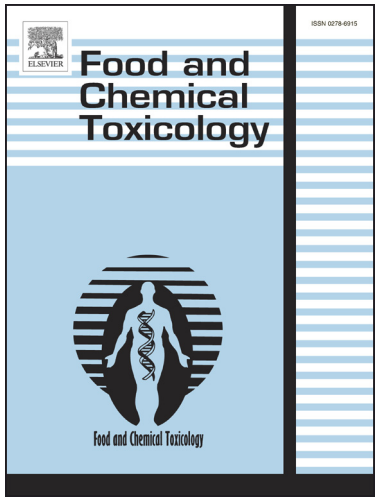

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PII: S0278-6915(13)00528-0
DOI: http://dx.doi.org/10.1016/j.fct.2013.07.073
Reference: FCT 7493

To appear in: *Food and Chemical Toxicology*

Received Date: 2 July 2013
Accepted Date: 27 July 2013

Please cite this article as: Monteiro, J.P., Silva, A.M., Jurado, A.S., Oliveira, P.J., Rapeseed oil-rich diet alters *in vitro* menadione and nimesulide hepatic mitochondrial toxicity, *Food and Chemical Toxicology* (2013), doi: http://dx.doi.org/10.1016/j.fct.2013.07.073

Rapeseed oil-rich diet alters *in vitro* menadione and nimesulide hepatic mitochondrial toxicity

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Abstract

Diet-induced changes in the lipid composition of mitochondrial membranes have been shown to influence physiological processes. However, the modulation effect of diet on mitochondrially-active drugs has not yet received the deserved attention. Our hypothesis is that modulation of membrane dynamics by diet impacts drug-effects on liver mitochondrial functioning. In a previous work, we have shown that a diet rich in rapeseed oil altered mitochondrial membrane composition and bioenergetics in Wistar rats. In the present work, we investigated the influence of the modified diet on hepatic mitochondrial activity of two drugs, menadione and nimesulide, and FCCP, a classic protonophore, was used for comparison. The results showed that the effects of menadione and nimesulide were less severe on liver mitochondria for rats fed the modified diet than on rats fed the control diet. A specific effect on complex I seemed to be involved in drug-induced mitochondria dysfunction. Liver mitochondria from the modified diet group were more susceptible to nimesulide effects on MPT induction. The present work demonstrates that diet manipulation aimed at modifying mitochondrial membrane properties alters the toxicity of mitochondria active agents. This work highlights that diet may potentiate mitochondrial pharmacologic effects or increase drug-induced liabilities.

Keywords: Toxicology; Diet; Menadione; Nimesulide; Mitochondria; Membranes

1 - Introduction

Dietary fat manipulations modify the expression (Ochoa et al., 2011) and the activity of mitochondrial electron transport chain complexes, including complex III (Barzanti et al., 1994) and IV (Barzanti et al., 1994; Battino et al., 2002; Yamaoka et al., 1988), as well as for the ATP synthase (Barzanti et al., 1994; Clandinin et al., 1985). The activity of the electron transport chain can also be modified by diet through modulating coenzyme Q, a mobile electron carrier in mitochondrial membranes (Huertas et al., 1991a; Mataix et al., 1997). Generation of ROS by the respiratory chain (Ramsey et al., 2005), as well as the mitochondrial permeability transition (O'Shea et al., 2009) can also be affected by diet. In a previous study, we demonstrated that Wistar rats fed a diet containing 20% rapeseed oil underwent alterations in terms of hepatic mitochondrial membrane composition, bioenergetics and oxidative stress (Monteiro et al., 2013b), with a general worsening of mitochondrial function, including decreased respiratory activity. An increased susceptibility of the calcium-induced mitochondrial permeability transition (MPT) was also found after 33 days of modified diet intake (Monteiro et al., 2013b). Our next experimental question was whether liver mitochondria from rats fed the 20% rapeseed oil-rich diet exhibited altered susceptibility to several mitochondria toxicants. To address this question, two agents (menadione and nimesulide) and the classical uncoupler FCCP (Brennan et al., 2006; Starkov, 1997) were chosen, since we have previously shown (Monteiro et al., 2011a; Monteiro et al., 2011b; Monteiro et al., 2013a) that these hepatotoxic drugs, besides impairing mitochondrial function, disturbed membrane physical properties. Our hypothesis is that mitochondrial alterations caused by the rapeseed oil-containing diet, which should be mediated, at least in part, by membrane lipid modifications, affect liver mitochondrial susceptibility to menadione, nimesulide and FCCP. Therefore, in the present study, Wistar rats were

fed a 20% rapeseed oil-enriched diet for the longer time period, 33 days, and several markers of mitochondrial function were evaluated in the absence and presence of menadione, nimesulide and FCCP. Interestingly, we demonstrate here that the *in vitro* mitochondrial toxicity of these compounds was attenuated by the modified diet.

2 - Materials and Methods

2.1 - Reagents

All reagents used were of the highest grade of purity commercially available (analytical grade or better), and all aqueous solutions were prepared in ultrapure (type I) water. FCCP, menadione and nimesulide were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA). FCCP was prepared in ethanol at a final stock concentration of 1 mM. Menadione and nimesulide were prepared in ethanol and dimethylformamide, respectively, at a final stock concentration of 40 mM.

2.2 - Animal feeding protocol and ethics statement

This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocol was approved by the Animal Ethics Committee of the Center for Neuroscience and Cell Biology (AEC034-22062011, June 20, 2011). All animal handlers, including the senior author, are accredited by the Federation for Laboratory Animal Science Associations (FELASA). The animal facility at the CNC is accredited by the National Office of Veterinary ("Direcção Geral de Veterinária, DGV).

Two months-old male Wistar-Han rats were maintained on a reverse 12-h light–dark cycle with food and water *ad libitum*. The control group had access to a standard rodent diet, while the treated group was fed with a modified diet, which had the same composition as the control diet but containing 20% rapeseed oil calorically adjusted for high fat. Both diets were purchased from Dyets Inc. (Bethlehem, PA) and their composition has been described previously by us (Monteiro et al., 2013b). After 33 days of diet consumption, the animals were sacrificed by cervical dislocation followed by decapitation and livers were harvested for mitochondria isolation. Control and modified-diet animals were sacrificed daily in a paired basis.

2.3 - Isolation of liver mitochondria

Mitochondria were isolated from the livers of male Wistar-Han rats by differential centrifugation, as previously described elsewhere (Pereira et al., 2007).

2.4 - Determination of respiratory parameters

The oxygen consumption of isolated mitochondria was determined polarographically with a Clark-type oxygen electrode connected to a suitable recorder in a 1 mL thermostated water-jacketed chamber with magnetic stirring, at 30°C (Pereira et al., 2007). The standard respiratory medium consisted of 125 mM sucrose, 65 mM KCl, 2.5 mM $MgCl_2$, 5 mM KH_2PO_4 and 5 mM HEPES (pH 7.4), to which 3 μM rotenone was added when succinate was used as substrate. Mitochondria (1 mg of protein/mL) and substrate (glutamate/malate, 10 mM and 5 mM respectively, or succinate, 10 mM) were added to the respiration medium and state 3 respiration was initiated by addition of 125 nmol ADP. Addition of FCCP (1 μg/mL) uncoupled mitochondrial respiration (FCCP state). The chemicals used (20 nmol menadione/mg of protein or 40 nmol

nimesulide/mg of protein) or the respective vehicles (ethanol or DMF) were incubated with the mitochondrial suspensions for 3 min, before inducing state 3 respiration by addition of ADP.

2.5 - Determination of mitochondrial transmembrane electric potential

The mitochondrial $\Delta\Psi$ was estimated using an ion-selective electrode to measure the distribution of tetraphenylphosphonium (TPP^+) according to a previously established method (Oliveira et al., 2001). Mitochondria (1 mg/mL) were suspended with constant stirring, at 30°C, in 1 mL of the standard respiratory medium supplemented with 3 μM TPP^+ and were energized by adding glutamate/malate, 10 mM and 5 mM respectively, or succinate, 10 mM. The distribution of TPP^+ was allowed to reach a new equilibrium (2 min) before making any further addition. ADP (125 nmol) was added to initiate phosphorylation. The electrode was calibrated with TPP^+ assuming Nernstian distribution of the ion across the synthetic membrane, and $\Delta\Psi$ was expressed in mV. A matrix volume of 1.1 μl/mg protein was assumed. The mitochondrial suspensions were incubated with the chemicals (20 nmol menadione/mg of protein; 40 nmol nimesulide/mg of protein) or respective vehicles (ethanol or DMF) for 3 min, after substrate and before ADP addition. FCCP was added through a sequence of 3 consecutive titrations of 10 pmol FCCP/mg of protein after establishment of the initial maximal membrane potential value, with 1 min elapsing between each addition. Equal volumes of ethanol (the solvent used for FCCP) had no effects on mitochondrial $\Delta\Psi$.

2.6 - Induction of mitochondrial permeability transition

Mitochondrial swelling was determined as a means to follow MPT induction (Oliveira et al., 2001). Mitochondrial osmotic volume increase was followed as a decrease of absorbance at 540 nm, using a Perkin-Elmer Lambda 45 UV/VIS Spectrometer. An aliquot of mitochondrial suspensions corresponding to 1.5 mg of protein was added to 2 mL of swelling buffer (200 mM sucrose, 10 mM Tris-MOPS, pH 7.4, 10 μM EGTA, 1 mM KH_2PO_4, 1.5 μM rotenone, and 2.5 mM succinate) under constant stirring, at a temperature of 30ºC. Calcium (66.7 nmol /mg of protein) was added to the preparation upon 1 min of recording basal absorbance after the start of the experiment. Assays were also performed in the presence of cyclosporin A (1 μM), the specific MPT de-sensitizer (Broekemeier and Pfeiffer, 1989). Drugs (200 nmol menadione/mg of protein or 100 nmol nimesulide/mg of protein) were incubated with the mitochondrial suspensions for 3 min before calcium addition.

2.7 - Statistical analysis of data

Results were expressed as means ± standard error of mean for a number of independent experiments, specified for each case. Comparisons were performed using two-way ANOVA with Bonferroni post-test, with diet and treatment with the toxicants as independent factors. One-way ANOVA with the Student-Newman-Keuls as a post-test was used when comparing the extension of chemical action in control vs. modified diet rats sacrificed and processed in the same day. A value of $p<0.05$ was considered significant.

3 - Results

3.1 - The modified diet altered menadione and nimesulide in vitro effects on mitochondrial respiration

For the determination of mitochondrial bioenergetic endpoints, menadione and nimesulide concentrations of 20 nmol/mg of protein and 40 nmol/mg of protein, respectively, were chosen, since they fall within the range that induces alterations of mitochondrial bioenergetics (Berson et al., 2006; Klöhn and Neumann, 1997).

In rats fed the normal diet, menadione caused an increase in state 4 respiration and a decrease in the respiratory control index (RCI) and ADP/O ratio (Fig. 1A, B and S1A), consistent with the literature (Klöhn and Neumann, 1997). Menadione also caused an increase in state 4 and a decrease in uncoupled respiration when succinate was used as respiratory substrate (Fig. 1B). In a previous study (Monteiro et al., 2013b) using this feeding protocol, we showed that the modified diet promoted, per se, an increase in state 4 respiration and a decrease in state 3, uncoupled respiration, RCI and ADP/O ratio. These results were generally reproduced in the present study, as deduced from the comparison of data from control preparations (incubated with ethanol) in Fig. 1A and B. Diet-chemical interaction was significant for RCI, as determined by the two-way ANOVA (Fig. 1A and B). The effects of menadione on respiratory parameters were less pronounced in mitochondria from rats fed the rapeseed oil diet than in rats fed the standard diet (Fig. 1C and D). Thus, differences between the respiratory parameters determined in the presence of the chemical and in its absence (menadione control) were significantly lower in the group fed the modified diet than in the group fed the control diet (diet control group), namely concerning the respiratory state 3 and RCI when

glutamate plus malate were used as substrates, and respiratory state 4, uncoupled respiration, RCI and ADP/O ratio, when succinate was used as substrate.

The effects induced by nimesulide on liver mitochondria from control rats (Fig. 1E, F and S1B) were also consistent with those previously reported in literature (Berson et al., 2006; Tay et al., 2005). Similarly to menadione, nimesulide also promoted a significant increase of respiratory state 4 and a decrease of uncoupled respiration (although the difference was only significant when using succinate as substrate), RCI and ADP/O ratio (Fig. 1E and F). Diet-drug interaction was significant for RCI and ADP/O ratio, when glutamate plus malate were used as respiratory substrates (Fig. 1E). Regarding the effects of nimesulide on both experimental groups (Fig. 1G and H), significant differences were found only when glutamate plus malate were used as substrates. Thus, mitochondrial complex I from rats fed the alternative diet was less susceptible to the toxic action of nimesulide on respiratory state 3, uncoupled respiration, RCI and ADP/O ratio (Fig. 1G).

3.2 - The modified diet altered menadione and nimesulide in vitro effects on mitochondrial transmembrane electric potential

Menadione decreased the initial $\Delta\Psi$ and the resting membrane potential after repolarization of glutamate plus malate-energized mitochondria from control diet-fed rats (Fig 2A). When succinate was used as substrate, no statistical differences were found for drug effects on transmembrane potential parameters (Fig. 2B). Comparing the extent of menadione effects on glutamate/malate-energized mitochondria from rats fed the normal versus the rapeseed oil-containing diet (Fig. 2C), it is noticeable that mitochondria from the latter group showed a lower susceptibility to menadione adverse

action. For succinate-energized mitochondria, no significant differences were observed between the two experimental groups (Fig. 2D).

Nimesulide also caused dissipation of the initial ΔΨ, as previously reported (Tay et al., 2005). Moreover, it decreased the repolarization potential post-ADP phosphorylation in glutamate plus malate-energized mitochondria from rats fed the control diet(Fig. 2E). For rats fed the modified diet, the initial ΔΨ was also decreased, but only when glutamate plus malate were used as respiratory substrates. Concerning the extent of nimesulide toxicity in the two experimental groups, the less severe effects were once more observed on mitochondria from rats fed the rapeseed oil-containing diet, and similarly to menadione, significant differences were only observed when complex I substrates were used (Fig. 2G and H).

After observing that both nimesulide and menadione affected differently the maximal ΔΨ in the control and the modified diet-group, FCCP, a classic protonophore (McLaughlin and Dilger, 1980), was used to investigate whether it also act differently in mitochondria from the two experimental groups. With this purpose, we performed a sequential titration of mitochondrial ΔΨ with FCCP to evaluate diet-induced effects on FCCP-promoted mitochondrial depolarization. The results revealed that mitochondria from rats fed the diet containing 20% rapeseed oil were less depolarized by the same amount of the uncoupler as compared to mitochondria from rats from the control group (Fig. 3). Similar findings were obtained using both respiratory substrates.

3.3 – The modified diet modulated menadione and nimesulide effects on the induction of the permeability transition pore

The effects of menadione and nimesulide as inducers of MPT pore have been previously reported (Singh et al., 2010; Toninello et al., 2004). On the other hand, we have

demonstrated that mitochondria from rats fed the modified rapeseed oil diet displayed increased susceptibility to calcium-induced mitochondrial permeability transition (Monteiro et al., 2013b). Similar diet-induced effects were observed here, with nimesulide treatment increasing the differences between control and modified diet groups, while menadione attenuated the differences between them (Fig. 4A). Comparing the extent of the effects of both nimesulide and menadione in the two experimental groups, it becomes evident that the ability of nimesulide to promote calcium-induced mitochondrial permeability transition was significantly potentiated in mitochondria from rats fed the modified diet, while menadione exerted a smaller effect on this group of rats as compared to the control group, although differences between them were not statistically significant (Fig. 4B).

4 - Discussion

Although the great majority of clinically used drugs have been designed to exert a specific action towards target membrane proteins at relatively low concentrations, some have shown to hold non-target effects at higher concentrations (Lundbaek et al., 2004). The close relationship between lipid bilayer properties and membrane protein conformational dynamics and function (Andersen and Koeppe, 2007; Lundbaek, 2008; Lundbaek et al., 1996; Lundbaek et al., 2004) implies that changes in bilayer lipids (e.g. induced by diet) may influence drug action at membrane protein targets. On the other hand, lipophilic drugs may influence membrane protein activity by interfering with bilayer biophysical properties, this mechanism contributing to their pharmacological effects and/or being responsible for unwanted nonspecific side effects (Adachi et al., 2007; Hwang et al., 2003; Lundbaek, 2008; Lundbaek et al., 2005; Özdirekcan et al.,

2008). Disruption of lipid rafts, with redistribution of proteins between raft and non-raft domains, alterations of membrane curvature stress or bilayer thickness are some of the consequences of the incorporation of amphiphiles into the lipid bilayer with predictable repercussions in membrane protein activity (Szabo et al., 2007).

Since mitochondria display a complex physiology, in which membranes play a crucial role, and are involved in numerous cell processes, these organelles are very susceptible targets for drug-induced membrane-mediated toxicity (Wallace and Starkov, 2000). In fact, it is well known that a large number of natural, commercial, pharmaceutical, and environmental chemicals exert their hepatotoxicity by interfering with mitochondrial bioenergetics (Chan et al., 2005; Pereira et al., 2009; Wallace and Starkov, 2000). On the other hand, the influence that diet fat content exerts on mitochondria functional performance has been also reported. A previous study (Zsigmond and Clandinin, 1986) showed that the ATPase of heart mitochondria from animals fed diets enriched in fatty acids with increasing chain length exhibited increased oligomycin sensitivity and decreased 2,4-dinitrophenol-induced stimulation. The authors suggested that *in vivo* changes in the thickness of the lipid bilayer might alter mitochondrial ATPase functioning.

With this background in mind, the objective of the present study was to investigate whether a modified diet rich in rapeseed oil, previously shown to impact on hepatic mitochondrial function (Monteiro et al., 2013b), modulates the susceptibility of liver mitochondria to menadione, nimesulide and FCCP (see also Table I).

The use of hepatic mitochondria in this study is justified by the fact that the liver is an important organ involved in drug detoxification (Grant, 1991) and the drugs used in this study, namely menadione and nimesulide, were shown to cause liver injury (Ip et al., 2000; Tan et al., 2007). In fact, liver mitochondria permeabilization and impairment has

been proposed as an unsafe event potentially evoked by hepatotoxic drugs, representing an effective risk to human health (Singh et al., 2010).

Menadione (2-methyl-1,4-naphtoquinone) is a quinine derivative which can undergo redox cycling, generating intracellular ROS at multiple cellular sites and eliciting rapid oxidation in both the mitochondrial matrix and cytosol. Menadione is often used to simulate *in vitro* oxidative stress in mitochondrial preparations (Criddle et al., 2006; Han et al., 2000). In the present study, menadione affected mitochondrial respiration (Fig 1A and B) and $\Delta\Psi$ (Fig. 2A and B), consistently with data reported in literature (Klöhn and Neumann, 1997). Mitochondria from rats fed the alternative diet were generally more resistant to menadione-induced toxicity, especially when complex I substrates were used (Figs. 1 and 2). This fact suggests that diet-induced modulation of menadione toxicity should be exerted mostly at the complex I level, putatively involving modulation of its lipid environment. In a previous work, we demonstrated that the rapeseed oil-containing diet induces significant changes in liver mitochondrial membrane lipid composition, affecting the relative percentages of the three major phospholipid classes (PC, PE and CL) and the fatty acid content (with the saturated/unsaturated index being decreased) (Monteiro et al., 2013b). The phospholipids PE and CL, whose content decreased in mitochondria from animals fed the rapeseed oil diet, were pointed as being essential lipid components for the optimal functioning of complex I (NADH:ubiquinone oxidoreductase) (Sharpley et al., 2005). Tightly bound CL is required for the structural integrity of the complex I (Schlame, 2013) and PC and PE were found to bind to this complex as well, although more weakly than CL, determining its catalytic activity (Sharpley et al., 2005). Thus, one could speculate that the decrease in CL and PE content in mitochondrial membranes of rats fed the modified diet may lead to a deficit in the association of these lipids with

complex I, resulting in a decrease of mitochondria performance. Therefore, menadione effects on mitochondria bioenergetic parameters, which follow the same trend of diet-induced changes, may have a lower impact on mitochondria from rats fed the modified diet than on more efficient mitochondria from rats fed the control diet. Another way by which diet-induced lipid composition changes may influence menadione activity could be related with differences in mitochondria susceptibility to drug-induced effects on the lateral pressure profile across the membrane lipid bilayer, affecting the conformational dynamics of proteins, namely complex I. In fact, the activity of some membrane enzymes is influenced by the proneness of the surrounding lipids to form hexagonal II structures (Li et al., 1995). This eventually beneficial lipid influence may be compromised in mitochondria from rats fed the modified diet due to the decrease of PE content (Monteiro et al., 2013b), a phospholipid that promotes such non-lamellar arrangements. Since menadione increases the proneness of a model mimicking the mitochondrial membrane to adopt H_{II} arrangements (Monteiro et al., 2013a), it is possible that the compound could compensate the loss of non-bilayer phospholipids (PE and CL) in mitochondria from rats fed the modified diet. Therefore, menadione would exert less toxic effects in mitochondria from the modified diet group, to which the decrease in PE content might be detrimental for their function. In contrast, in normal mitochondria, menadione could create an unbalance between conditions that favour the curvature stress and those that confer stability to the lipid bilayer, eventually compromising mitochondrial normal functioning.

We also observed that menadione increases the susceptibility to calcium-induced mitochondrial swelling, consistently with previous reports (Toninello et al., 2004), but no significant differences in the action of the chemical were found between mitochondria obtained from both dietary groups (Fig. 4). Menadione-induced

mitochondrial permeability transition has been assigned to its oxidant activity, leading to the direct oxidation of mitochondrial pyridine nucleotides and the modification of critical thiols of the MPT pore components (Kruglov et al., 2008). Such events should not be significantly affected by diet manipulation. Since MPT was suggested as being essential for menadione-elicited apoptosis, promoting efflux of cytochrome c into the cytoplasm and subsequent activation of caspases 9 and 3 (Gerasimenko et al., 2002), differences in menadione-induced cell death between rats from the two experimental groups should not be expected.

In agreement with our previous study (Monteiro et al., 2013b), mitochondria from rats fed the rapeseed oil diet showed to be more susceptible to calcium-induced swelling (Fig. 4), which could be related with the detected decrease (Monteiro et al., 2013b) in the ratio of saturated/unsaturated lipid acyl chains (Cavalcanti et al., 1996; Haeffner and Privett, 1975).

Nimesulide, a non steroidal anti-inflammatory drug (NSAID) that acts by preferential inhibition of cyclooxygenase-2 (Garcia-Nieto et al., 1999), also affected mitochondrial respiratory parameters (Figs. 1E, F and 2E, F) in agreement with previous reports (Caparroz-Assef et al., 2001). The above mentioned prediction that diet-induced modulation of drug effects takes place at the complex I level was also supported by nimesulide data. In fact, when comparing the severity of nimesulide effects on mitochondria from control rats and rats fed the rapeseed oil diet, differences were only significant when glutamate and malate were used as respiratory substrate (Fig. 1G, H and Fig. 2G, H). The same mechanisms we proposed for dietary modulation of menadione toxicity may be applied in this case. In a previous study (Monteiro et al., 2011b), we have shown that nimesulide, unlike menadione (Monteiro et al., 2013a), stabilized lamellar arrangements in a mitochondrial membrane mimicking model

composed of PC, PE and CL (1:1:1). Assuming that the depressed mitochondrial function in the presence of nimesulide resulted from a decrease in curvature stress surroundings critical membrane proteins, the mitochondrial membranes from rats fed the 20% rapeseed oil diet, with a lower content of PE and CL (Monteiro et al., 2013b), would probably be less susceptible to the influence of this drug, since their lipid composition by itself would promote lamellar arrangements with low stress of curvature. This could be the explanation for the less severe effects of nimesulide on mitochondria from rats fed the rapeseed oil diet, as compared to mitochondria from control rats.

Regarding nimesulide activity on inducing the MPT, confirming previous reports (Berson et al., 2006; Mingatto et al., 2000), differences were observed between the two experimental groups (Fig. 4A and B), with mitochondria from rats treated with the 20% rapeseed oil diet being more affected by the chemical agent. Since nimesulide is an anionic drug, a putative competition with negatively charged lipids, such as CL, for electrostatic interactions with protein components of the MPT pore, preferentially localized in CL-enriched domains, may occur, thus interfering with the MPTP,. As liver mitochondria seem to be particularly sensitive to MPT compared to mitochondria from other organs (Berman et al., 2000), the stimulation of this process by nimesulide is thought to play a role in the idiosyncratic hepatic toxicity of this and other NSAIDs (Tay et al., 2005). Mitochondrial uncoupling induced by nimesulide (Mingatto et al., 2000) has been proposed as initiating MPT, concurring with a secondary ROS production (Tay et al., 2005). Since state 4 respiration was shown to be higher in mitochondria from rats fed the rapeseed oil diet (Fig. 1), the uncoupling effect of nimesulide that reportedly leads to MPT may be potentiated by the uncoupling effect of the diet treatment itself. It would also be of great interest to study how dietary treatment

may modulate nimesulide action on cyclooxigenase-2 activity. The potency of this anti-inflammatory drug might be largely influenced by diet-induced modulation of membrane lipid composition since NSAID-induced biophysical changes in membranes have been considered to contribute to their toxicological/pharmacological activity (Abramson et al., 1990).

FCCP, a classical uncoupler of oxidative phosphorylation (Berman et al., 2000; Starkov, 1997), that acts at the inner mitochondrial membrane disrupting mitochondrial transmembrane potential, displayed a decreased activity on mitochondria from rats fed the rapeseed oil diet as compared to rats fed the control diet, regardless of the respiratory substrate used (Fig. 3). The extent of the uncoupling promoted by FCCP in mitochondria from the two groups of rats may be correlated with their different lipid composition (Monteiro et al., 2013), since this was proposed to modulate FCCP-induced proton translocation across the inner mitochondrial membrane (Benz and McLaughlin, 1983). Biophysical studies of FCCP effects on membrane models showed that FCCP, besides increasing membrane fluidity and promoting lateral phase separation in membranes consisting of CL and PE (3:7), increased the proneness of membranes composed of PC, PE and CL (1:1:1) to form hexagonal II arrangements (Monteiro et al., 2011a). FCCP uncoupling activity may depend on membrane physical effects and, hence, dietary modulation of its activity should be considered when investigating pharmacological uses of FCCP, as already described for mild-uncoupling-based neuroprotection (Maragos and Korde, 2004), or cardioprotection (Brennan et al., 2006). It is indeed reasonable to assume that diet may modulate the pharmacological activity of FCCP, by modulating FCCP-induced uncoupling.

Altogether, our data illustrate the complex interplay between membrane lipid composition and drug action at the mitochondrial level. Diet, besides affecting drug

metabolism (Wade, 1986), may affect drug action at membrane protein targets due to an indirect interaction with the membrane lipid environment. Diet-induced changes in lipid composition may also influence the access of lipophilic drugs to membrane protein sites by altering the partitioning of drugs into the lipid bilayer, while interfering with membrane biophysical properties. This hypothesis may also explain, at least to certain extent, the nonspecific action of lipophilic drugs on non-target membrane proteins, which constitutes a significant problem in novel drug design (Andersen and Koeppe, 2007; Lundbaek, 2008; Lundbaek et al., 1996; Lundbaek et al., 2004).

Despite the pharmacological interest of the findings included in this work, we must acknowledge that present study would be strengthened if extended to other mitochondriotoxic drugs. For example, it would have been relevant to study drugs with different mitochondrial toxicological profiles, including those which act through state 3 inhibition or even by disturbing fatty acid beta-oxidation.

In conclusion, diet may modulate the pharmacological activity of membrane-active drugs by modifying drug-membrane interactions, from which either non-specific toxicity or increased pharmacological effect may result. On the other hand, we should be aware that changes in lipid composition do occur during development and/or disease states, which can alter drug action *in vivo* (Baenziger et al., 2008). Membrane-lipid therapy, a novel therapeutic approach in which membrane lipids constitute the main molecular target (Escribá, 2006), highlights the importance that membrane lipid composition and dietary interventions may hold in a therapeutic context. Our work is a framework to a new therapeutic paradigm in which modulation of diet composition may constitute a tool in personalized medicine or mitigate drug-induced idiosyncratic reactions.

5 - Acknowledgements

The project was supported by the Foundation for Science and Technology (FCT, Portugal) with FEDER/COMPETE/National Budget funds (research grants PTDC/QUI-QUI/101409/2008 to P.J.O., PTDC/QUI-BIQ/103001/2008 to A.S.J. and PEst-C/SAU/LA0001/2013-2014 to the CNC). J.P.M. and A.M.S acknowledge FCT for Ph.D. grants SFRH/BD/37626/2007 and PTDC/AGR-ALI/108326/2008 respectively.

6 – Conflict of interests

The authors declare that they have no conflict of interests. The funding agency (FCT Portugal) had no role in deciding the manuscript contents or in any decision to publish.

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Table I: Reported mitochondrial effects of the drugs used in this study.

	Mitochondrial Oxidative Phosphorylation	**Mitochondrial Permeability Transition**	**Oxidative Stress**	**Apoptosis**
FCCP	Uncoupler (Starkov, 1997)	Induction or inhibition, depending on concentration (Aronis et al., 2002; Petronilli et al., 1993)	Stimulation (Scorrano et al., 1997)	Inducer (Demasi et al., 2006)
Menadione	Stimulation in state 4; decrease in RCI and ADP/O ratio (Klöhn and Neumann, 1997)	Stimulation (Gerasimenko et al., 2002)	Stimulation (Criddle et al., 2006)	Inducer (Gerasimenko et al., 2002)
Nimesulide	Uncoupler (Caparroz-Assef et al., 2001)	Stimulation (Berson et al., 2006)	Stimulation (Ong et al., 2006)	Inducer (Li et al., 2003)

Legends for figures

Fig. 1: Effects of menadione (A - D) or nimesulide (E - H) on the oxygen consumption rate of liver mitochondria from rats fed a control diet (black and dark grey bars) or a diet containing 20% rapeseed oil (white and light grey bars). Mitochondria were incubated with 20 nmol menadione or 40 nmol nimesulide per mg of protein (dark and light grey bars) or with the corresponding volume of ethanol vehicle (black and white bars), as controls, for 3 min before the beginning of the assay. Substrates used for the respiratory studies were a mixture of glutamate plus malate (Complex I) or succinate (Complex II). The parameters represented in the figure are respiratory states 3 and 4 (St. 3 and St. 4, respectively), uncoupled respiration (St. FCCP), RCI and the ADP/O ratio. The extent of the action of the drug (C and D for menadione, and G and H for nimesulide) was evaluated by subtracting from the control value (incubation with ethanol) the corresponding value in the presence of drug (black bars for the difference between black and dark grey bars from A and B or E and F; white bars for the difference between white and light grey bars from A an B or E and F). Values depicted are means ± standard error of mean for 8 animals after 33 days of dietary treatment. Two-way ANOVA test with Bonferroni post-test was performed using drug presence (***, $P < 0.001$; **, $P < 0.01$; *, $P < 0.05$) and diet (^{ooo}, $P < 0.001$; ^{oo}, $P < 0.01$; ^{o}, $P < 0.05$) as variables ($^{\#\#}$, $P < 0.01$; $^{\#}$, $P < 0.05$). Comparisons between the extension of the action of the drug (C, D, G and H) were performed using one-way ANOVA, with the Student-Newman-Keuls as a post-test for diet control rats versus rats fed the modified diet, for the same parameter (***, $P < 0.001$; **, $P < 0.01$; *, $P < 0.05$).

Fig. 2: Menadione (A – D) and nimesulide (E – H) effects on mitochondrial transmembrane potential (ΔΨ) of liver mitochondria from rats fed a control diet (black and dark grey bars) or a diet containing 20% rapeseed oil (white and light grey bars). Mitochondria were incubated with 20 nmol menadione or 40 nmol nimesulide per mg of protein (dark and light grey bars) or with a corresponding volume of ethanol vehicle (black and white bars), as controls**,** for 3 min before ADP addition. Substrates used to energize mitochondria were a mixture of glutamate plus malate (Complex I) or succinate (Complex II). The parameters represented in the figure are the initial maximal transmembrane electric potential ($\Delta\Psi_i$) developed in the presence of the substrate and drug (or ethanol in the case of controls), depolarization induced by ADP ($-\Delta\Psi_{ADP}$), potential after repolarization ($\Delta\Psi_{rep}$) and phosphorylative lag phase. The extent of the action of the drug (C and D for menadione, and G and H for nimesulide) was evaluated by subtracting from the control value (incubation with ethanol) the corresponding value in the presence of drug (black bars for the difference between black and dark grey bars from A and B or E and F; white bars for the difference between white and light grey bars from A and B or E and F). Values depicted are means ± standard error of mean for 8 animals after 33 days of dietary treatment. Two-way ANOVA test with Bonferroni post-test was performed using drug presence (***, $P < 0.001$; **, $P < 0.01$; *, $P < 0.05$) and diet (^{ooo}, $P < 0.001$; ^{oo}, $P < 0.01$; ^{o}, $P < 0.05$) as variables ($^{\#\#}$, $P < 0.01$; $^{\#}$, $P < 0.05$). Comparisons between the extension of the action of the drug (C, D, G and H) were performed using one-way ANOVA, with the Student-Newman-Keuls as a post-test for diet control rats versus rats fed the modified diet, for the same parameter (***, $P < 0.001$; **, $P < 0.01$; *, $P < 0.05$).

Fig. 3: FCCP-induced depolarization of liver mitochondria from rats fed a control diet (black bars) or a diet containing 20% rapeseed oil (white bars). Three consecutive additions of small pulses of FCCP (10 pmol FCCP/mg of protein) were performed on energized mitochondrial fractions, using either a mixture of A) glutamate plus malate (complex I) or B) succinate (complex II) as respiratory substrates, and the severity of the effects found was compared between experimental groups. Assays with ethanol (FCCP solvent) were performed for control purposes, but they are not represented since ethanol had no noticeable effects on $\Delta\Psi$. Values depicted are means ± standard error of mean for 8 animals after 33 days of dietary treatment. Comparisons were performed using one-way ANOVA, with the Student-Newman-Keuls as a post-test for diet control rats vs. rats fed the modified diet. ***, $P < 0.001$; **, $P < 0.01$; *, $P < 0.05$.

Fig. 4: Effect of menadione and nimesulide on calcium-induced permeability transition of rat liver mitochondria from rats fed a control (black bars; full lines) or a rapeseed oil-enriched diet (white bars; dashed lines) for 33 days, determined by absorbance decrease as a result of mitochondrial swelling. The rates of absorbance decrease are represented in A. The extent of drug action, as determined by subtracting the rates of the controls (incubation with solvent) to the corresponding values in the presence of drugs, is represented in B. Succinate-energized mitochondria were incubated with 200 nmol menadione per mg of protein or 50 nmol nimesulide per mg of protein for 3 min before the addition of Ca^{2+} (66.7 nmol/mg protein). Typical traces for the mitochondrial swelling experiments are represented in C. Cyclosporin A, a classic MPT pore inhibitor, completely halted calcium-induced mitochondrial swelling, as shown in C. Values depicted are means ± standard error of mean for 7 independent experiments (provided by 7 different mitochondrial preparations from 7 different animals). Comparisons were

performed using one-way ANOVA, with the Student-Newman-Keuls as a post-test for diet control rats vs. rats fed the modified diet. ***, P <0.001; **, P <0.01; *, P <0.05.

Fig. S1: Typical oxygen consumption traces for complex II, as followed with a Clark type electrode, in the presence of menadione (A) or nimesulide (B); for complex I similar profiles were obtained. Typical $\Delta\Psi$ traces for mitochondrial preparations of control diet-fed rats, as followed by a TPP^+ electrode, are represented in C (with menadione) and D (with nimesulide); for mitochondrial preparations from rats fed the modified diet, similar profiles were obtained.

Figure 1

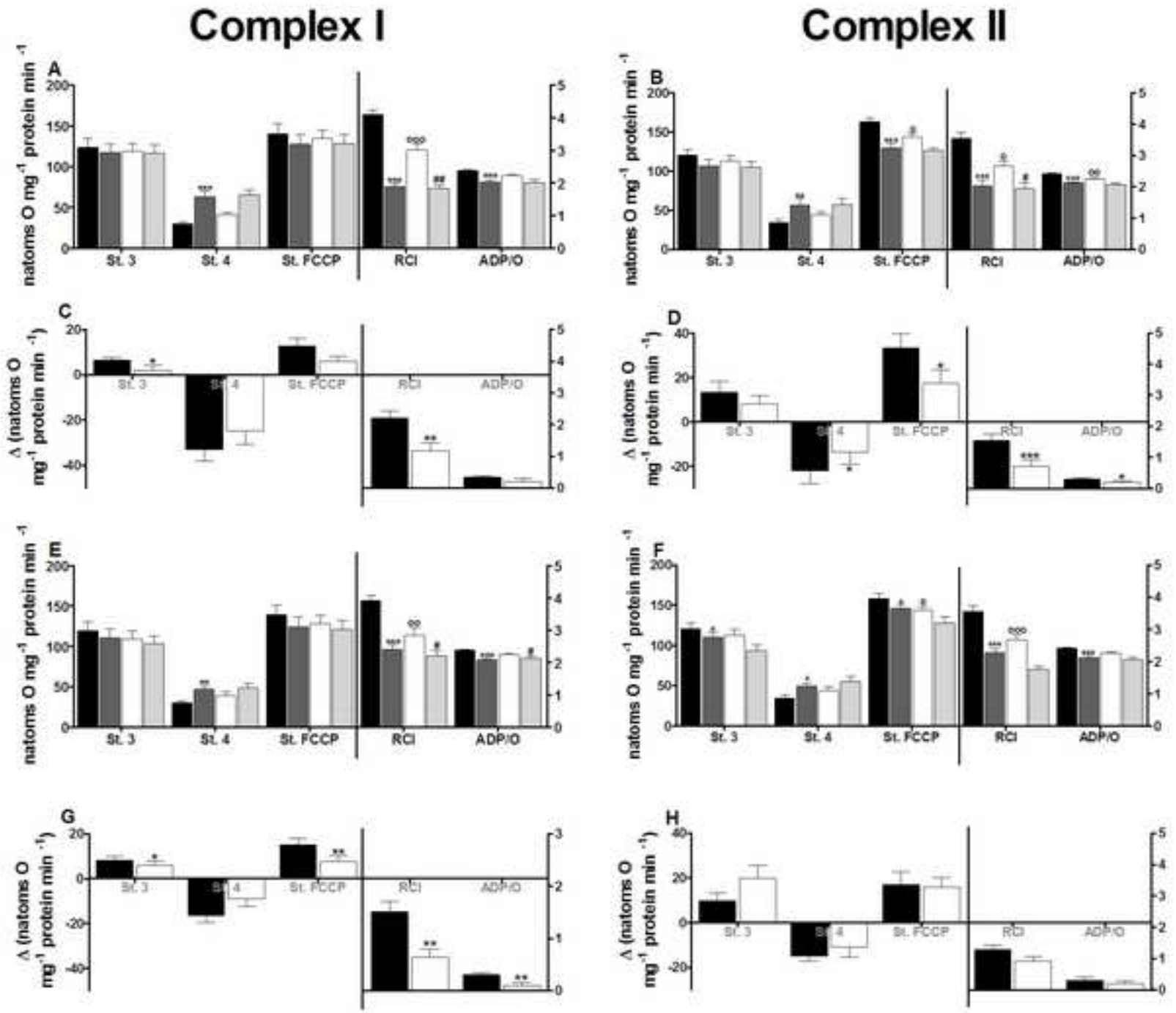


Figure 2

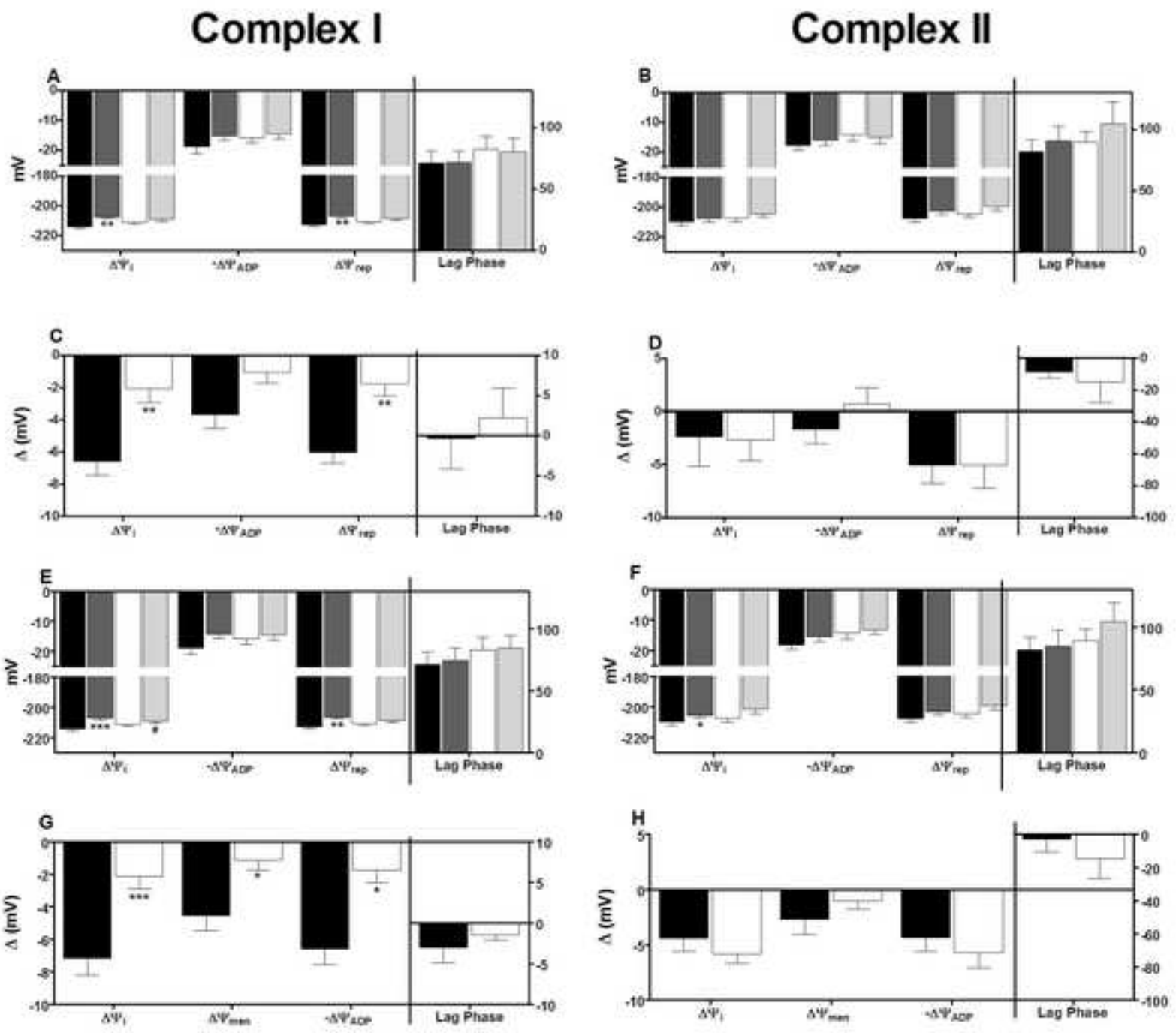


Figure 3

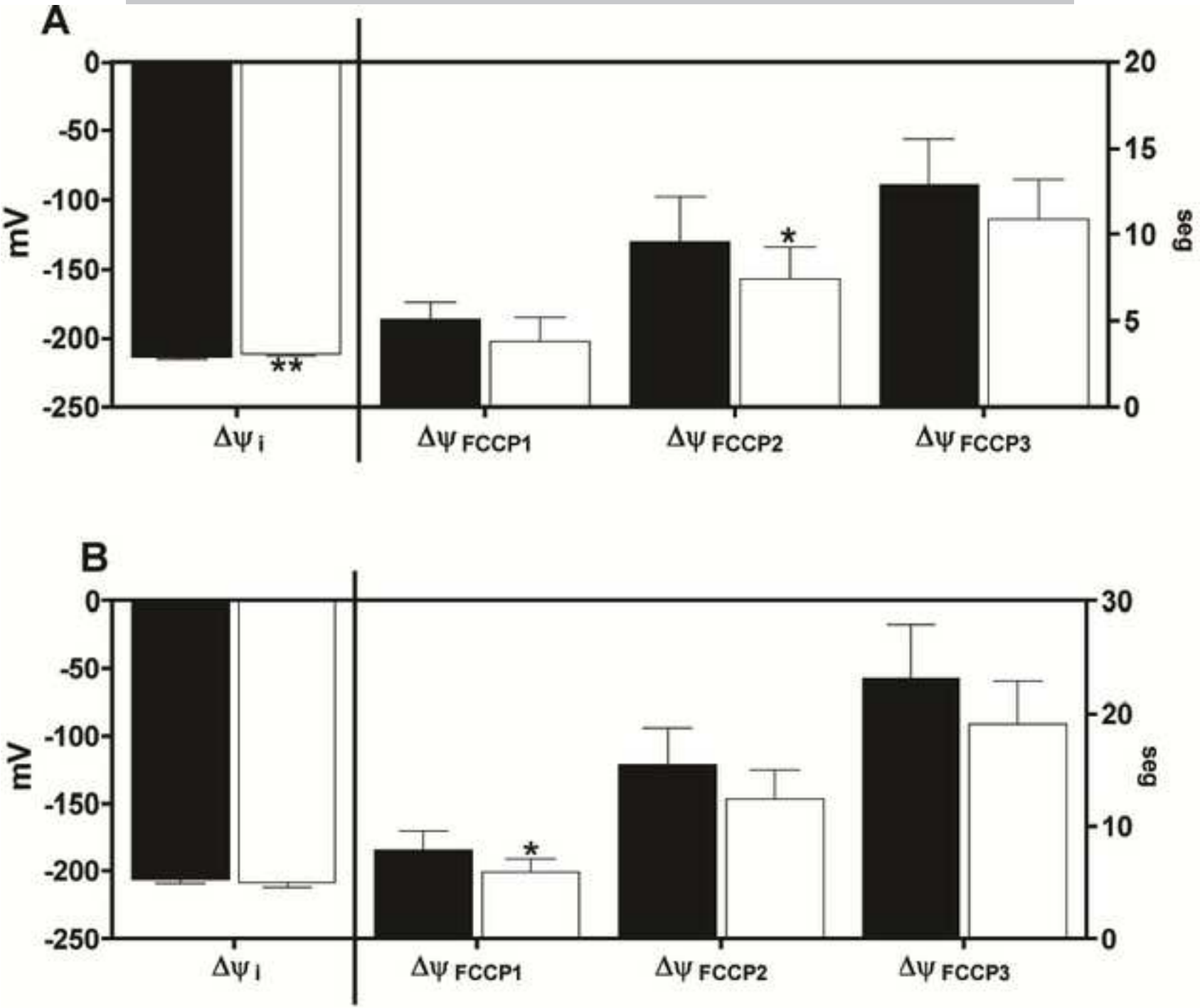
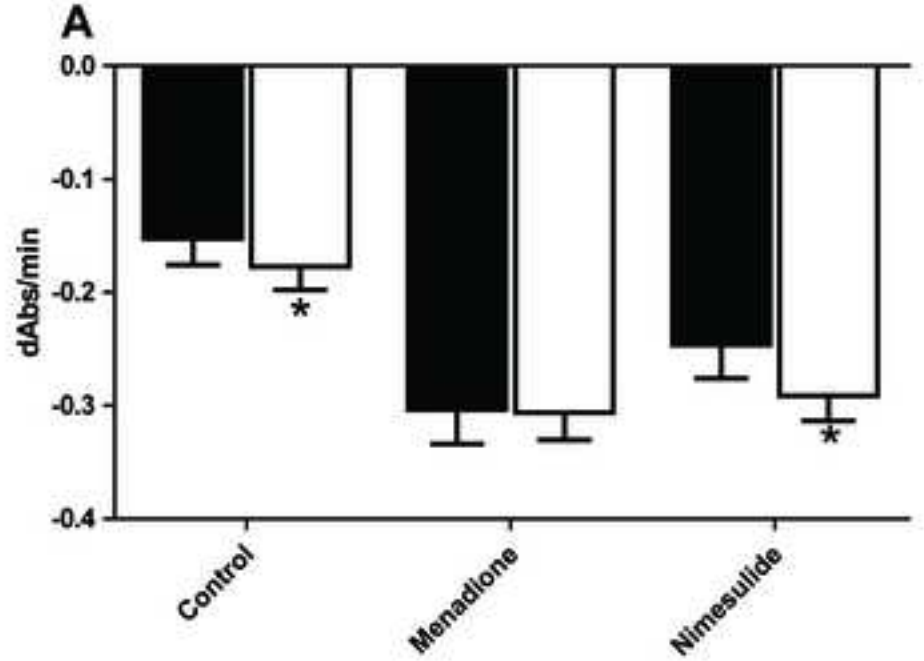
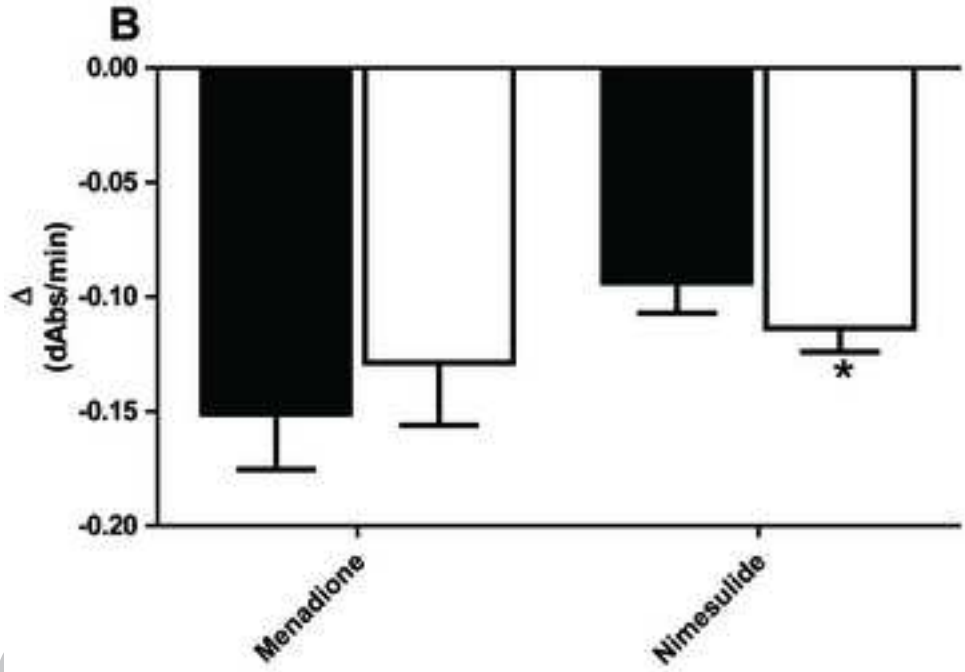
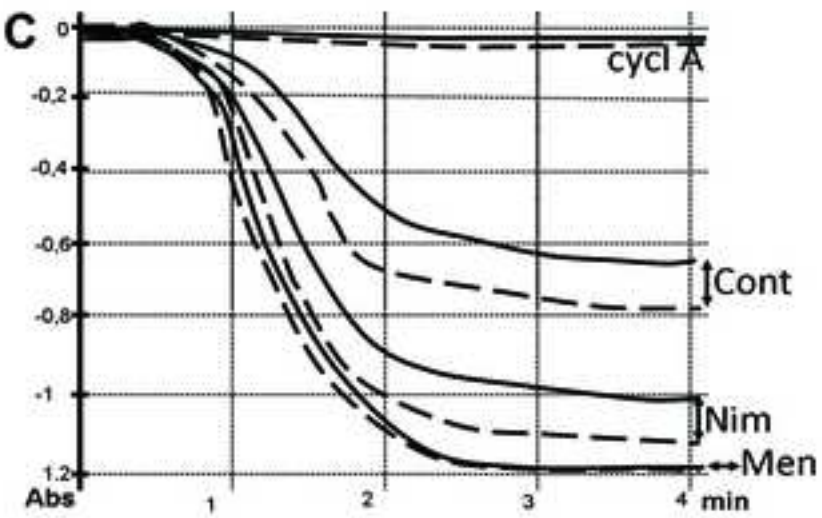


Figure 4







Highlights

- Rapeseed oil-rich diet modifies mitochondrial bioenergetics
- *In vitro* menadione and nimesulide mitochondrial toxicity was lower in the modified diet group
- FCCP showed a lower mitochondrial uncoupling effect in the modified diet group
- Diet manipulation can alter the activity of hepatic mitochondrial toxic agents