Ethanol drinking, brain mitochondrial DNA, polyunsaturated fatty acids and effects of dietary anthocyanins

Short title:

Ethanol, brain fatty acids and mitochondria

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ABSTRACT

Background: This study aimed at exploring whether moderate ethanol drinking may have adverse effects on the fatty acids composition and on mitochondrial DNA (mtDNA) of rat brain. A secondary aim was to examine whether dietary antioxidant anthocyanins (ACN) can be protective.

Methods: One group of rats received ethanol 12% and another water as an exclusive liquid to drink for 8 weeks. In order to test the impact of ACN consumption, two other groups of rats were fed an ACN-rich diet in combination with either ethanol or water. Brain fatty acids were measured by gas chromatography and mtDNA alterations, markers of mitochondrial suffering, were studied through an original real-time qPCR-based protocol.

Results: Linoleic acid (LA, 18:2n-6) and eicosadienoic acid (20:2n-6) were significantly decreased, by 12% and 31% respectively, in the brains of both ethanol groups. The other brain lipids, including arachidonic acid (20:4n-6) and n-3 polyunsaturated fatty acids, were not modified. These changes were associated with a significant increase in deleted mtDNA (by 28%) in the ethanol group, without total mtDNA depletion. The ACN-rich diet prevented the increase in mtDNA common deletion (mtDNA CD).

Conclusion: These data demonstrate that moderate ethanol drinking reduces certain brain n-6 and results in mtDNA injury. The antioxidant anthocyanins protect brain mtDNA but do not restore normal n-6 levels. Further studies are required to investigate the consequences of a decrease in n-6 levels in brain.

Keywords: ethanol; brain; polyunsaturated fatty acids; mitochondrial DNA; anthocyanins

ABBREVIATIONS

AA: Arachidonic Acid

ACN: Anthocyanins

DHA: DocosaHexanoic Acid

DPA: DocosaPentaenoic Acid

CONT: Control

EDA: EicosaDienoic Acid

EPA: EicosaPentanoic Acid

ETH: Ethanol

LA: Linoleic acid

mtDNA: mitochondrial DNA

mtDNA-CD: mitochondrial DNA common deletion

INTRODUCTION

Heavy ethanol drinking is thought to result in adverse effects on the brain (Guerri and Pascual, 2010; Lamarche et al., 2013; Mansouri et al., 2001; Qin et al., 2008; Volkow et al., 2008). Among these effects, ethanol may alter the metabolism of some brain fatty acids. Pawlosky reported that the brains of chronic alcohol-exposed cats (Pawlosky and Salem, 1995) and rhesus monkeys (Pawlosky et al., 2001) exhibit reduced levels of docosahexanoic acid (DHA or 22:6n-3), the main brain n-3, while docosapentaenoic acid (DPA or 22:5n-6) is increased, maybe due to a compensatory mechanism. This reciprocal change in the ratio of 22:6n-3 to 22:5n-6 is known to be associated with a loss in nervous system function (Uauy et al., 1992) and may provide a biochemical mechanism underlying some of the neuropathology associated with alcoholism (Pawlosky et al., 2001). Arachidonic acid (AA or 20:4n-6), the main brain n-6, was unchanged in these two studies (Pawlosky and Salem, 1995; Pawlosky et al., 2001).

Mitochondria are major targets for ethanol toxicity in different tissues (Demeilliers et al., 2002; Mansouri et al., 2001; Marin-Garcia et al., 1995), including the brain (Lamarche et al., 2013; Mansouri et al., 2001; Marin-Garcia et al., 1995). Oxidative stress due to ethanol metabolization causes extensive degradation and depletion of brain, heart, liver, and skeletal muscle mitochondrial DNA (mtDNA) in mice. MtDNA, which codes for 13 of the oxidative phosphorylation proteins, is more susceptible to oxidative damage than nuclear DNA, due to the absence of protective histones and to its proximity with the mitochondrial respiratory chain, which is the main cellular site of reactive oxygen species formation in the cells. Thus ethanol-induced oxidative stress causes diverse mtDNA lesions, including oxidized DNA bases, apurinic/apyrimidinic sites, as well as mtDNA strand breaks, resulting in mtDNA depletion (Demeilliers et al., 2002; Mansouri et al., 2001, 1999, 1997). Among these lesions, the so-called mitochondrial mtDNA “common deletion” (mtDNA-CD) is the most frequent and best characterized mutation in mtDNA. It is a large deletion of 4977 bp in humans (4834 bp in rats), affecting several genes coding for several subunits of NADH

dehydrogenase and one subunit of cytochrome oxidase. Even though deleted mtDNA represents only a small fraction of the damage to mtDNA, a quantitative analysis of mtDNA-CD is considered to be a sensitive and early marker for mitochondrial suffering (Peinnequin et al., 2011). Importantly, the ethanol-induced mtDNA alterations might be prevented by antioxidants as vitamin E, melatonin or coenzyme Q10 (Demeilliers et al., 2002; Mansouri et al., 2001, 1999).

The main goal of the present study was to test whether moderate chronic ethanol drinking could induce a change in fatty acid metabolism and/or alterations of mtDNA in rat brains.

The second goal was to evaluate whether an anthocyanin (ACN)-rich diet can be protective. Despite there is an emerging view that ACN may also act by modulating signalling pathways thereby impacting the activity of metabolic pathways (Martin et al., 2013, 2011), the health benefits of ACN have been mostly attributed to their antioxidant properties (Terao, 2009). Therefore, they may have a protective effect against mtDNA lesions induced by ethanol-oxidative stress. Our previous study on a rat model showed that an ACN-rich diet induces a significant increase in eicosapentaenoic acid (EPA or 20:5n-3) and DHA levels in plasma (Toufektsian et al., 2011), indicating that ACNs interact with the metabolism of n-3s. It was therefore hypothesized that ACN may compensate for the possible decrease in brain DHA induced by ethanol, as previously described in cat and monkey models (Pawlosky and Salem, 1995; Pawlosky et al., 2001).

MATERIAL AND METHODS

Animals and experimental protocols

Sixty male Wistar rats (1 month old, initial body weight 75-100 g) were purchased from Charles River Laboratories. The animals were cared for according to the European Community Council Directive L358-86/609/EEC on the care and use of laboratory animals. The protocols were performed under license from the French Ministry of Agriculture (license No. A380727) and approved by the local animal ethics committee. The rats were housed under conditions of constant

temperature, humidity and standard light-dark cycle (12h/12h). Food and tap water were consumed *ad libitum*.

The rats were fed a standard diet (A04) while acclimating, before being distributed into the experimental groups. In order to evaluate the effects of alcohol on brain fatty acid composition and mtDNA, they received either tap water (CONT) or ethanol 12% (v/v in water) (ETH) as sole drinking liquid for a period of 8 weeks.

In order to test the impact of ACN consumption on the same parameters, two other groups of rats were fed an ACN-rich diet in combination with either tap water (ACN) or ethanol 12% (v/v) (ACN-ETH) for 8 weeks. At the end of the 8-week dietary trials, the animals were anaesthetized with an intraperitoneal injection of pentobarbital (60 mg/kg). The whole brains were then rapidly excised and the cerebellums were discarded. Brain samples were frozen and homogenized in a metallic mortar cooled down in liquid nitrogen. The homogenates were aliquoted and stored at –80°C for subsequent fatty acid and mtDNA analysis.

ACN content of the food pellets.

These experimental diets containing (ACN-rich) or not ACN (ACN-free) were prepared as previously reported (Toufektsian et al., 2008). Briefly, the ACR genotype carried the *R-r* allele, conferring high anthocyanin accumulation to the aleurone of the seed, whereas the *r-Δ902* genotype (here referred to as *r1*) carried a deletion containing the *r1* locus and is totally devoid of pigment (Toufektsian et al., 2008). The *ACR* and *r1* genotypes previously in a W22 background were crossed to a commercial hybrid stock and the F1 progeny seeds were used to produce 2 synthetic populations characterized by a high level (ACN-rich) or an absence of ACN. Maize content from a standard pellet formula (A04, SAFE, France) was replaced by maize seed powder obtained from either the ACR (ACN-rich) or the *r1* genotype (ACN-free). Both diets were equivalent in energy, with macronutrient concentrations of 67% carbohydrates, 23% protein and 10% lipids (SAFE, France). Both the ACN-rich and ACN-free diets were similar in terms of fatty acid composition

(Toufektsian et al., 2011). Moreover, as previously reported (Toufektsian et al., 2008), HPLC analyses showed that ACN were detected in the ACN-rich seeds but were entirely absent from the ACN-free maize seeds. The same ACN remained in the food pellets. Quantitative analyses indicated that the ACN -rich diet contained ~0.24±0.01mg of ACN/g of food pellets.

Brain fatty acids analysis

Brain lipids were extracted in hexane/isopropanol as previously described (Guiraud et al., 2008). Briefly, methylated fatty acids were extracted with hexane, separated and quantified by gas chromatography using an Agilent Series Gas chromatography apparatus. Methyl ester peaks were identified by comparing their retention time to those of a standard mixture. Saturated, mono- and poly-unsaturated fatty acid levels were expressed as a percentage of total fatty acid content.

Brain mtDNA analysis

The procedures used to perform a semi-quantitative analysis of total mtDNA and of mtDNA-CD have been described previously (Peinnequin et al., 2011). Briefly, brain tissue was disrupted using a Retsch MM 301 mixer mill (2 min, 30 Hz, 2-mm tungsten carbide bead) in 1mL of 1X lysis buffer (Tween 20 0.05% v/v; NP40 0.05% v/v; Tris HCl 10mM pH 8) and proteinase K was added to a final concentration of 0.1mg/mL. The samples were incubated at 56°C for 30 min and the proteinase K inactivated by heating at 98°C for 15 min.

The lysate was diluted as previously described (Peinnequin et al., 2011). These dilution steps were performed in order to homogenize the detergent present in the lysis buffer and to obtain reproducible qPCR efficiency. The LightCycler FastStart DNA Master SYBR Green I kit (Roche) was used to perform qPCR analysis. Each qPCR reaction was carried out using 5µl of final lysate, 7mM of $MgCl_2$ and 0.4µM of both forward and reverse primers. Forward and reverse primers were as follows: for mtDNA 5'-GGGTTAAAAACCGACGCAATC-3' and 5'-AATGGGTATGAAGCTGTGATTTGAG-3'; for deleted mtDNA 5'-

TCAGCAACCGACTACACTCATTTC-3' and 5'- AGTTATGGATGTGGCGATTAAAGTG-3'; for GAPDH 5'-CCTGTTCATCCCTCCACACATC-3' and 5'-CCAGTGATTTTCCAGCCCTAATC-3'. The PCR was performed using a Lightcycler (Roche) for 45 cycles at 95°C for 20 s, 54°C for 5 s, and 72°C for 8 s. All assays were carried out in duplicate. For each sample, PCR efficiency was assessed using the LinRegPCR software. The relative quantification was then achieved using the comparative threshold cycle method, with efficiency correction using the average value of measured PCR efficiencies. The data are expressed using arbitrary relative units, depending on the calibrator value (Peinnequin et al., 2011).

Statistical analysis

Statistical analysis was done with Stata 12^{TM} (Stata Corp, College Station, Texas). The data are expressed as mean ± SEM. Comparisons were performed using one-way ANOVA followed, when authorized, by contrast analysis using Bonferroni correction. P values < 0.05 were considered statistically significant.

RESULTS

Food consumption and body weight

The food consumption is 22,5±0,6g/j (i.e ~64kcal), 21,8±0,2g/j (i.e ~62kcal), 16,6±0,5g/j (i.e ~47kcal) and 18,8±1,1g/j (i.e ~53kcal) respectively for control, ACN, ethanol and ethanol+ACN. The reduction of food intake on the rats exposed to alcohol is balanced by the alcohol intake which provides ~12 kcal per day.

The evolution of the body weight did not differ significantly between the control and the ACN-rich diet groups (Figure 1). However, a longitudinal analysis show that ethanol has significant negative effect on body weight evolution (p=0.001 by a GEE population-averaged model). For example, at the 8th week, the weight gain is 266g +/-8 for the control rats, whereas is 246g +/-7 for the rats

receiving ethanol ($p=0.046$). Interestingly, the group receiving ethanol and ACN did not differ significantly from the control.

Effects of ethanol and an ACN-rich diet on brain fatty acids

Saturated and monounsaturated fatty acids did not differ between groups (table 1). Regarding polyunsaturated fatty acids, in particular AA (20:4n-6) and DHA (22:6n-3), we found no difference between groups (table 1). However, two minor brain n-6 fatty acids, linoleic acid (LA or 18:2n-6) and eicosadienoic acid (EDA or 20:2n-6), were significantly decreased in the two groups that had consumed ethanol (table 1). LA decreased by 12% and 20% respectively in the ETH and ACN-ETH groups, whereas EDA decreased by 31% and 38%. No other significant difference was observed (table 1).

Effects of ethanol and an ACN-rich diet on brain mtDNA

We observed no significant change in the mtDNA/GAPDH ratio, and therefore no mtDNA depletion in the ethanol groups (Figure 1A). However, the deleted mtDNA /total mtDNA ratio increased significantly in the ethanol group (by 28%, Figure 1B) as compared with the control ($p<0.05$). There was no significant difference between CONT and both the ACN and ACN-ETH groups.

DISCUSSION

In this study in rats, we examined whether moderate ethanol drinking may be harmful for the brain. Two issues were examined: brain fatty acid composition and mtDNA alterations. Ethanol exposure over a period of 8 weeks resulted in a minor but significant changes in two n-6 fatty acids and in an increase in mtDNA-CD in the brain. The metabolism of other fatty acids, including n-3s, was not modified. We also observed that an ACN-rich diet could prevent the apparition of mtDNA-CD, but did not result in any change in n-6.

Ethanol and brain fatty acid composition

In our rat model, moderate ethanol drinking did not result in any significant variation in ALA (18:3n-3), DHA and total n-3s. However, two minor brain n-6 fatty acids (LA or 18:2n-6 and EDA or 20:2n-6) significantly decreased in the two groups following ethanol consumption (table 1). These results are partly in line with those observed in humans (de Lorgeril et al., 2008). We actually showed a progressive decline in LA plasma levels with increased wine ethanol intake, whereas ALA and DHA were unchanged regardless of ethanol consumption. In the present study, the decrease of LA (18:2n-6) and EDA (20:2n-6) was probably a compensatory mechanism with no decrease in AA (20:4n-6), a major second messenger in brain (Bosetti, 2007; Kiso, 2011; Rapoport, 2008). Ethanol was shown to induce a release of AA and its metabolites (Basavarajappa et al., 1998; Lin et al., 1988) by the activation of phospholipase A2 in brain rodents (Basavarajappa et al., 1998). This could alter membrane physiology and be involved in ethanol tolerance and dependence. We hypothesize that LA (18:2n-6) was preferentially consumed to synthesize AA in order to maintain a constant AA level. Thus, LA slightly decreased in brain lipids, while the AA concentration remains unchanged at least in the short term.

Our study was in apparent contradiction with the results of Pawlosky, who showed that ethanol intoxication significantly decreases DHA concentration in the brains and retinas of felines and in the

brains of rhesus monkeys (Pawlosky et al., 2001). In addition to the loss of DHA, a significant increase in DPA was also observed. This lipid change is associated with a loss in nervous system function (Uauy et al., 1992) and may provide a biochemical mechanism underlying some of the neuropathology associated with alcoholism (Pawlosky et al., 2001). In these two studies, however, the duration of alcohol intake was much longer than in our 8-week study, namely 2,5 years (Pawlosky et al., 2001) and 6 to 8 months (Pawlosky and Salem, 1995). Finally, the present study examined fatty acids in the whole brain; however, dissection of the various brain areas might have allowed detecting changes in fatty acid composition following treatments.

Finally, the ACN-rich diet had no protective effect regarding the modification of brain fatty acids. This does not contradict our previous study, in which we observed an interaction between ACN and omega 3, but not between ACN and omega 6 metabolism (Toufektsian et al., 2011).

Ethanol and brain mtDNA

In this study, mtDNA-CD in the brain increases after ethanol exposure, suggesting that brain mitochondria have suffered.

Ethanol exposure increases the formation of reactive oxygen species by the mitochondria (Demeilliers et al., 2002; Mansouri et al., 1999), and this may lead to oxidative damage of mtDNA (Demeilliers et al., 2002; Mansouri et al., 1999). Oxidative damage of DNA produces single-strand breaks and favors slipped mispairing of repeated sequences during replication. This may explain the occurrence of mtDNA-CD, which removes the DNA between two repeated sequences as well as one of the repeated sequences. This mtDNA-CD cannot be repaired by the mitochondria, and therefore they accumulate. Although mtDNA-CD has been extensively studied, it may be just one among many other mtDNA mutations that are difficult to detect because of the low ratio of mtDNA with a given mutation (i.e. oxidized DNA bases, apurinic/apyrimidic sites). Considering this "tip-of-the-iceberg" hypothesis and the accumulation of this mtDNA-CD, a quantitative analysis of mtDNA-CD is considered to be a sensitive and early marker for mitochondrial mutations and

suffering and has been detected in the liver of alcoholic patients (Mansouri et al., 1997). Moreover, these diverse mtDNA lesions cause mtDNA depletion in experimental animals (Demeilliers et al., 2002; Mansouri et al., 2001, 1999). Thus, in comprehensive molecular studies where mitochondrial disorders can be involved, there is growing interest in a quantitative analysis of mtDNA-CD, in addition to the determination of the total mtDNA content.

In our rat model, there is no depletion of mtDNA in any group. An adaptive synthesis of mtDNA probably contributes in maintaining the mtDNA level, as already observed in other ethanol intoxication model (Demeilliers et al., 2002; Mansouri et al., 2001). However, the mtDNA-CD/total mtDNA ratio significantly increased after ethanol drinking as compared with controls, which may be due to an alcohol-induced oxidative stress. As a matter of fact, when ethanol was associated with an ACN-rich diet known for its antioxidant effect (Terao, 2009), the accumulation of mtDNA-CD was not significantly different from the control.

Limitations of the study

First, the amount, duration and route of ethanol administration may influence the tissue concentration of polyunsaturated fatty acids. This may in turn explain the apparent contradiction with Pawlosky's findings (Pawlosky and Salem, 1995; Pawlosky et al., 2001). Also, the animal species, the amount of ethanol and the duration of the ethanol exposure were not the same in this study and in Pawlosky's, and thus the adaptive mechanism was probably different.

Second, we did not investigate the effect of ethanol intoxication on mitochondrial function. Taking mitochondrial heteroplasmy into account, we cannot say if there is a significant consequence of the accumulation of mtDNA-CD on the mitochondrial respiration rate or on the permeability transition pore. Our aim was to identify a possible harmful effect on the brain, as shown through measurements of a marker of mitochondrial suffering, but not a specific functional parameter. Further studies are required to explore these functional issues.

Third, additional studies are required to understand at which level of their metabolism (intestinal absorption, regulation of lipolysis and lipogenesis, whole-body fuel utilization, regulation of n-6 elongation and desaturation) ethanol and n-6 interact.

Conclusions

In this study, we have shown that ethanol leads to a decrease in AA (18:2n-6) and EDA (20:2n-6) levels in the brain. These changes were associated with increased mtDNA-CD, a marker of mitochondrial suffering. When ethanol was associated with an ACN-rich diet, there was no accumulation of mtDNA-CD. However, the ACN-rich diet had no effect on the modification of brain fatty acid composition. Further studies are required to investigate the functional consequences of this decrease in brain LA levels.

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There are no conflicts of interest.

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M.C.T., P.S and M.d.L designed and conducted the research; C.D provided the measure of deleted and total mtDNA and wrote the paper; F.L was in charge of the fatty acid measurements. K.P. provided the ACN-rich and ACN-free corn lines and designed the experimental diets. H.R and C.D performed the statistical analysis. All authors read and approved the final manuscript.

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LEGENDS

Figure 1. Effect of 8-week water (CONT) or ethanol 12% drinking (ETH) with/or not ACN rich diet on body weight. ♦ CONT (n=14); ▲ ACN (n=12); ■ ETH (n=14); × ETH-ACN (n=12).

Figure 2. Brain total mitochondrial DNA (total mtDNA) and mitochondrial DNA-common deletion (mtDNA-CD) levels assessed by real-time PCR as previously described (Peinnequin et al., 2011) after 8 -weeks with water (CONT) or ethanol 12% (ETH) drinking and/or with anthocyanins (ACN) rich diet. (A) Relative quantification of total mtDNA normalized by GAPDH, (B) Relative quantification of mtDNA-CD normalized by total mtDNA.

Data are mean ± SEM.

* $p<0.05$, versus control

400 **Table 1: Brain fatty acids composition**

Fatty acids		CONT (n=14)	ETH (n=16)	ACN (n=12)	ACN+ETH (n=12)
		(% total fatty acids)	*(% total fatty acids)*	*(% total fatty acids)*	*(% total fatty acids)*
Myristic	14:0	0.14 ± 0.01	0.13 ± 0.01	0.13 ± 0.01	0.12 ± 0.01
Palmitic	16:0	20.94 ± 0.43	21.24 ± 0.21	20.96 ± 0.24	20.75 ± 0.27
Stearic	18:0	19.97 ± 0.12	19.77 ± 0.09	20.34 ± 0.31	20.04 ± 0.15
Arachidic	20 :0	0.39± 0.15	0,31± 0.04	0,37± 0.07	0,31± 0.05
Total SFA		41.44 ± 0.49	41.45 ± 0.23	41.82 ± 0.45	41.21 ± 0.27
Oleic	18:1n-9	21.03 ± 0.52	20.47 ± 0.21	20.36 ± 0.28	20.14 ± 0.35
Vaccenic	18:1n-7	4,39± 0.44	4,19± 0.37	4,07± 0.47	4,13± 0.17
Total MUFA		25.86 ± 0.60	25.11 ± 0.28	24.86 ± 0.34	24.70 ± 0.39
Linoleic (LA)	18:2n-6	0.71 ± 0.02	**0.62 ± 0.02***	0.72 ± 0.05	**0.57 ± 0.03***
α-linolenic (ALA)	18:3n-3	1.52 ± 0.43	1.43 ± 0.22	1.43 ± 0.32	1.78 ± 0.10
Arachidonic (AA)	20:4n-6	11.11 ± 0.22	11.40 ± 0.09	11.25 ± 0.16	11.50 ± 0.18
Tetracosatetraenoic	22:4n-6	3,53±0,18	3,52±0,16	3,51±0,21	3,48±0,18
Docosahexanoic (DHA)	22:6n-3	14.85 ± 0.36	15.52 ± 0.16	15.38 ± 0.22	15.81 ± 0.21
Eicosadienoic	20:2n-6	0,16 ± 0.12	**0,11 ± 0.004***	0,16 ± 0.02	**0,1 ± 0.004***
Total omega 3		16.64 ± 0.40	17.23 ± 0.31	17.11 ± 0.43	17.88 ± 0.17
Total omega 6		15.96 ± 0.23	16.09 ± 0.09	16.11 ± 0.23	16.08 ± 0.20
Total PUFA		32,6 ± 0.39	33,22 ± 0.33	33,33 ± 0.32	33,96 ± 0.21
Omega 3/omega 6		1.05 ± 0.03	1.07 ± 0.02	1.07 ± 0.04	1.11 ± 0.02

401 Mean values were significantly different from those of the control group. p<0,05

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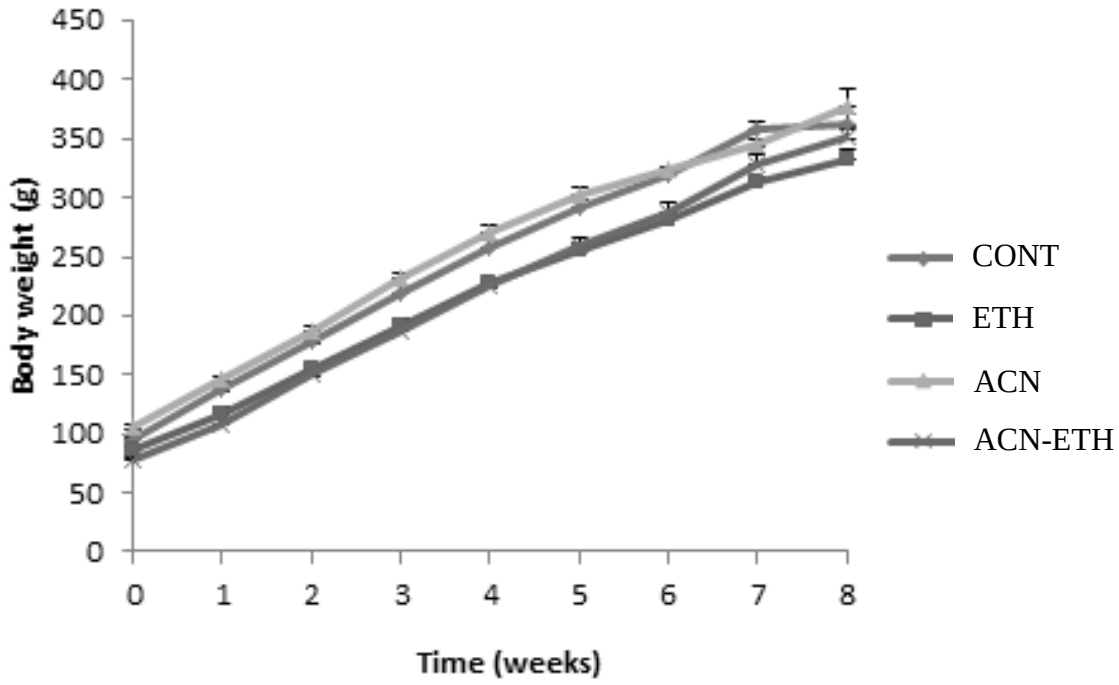
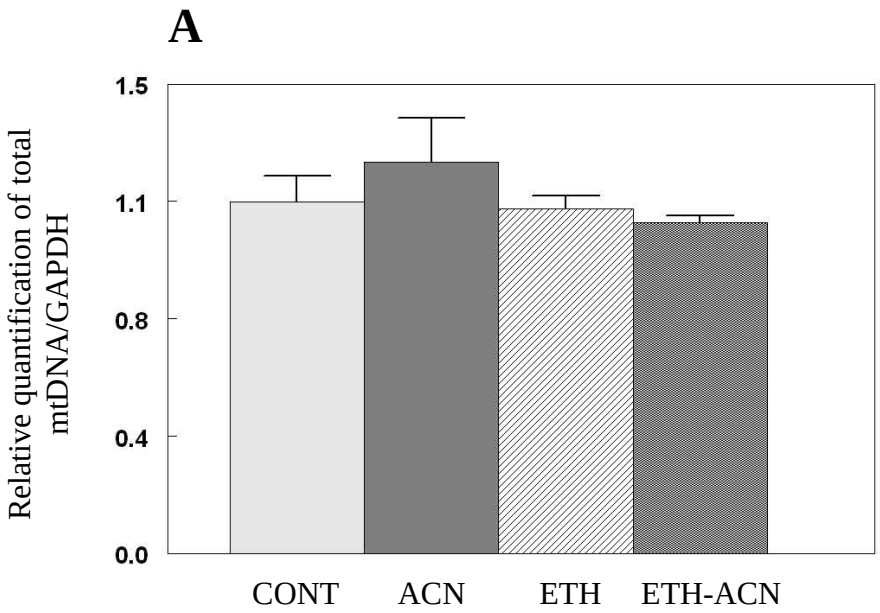
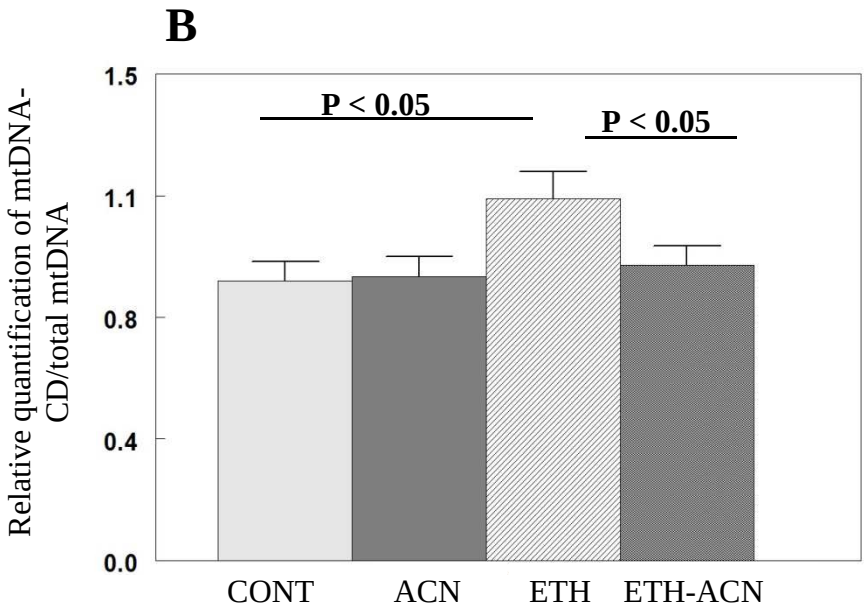
Table 1. **Brain fatty acid composition.** Brain fatty acid composition (expressed as % total fatty acids) after 8 -weeks with water (CONT) or ethanol 12% (ETH) drinking and/or with anthocyanins (ACN) rich diet (mean and SEM).


Body weight (g)
450
400
350
300
250
200
150
100
50
0
0
1
2
3
4
5
6
7
8
Time (weeks)
CONT
ETH
ACN
ACN-ETH


A
Relative quantification of total mtDNA/GAPDH
1.5
1.1
0.8
0.4
0.0
CONT
ACN
ETH
ETH-ACN


B
Relative quantification of mtDNA-CD/total mtDNA
P < 0.05
P < 0.05
1.5
1.1
0.8
0.4
0.0
CONT
ACN
ETH
ETH-ACN