosis/survival (Sen et al., 2012). In addition, this protein is responsible for reducing the production of ROS (Kim et al., 2013). The group treated with the higher dose for 20 days presented an increase in the activity of complex I, which might suggest an inhibition of Rps53 in attempt to reestablish the energetic flow. Increase in the activity of Complex I is expected to improve mitochondrial respiration, resulting in enhanced ATP production and less accumulation of free radicals (Yang et al., 2015). Moreover, when the cellular component was analyzed, the proteins with major alterations were all related to mitochondrion, indicating the general damage in this period.

Exposure to the lower dose of F for the shorter period of time increased the protein Dlat (Figure 4A), present in the pyruvate dehydrogenase complex, which is responsible for the energy flow of the TCA and consequently increases the production of coenzymes responsible for the OXPHOS. This might be related to the trend for increase in the activity of the complex I-III observed for the group treated with the lower dose of F for 20 days, despite this increase was significant only in the long term (60 days). .

On the other hand, in the group treated with the higher dose of F for the shorter period (20 days), a protein involved in the maintenance of the complex I, Ndufa10 (Q561S0) that belongs to the accessory subunits of this complex, had its expression reduced. This protein is mainly related to mitochondrial regulation, in addition to being important for promoting oxidation and mitochondrial ATP generation. The reduction of this key protein may indicate an attempt to maintain balance in OXPHOS, as seen by the increased activity of this complex (Yang et al., 2015; Roy Chowdhury and Banerji 2018; Shimada et al., 2018). TCA proteins like Fh and Aco2 were increased. This increase may explain why the electron transport chain presented an increase in the expression of proteins belonging to complexes I, II and III. Thus, this possible increase in the energy production in the mitochondria can also be responsible for the oxidative stress observed upon exposure to F (Barbier et al., 2010; Pereira et al., 2018; Araujo et al., 2019). The increased expression of the protein Immt (Table S2- group treated with the higher dose of F for 20 days) is an important finding. This protein maintains the mitochondrial membrane potential by preserving the cytochrome c inside and avoiding the activation of the apoptotic pathway (Van Laar et al., 2016). Another interesting finding of the present study was the increase in the protein Stoml2, which regulates the biogenesis and activity of the mitochondria. Through the regulation of the mitochondrial function, it can play a role in processes such as cell migration, cell proliferation, T cell activation, calcium

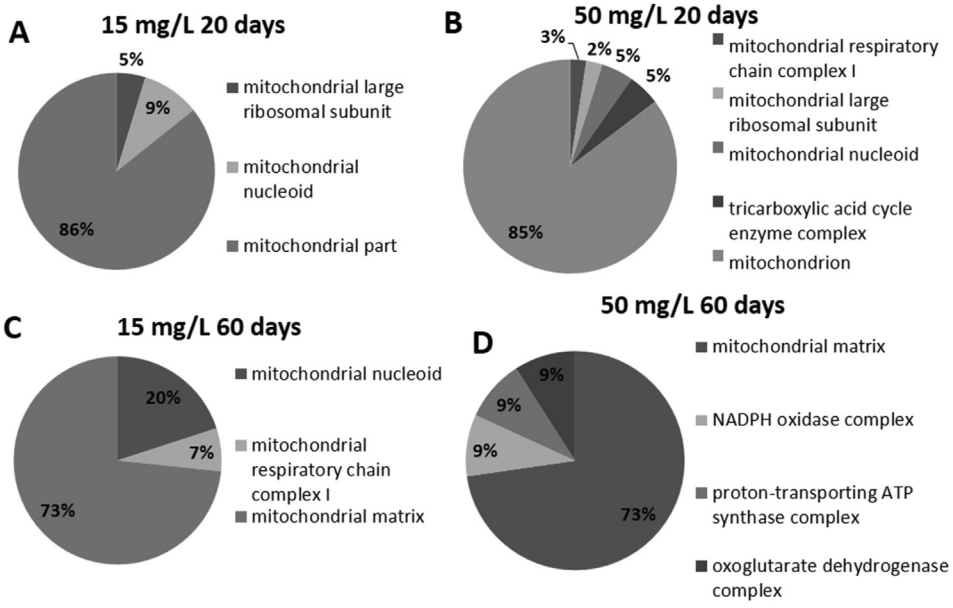

Figure 3. Functional distribution of proteins identified with differential expression in the liver of rats chronically treated with fluoride (15 or 50 mg/L) or not for 20 days with cellular component relational the mitochondria. Categories of proteins based on GO annotation (Cellular Component, Molecular function and Biological Process). Terms significant (Kappa = 0.03) and distribution according to the percentage of number of genes association. (3A) Control group *vs.* 15 mgF/L group 20 days. (3B) Control group *vs.* 50 mgF/L group 20 days, Control group *vs.* 15 mgF/L group 60 days. (3C) Control group *vs.* 50 mgF/L group 60 days (3D).

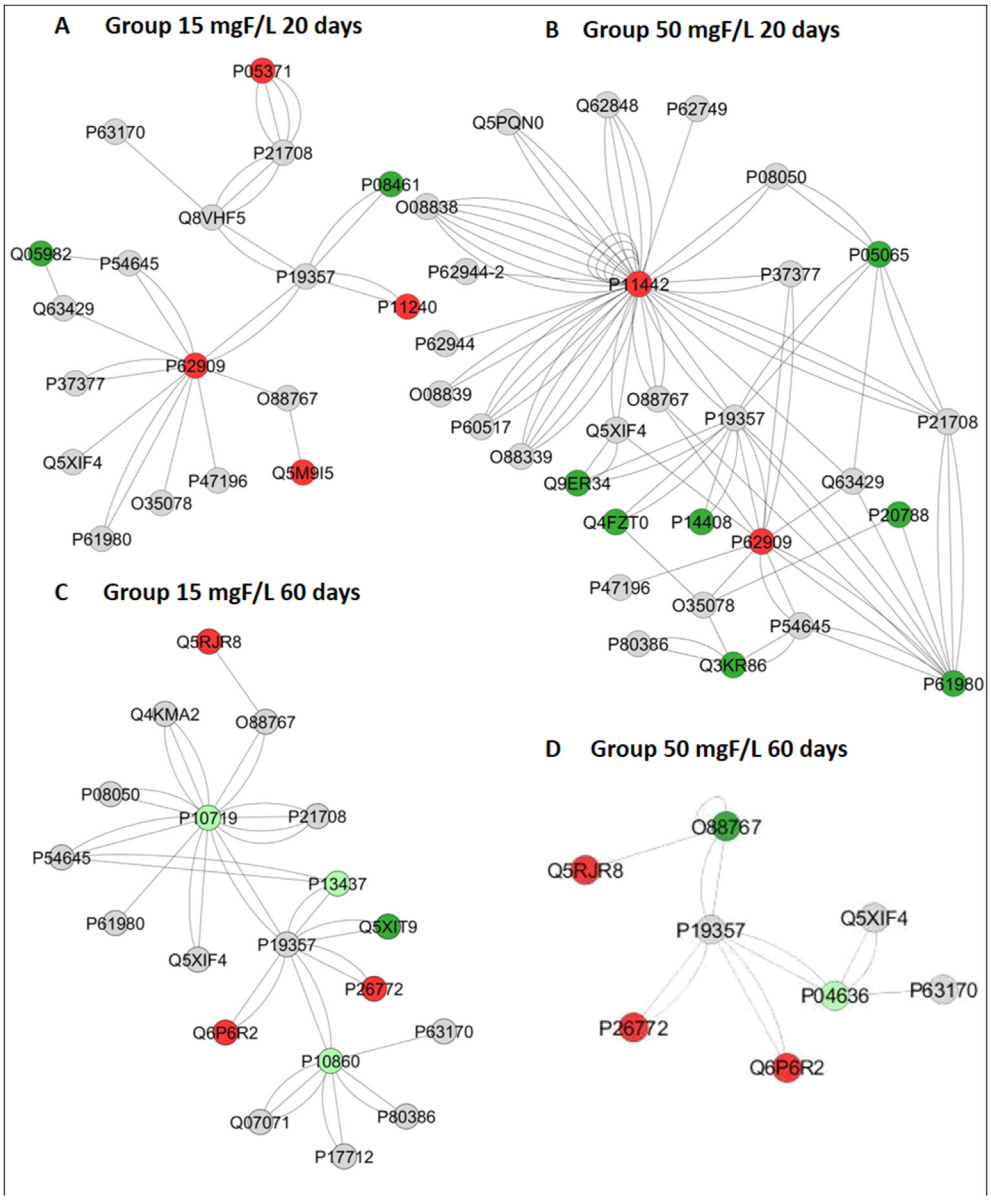

Figure 4. Networks generated by Cytoscape for each comparison – A Control group *vs.* 15 mgF/L 20 days; B Control group *vs.* 50 mg/L 20 days. C Control group *vs.* 15 mgF/L 60 days; D Control group *vs.* 50 mg/L 60 days. Color of node indicates the differential expression of the respective protein, for each comparison. Red and green nodes indicate protein down-regulation and up-regulation, respectively, in the treatment group and control of each comparison. Grey nodes indicate proteins presenting interaction but that were not identified in the present study.

homeostasis and cell response to stress (UNIPROT). The increase in this protein may indicate a cellular pro-survival response to stress that might result from increased production of mitochondrial ATP.

For the longer period (60 days), the lower dose of F led to an increase in the complex I. The internal energy status of the mitochondria suggests the formation of ROS, due to a slight reduction in complex II (Figure 2). This result corroborates with the increase in the expression of Acaa2 that can be associated with the production of ROS. This happens because when the mitochondria has a vigorous energy flux, part of this energy is dissipated through ROS (Cao et al., 2008), which might open the mitochondrial permeability transition pore (MPTP), thus leading to mitochondrial apoptosis. In addition, we recently reported that the Acca2 can prevent the opening of these canals (Araujo et al., 2019). On the other hand, we observed reduction in proteins related to complexes III and IV (Uqcrh and Cox5a) that are responsible for complex inhibition or production of ROS leading to reduction in expression of proteins. This could explain the discrete increase in the activity of the complex in the short time. According to Fina et al. (2014), chronic or acute exposure to F in vitro inhibits complexes I-III of the ETC, which may result in an increase in the release of superoxide anions, thus increasing ROS levels. In addition, studies show that the presence of F leads to a decrease in the expression or inhibition of mitochondrial complexes activity (Chauhan et al., 2013; Fina et al., 2014; Liang et al., 2016; Panneerselvam et al., 2017; Wang et al., 2017; Araujo et al., 2019).

Reduction of complex II-III was observed upon exposure to the higher dose of F for the longer period (60 days). The regulation of the mitochondrial respiratory complex II has the function of reserving respiratory capacity and cell survival (Dhingra and Kirshenbaum 2015). The classification analysis showed changes related to mitochondrial matrix proteins, which is consistent with alterations in the mitochondrial complex. The subnetwork indicated reduction in a protein related to complex I-III (Dld), which may indicate a reduction in the energetic flux, suggesting an adaptation to the effect of F that leads to oxidative stress and ROS generation (Pereira et al., 2018; Araujo et al., 2019). On the other hand, the proteins linked to the fatty acid biosynthesis were reduced (Crb4 and NADPH oxidase 1; Table S4). This reduction may indicate changes in the flow of acetyl-CoA to increase the energy flow in the TCA, indicated by an increase in ATP synthase (Atp5mc3) and

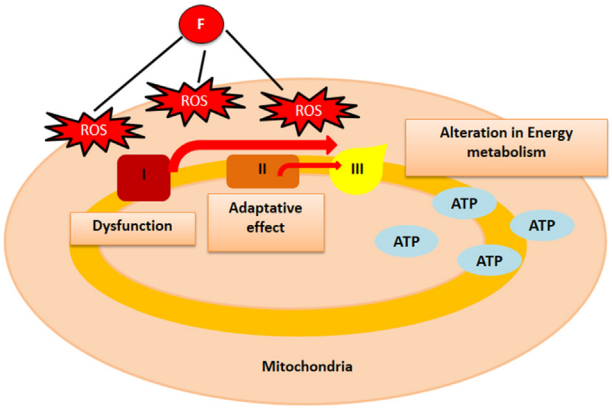

Figure 5. F action on complexes I-III and II-III. Oxidative stress provoked by F increases the activation of the complex I-III in the short term. The adaptation to the effect of F along time reduces the activation of complex II-III, thus reducing the proteins responsible for ATP and ROS production.

Mdh2, proteins involved in the production of ATP. The increase in the flow of the substrate coincides with the increase of the metabolic demand in the cell through the flow of TCA and ETC. However, the limiting factor for this increase in flux appears to be succinate dehydrogenase (Sdh), which is the only enzyme commonly shared between the TCA cycle and the ETC (Dhingra and Kirshenbaum 2015). The complex was reduced in this group, indicating a possible attempt to reduce the energy production, to fight oxidative stress. A recent study administrated different doses of F (25, 50 and 100 mg/L F) to mice for 90 days. Reduction of ATP in hepatocytes was observed upon administration of the highest doses, associated with increase in ROS. The authors suggested that disturbs in the mitochondrial respiratory chain were responsible for the damages in liver provoked by F (Wang et al., 2019; Wang et al., 2020). These results are in-line with ours, indicating the F-induced damage to mitochondria.

The oxidative stress induced by F provokes the dysfunction of the mitochondrial complexes. The increased activity in complex I-III might lead to a higher production of ATP, which in turn might increase ROS production (Wang et al., 2019; Wang et al., 2020). However, to confirm this increase in ATP and ROS production, further experiments should be conducted. Thus, the reduction in complex II-III in the long term suggests an attempt to reduce the energy production to fight the ROS increase and seems to be part of an adaptative mechanism of the organism to fight the deleterious effects of this ion (Figure 5). Additional studies evaluating the production of ATP an ROS in mitochondria upon exposure to F are needed to confirm the suggested adaptative mechanism.

Declarations

Author contribution statement

Heloisa Aparecida Barbosa Silva Pereira, Marilia Buzalaf: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Tamara Teodoro Araujo, Aline Dionízio: Performed the experiments; Analyzed and interpreted the data; Wrote the paper.

Juliana Sanches Trevizol: Contributed reagents, materials, analysis tools or data; Wrote the paper.

Fabrício Soares Pereira, Flávia Godoy Iano: Analyzed and interpreted the data; Wrote the paper.

Valdecir Faria Ximenes: Conceived and designed the experiments; Performed the experiments; Contributed reagents, materials, analysis tools or data.

Funding statement

Heloisa Aparecida Barbosa da Silva Pereira was supported by FAPESP (Proc. 2018/24073-0).

Data availability statement

Data included in article/supplementary material/referenced in article.

Declaration of interests statement

The authors declare no conflict of interest.

Additional information

Supplementary content related to this article has been published online at https://doi.org/10.1016/j.heliyon.2021.e06028.

Acknowledgements

The authors thank FAPESP for the concession of a fellowship to the first author (Proc. 2018/24073-0).

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