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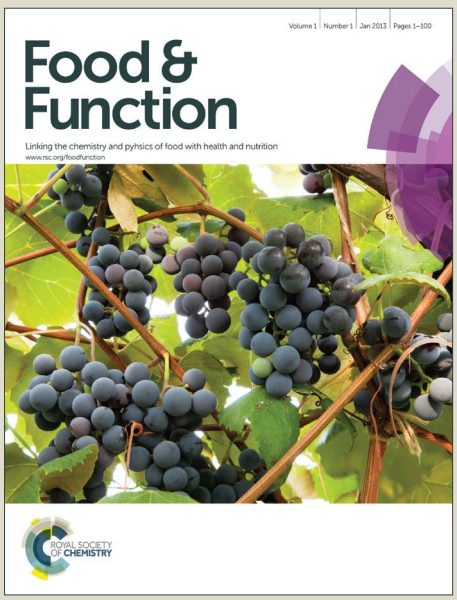

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Dietary docosahexaenoic acid-enriched glycerophospholipids exert cardioprotective effects in ouabain-treated rats via physiological and metabolic changes

Monique Bernard,[a*] Jean-Michel Maixent,[b] Alain Gerbi,[c] Carole Lan,[a] Patrick Jean Cozzone,[a] Gérard Pieroni,[d] Martine Armand [a,1] and Thierry Charles Coste [e,1]

[a] Aix-Marseille Université, CNRS, CRMBM UMR 7339, F-13385 Marseille, France

[b] EB2M-Protée, Université du Sud Toulon-Var, F-83957 La Garde, France

[c] RDVC Produits Santé, F-76600 Le Havre, France

[d] Application Santé des Lipides, ASL, Bioparc de Vichy, F-03270 Hauterive, France

[e] Novastell, F- 27150 Etrépagny, France

* Corresponding author at: CRMBM, CNRS UMR 7339 AMU, Faculté de Médecine Timone, 27 Boulevard Jean Moulin, 13385 Marseille cedex 05, France. Tel: + 33 4 91 32 48 18; Fax +33 4 91 25 65 39.

E-mail address: monique.bernard@univ-amu.fr (M. Bernard)

[1] Contributed equally as senior scientists

Short running title: cardioprotective effects of GPL-DHA

Abstract

The docosahexaenoic acid (DHA) might prevent heart failure or optimise drug treatments by improving cardiac contraction. We investigated whether DHA-enriched avian glycerophospholipids (GPL-DHA) exert cardioprotection in ouabain-treated rats after 4-weeks of dietary supplementation with 10, 35 or 60 mg DHA kg^{-1} body weight versus none (DHA10, DHA35, DHA60 and Control groups, respectively). Contractile responsiveness to different doses of ouabain (10^{-7} to 10^{-4} M), ouabain intoxication (at 3 x 10^{-4} M), and relative variations in cardiac energy metabolism were determined using ^{31}P NMR in isolated perfused rat hearts. The fatty acid composition of cardiac membranes was analysed by gas chromatography. DHA accretion in heart was dose-dependent (+8%, +30% and +45% for DHA10, DHA35 and DHA60, respectively). Cardiac phosphocreatine content significantly increased at baseline in DHA35 (+45%) and DHA60 groups (+85%), and at the different doses of ouabain in the DHA60 group (+73% to 98%). The maximum positive inotropy achieved at 10^{-4} M ouabain was significantly increased in all DHA groups versus control (+ 150%, + 122.5% and +135% for DHA10, DHA35 and DHA60, respectively), and ouabain intoxication was delayed. Increase in myocardial phosphocreatine content and the improved efficacy of ouabain on myocardial contraction without toxicity suggest the interest of GPL-DHA as a dietary supplement or ingredient for functional food, and possibly as a co-treatment with digitalis drugs in humans.

KEYWORDS Docosahexaenoic acid; Glycerophospholipids; Heart function; Phosphocreatine; ^{31}P NMR; Ouabain

Abbreviations: AA, arachidonic acid; DHA, docosahexaenoic acid; dp, developed pressure; EPA, eicosapentaenoic acid; PCr, phosphocreatine; pHi, intracellular pH; Pi, inorganic phosphate.

1. Introduction

Heart failure is the major cause of mortality in industrial countries. Numerous studies have supported the cardiovascular preventive and therapeutic effects of the intake of fish or fish oil supplements due to their high levels in n-3 long chain polyunsaturated fatty acids (LC-PUFA), mainly eicosapentaenoic (EPA, C20:5) and docosahexaenoic (DHA, C22:6) acids.[1-5] As a consequence, dietary recommendations have been set recently by several governmental and health organisations for preventing (minimum intake of 250 mg DHA + EPA per day for adults) or treating (450 mg to 4 g DHA+EPA per day) cardiovascular disorders.[4] Among their different cardiovascular effects, marine n-3 LC-PUFA might be beneficial against arrhythmia and sudden cardiac death although there are some inconsistent findings in the prevention of atrial fibrillation.[6-8] In addition, we previously found that fish oil supplementation in rats reduced the high toxicity and improved the positive inotropy of ouabain, as an example of digitalis drug administered to treat heart failure in humans.[9]

Discrepancies among studies could be explained in part by the heterogeneous ratio of EPA/DHA in fish or fish oils used in animal or clinical studies.[10] Furthermore, if EPA and DHA share common biological effects, some are quite specific (anti-thrombotic effect for EPA, anti-arrhythmic effect for DHA), or even dualist.[3,5,11-14] Moreover, a growing interest of DHA supplementation for myocardial function is related to the observation of a much higher level of DHA than EPA in cardiac tissue phospholipids[15-17], and of depressed levels of DHA in tissues of patients with coronary heart disease.[18]

In this study, we assessed the effects of an alternative source of DHA, i.e. DHA-enriched glycerophospholipids (GPL-DHA) obtained by hen's diet manipulation, on cardiac function in rats. We hypothesized that a 4-week supplementation with GPL-DHA will increase the DHA incorporation into cardiac membrane phospholipids leading to heart protection under ouabain through changes in its inotropic and toxic effects.

2. Materials and methods

2.1. Animals and diets

National guidelines for care and use of research animals were followed (agreement number A 13823, French Ministry of Agriculture). All animal procedures were approved by the Ethic Committee of Aix-Marseille University Medical School (agreement n° 40-10102012). Egg yolk powder containing glycerophospholipids (phosphatidylcholine and phosphatidylethanolamine) specifically highly enriched in DHA (GPL-DHA) was obtained from laying hens supplemented with fish oil containing DHA and EPA (ASL, Hauterive, France). Due to their specific metabolism, hens incorporate dietary DHA, but not EPA, from fish oil triglycerides into egg yolk mainly as phospholipids.[19] Eggs were collected, boiled, and yolks were pooled to prepare a dry powder for which more than 83% of the total DHA content was distributed to the sn-2 position of GPL-DHA. A regular egg yolk powder with GPL that contains naturally DHA, but at a much lower content, was obtained from hens receiving a standard diet. The fatty acid composition of both egg yolk powders is given in Table 1. Male Sprague Dawley rats (IFFA Credo, L'Abresle, France) weighing approximately 200 g were randomly divided into four groups of 13 animals (7 rats for NMR study and 6 rats for heart fatty acid composition analysis). Three experimental groups were fed a standard rat chow (A04 UAR, Villemoisson sur Orge, France; 16% protein, 60% carbohydrate, 3.5% fiber, 12.5% water, 3% fat with a ratio linoleic acid/linolenic acid of 13.1/1) supplemented with egg yolk powder and compared to a control group fed only the standard chow. DHA was provided daily for 4 weeks by regular egg yolk powder mixed with the chow at the dose of 10 mg DHA kg^{-1} body weight (DHA10), or by DHA-enriched egg yolk powder at the dose of 60 mg DHA kg^{-1} BW (DHA60), or by a mix (50/50) of these two egg yolk powders providing 35 mg DHA kg^{-1} BW (DHA35). To avoid any oxidation of DHA, powders were stored at + 4°C in separated bags filled with nitrogen after each opening, and DHA content

was controlled weekly by gas chromatography analysis; no change was observed in fatty acid composition over the study (data not shown). All rats were fed daily with 30 g of freshly prepared solid food with free access to water, and final rats body weight were similar suggesting no alteration in food intake by the diets (C: 381 ± 12g, DHA10: 350 ± 8g, DHA35: 374 ± 7g, DHA60: 380 ± 9g, means ± SEM).

2.2. Isolated working heart preparation and ^{31}P NMR experiments

The study of the cardioprotective effect of GPL-DHA during ouabain treatment was conducted *ex vivo* using the isolated perfused heart model. Hearts (n = 7 in each group) were quickly removed from intraperitoneally sodium pentobarbital anaesthetized rats and perfused with a modified Krebs-Henseleit buffer at low external concentration of Ca^{2+} to elicit biphasic positive inotropic response to ouabain, and paced at a frequency about 20% above the spontaneous heart rate, as previously described. [9] Langendorff isovolumic model was used and developed pressure (dP) and the first pressure derivative (dP/dt) were recorded as previously described. [9] Temperature was maintained at 37 °C throughout the protocol.

Perfused hearts were placed in a 20 mm sample tube and inserted into a ^{31}P probe that was seated in the bore of a superconducting wide-bore (89 mm), 4.7 Tesla magnet (Oxford instruments) and ^{31}P spectra were generated at 81 MHz using a Bruker-Nicolet WP-200 spectrometer as previously described. [20] The resonance areas of inorganic phosphate (Pi), phosphocreatine (PCr) and ATP were calculated using AMARES time domain fitting. Integrals of resonances were converted to concentrations by comparison with a standard reference. Values for intracellular pH (pHi) were derived from the chemical shift of the Pi resonance. [20] Cardiac function and ^{31}P NMR spectra were registered simultaneously every 4 min on the same hearts.

2.3. Experimental protocol

The basic experimental protocol consisted of a 12-min control period (without ouabain perfusion) followed by five successive 12-min periods of ouabain perfusion at 5 different concentrations, from 10^{-7} M for the first 12-min period to 3 x 10^{-4} M for the fifth one. Ouabain at 10^{-7}, 10^{-6}, 10^{-5}, and 10^{-4} M concentrations was used for measuring inotropic effect, and the $3x10^{-4}$ M dose was used for assessing ouabain intoxication. The perfusion of ouabain at $3x10^{-4}$ M followed immediately the 10^{-4} M dose, such as this last point was considered as the "zero time" of the intoxication test.

2.4. Heart membranes preparation and fatty acid composition analysis

Left ventricle and septum of 6 animals from each group were isolated, frozen in liquid nitrogen and stored at -80°C until analysis. Tissues were homogenized and purified cell membranes were obtained using differential centrifugations as previously.[9] The fatty acid composition of membrane lipid extract (Folch's method) was determined after methylation with BF3-methanol (Sigma, St Louis, MO, USA) by gas chromatography (Perkin Elmer Autosystem XL, flame ionization detector, Turbochrom software, Courtaboeuf, France) using a fused silica capillary column (Omegawax 250, 30 m x 0.25 mm i.d. x 0.25 µm, Sigma-Supelco) and hydrogen as carrier gas.[21] The oven temperature program ranged from 60°C to 215°C with a temperature rise of 45°C min^{-1}. Fatty acids were identified by their retention times on the column using appropriate standards (PUFA 2, Sigma-Supelco).

2.5. Statistical analysis

Animal weights were expressed in means ± SEM. All other data were analyzed using a nonparametric Kruskal-Wallis test and differences between groups were checked using the Dunn's Multiple Comparison Test. This test was chosen due to the non-Gaussian distribution of the data obtained from 6 to 7 animals per group. The data are expressed as medians, with first and

third quartile, and minimal and maximal values (box plot for Figures), or with interquartile range (Tables) instead of means ± SEM. A $p < 0.05$ was considered significant. All analyses were done using GraphPad Prism version 5.0 (GraphPad Software, San Diego, CA, USA).

3. Results

3.1. Fatty acid composition of myocardial membranes

The main PUFA in myocardial membrane phospholipids were linoleic acid (C18:2 n-6), arachidonic acid (C20:4 n-6, AA) and DHA (C22:6 n-3) (Table 2). EPA (C20:5 n-3) and linolenic (C18:3 n-3) acid levels were very low (< 0.25 % of total fatty acids), and AA levels were constant. The only substantial change in fatty acid composition after GPL-DHA supplementation was a dose-dependent increase in DHA with a trend for the 10 and 35 mg kg^{-1} DHA doses ($p < 0.10$; +8% and +30%, respectively), and a significant increase for the 60 mg kg^{-1} DHA dose ($p < 0.05$; +45%). It resulted in a significant decrease of the n-6/n-3 ratio and a significant increase of the DHA/AA ratio for the 35 and the 60 mg kg^{-1} DHA doses.

3.2. Inotropic effect and energy metabolism variations

Values of cardiac function, expressed as dP/dt, during the control period (first 12-min perfusion without ouabain) in isolated perfused hearts were consistent with a 0.5 mM Ca^{2+} perfusion[9], and were not statistically different between the groups (Fig. 1A). Dietary treatment with DHA significantly improved the positive inotropic effect of ouabain (Fig. 1B) for the 10^{-5} M ouabain dose in DHA35 and DHA60 groups, and for the 10^{-4} M ouabain dose in all DHA groups. No significant changes in diastolic pressure were observed (data not shown). Quantitative analysis of PCr in the different groups prior to ouabain infusion and during the positive inotropic effects of ouabain is given in Fig. 2. Interestingly, baseline concentrations of PCr were significantly higher in hearts of the DHA35 (+45%) and DHA60 (+85%) groups compared to control. Compared to

baseline values, PCr concentration decreased while increasing the ouabain dose in all groups, but remained significantly higher (+73-98%) at all drug doses in the DHA60 group (Fig. 2). ATP (Table 3) and pHi (data not shown) values were not significantly different between groups and at the different drug doses. Phosphocreatine-to-ATP ratio at baseline was significantly higher for DHA35 (+36%) and DHA60 (+47%) groups compared to the control and the DHA10 groups (Table 4). This ratio was also significantly higher at the 10^{-7}, 10^{-6} and 10^{-5} M doses of ouabain for the DHA60 group (+92%, +48%, +69%, respectively, with $p < 0.05$) compared to control (Table 4).

3.3. Ouabain intoxication

Ouabain intoxication was expressed as a function of the time (4, 8 and 12 min) of perfusion of 3 x 10^{-4} M ouabain for dP (Fig. 3A) and PCr (Fig. 3B). Consistent with the narrow therapeutic index of digitalis, ouabain intoxication was developing rapidly. In all DHA groups, at the beginning (4 min) of the perfusion of the 3 x 10^{-4} M dose of ouabain, the values obtained for the developed pressure expressed as % above the dose of 10^{-4} M ("point zero") were positive, while in the control group the intoxication by ouabain was evidenced by a negative value. After 8 and 12 min, medians reached negative values in all groups. As a sign of earlier toxicity in the control group, the increase in EDP (contracture) occurred at 4 min and reached significantly higher level when compared to DHA60 ($p < 0.05$) (C: 16±28, DHA10: 0±12, DHA35: 8±10, DHA60: 0±4 mmHg). Toxicity in the control group was also shown by the depletion in PCr concentrations while PCr remained at significantly higher values (+31-76%) in the DHA60 group. No significant change in ATP level (Table 3) or in PCr-to-ATP ratio (Table 4) was found between groups.

4. Discussion

This study shows for the first time that a dietary source of DHA from avian glycerophospholipids displays cardioprotective properties. Firstly, it increases cardiac phosphocreatine content, a high-energy phosphate compound of great interest for heart contractility. Secondly, this source of DHA promotes positive inotropy of ouabain without increased toxicity. These effects were associated with an increased incorporation of DHA into the cardiac membranes.

We report that DHA-enriched phospholipids are as efficient as fish oil used in our previous study (90 mg EPA + 60 mg DHA kg^{-1} BW provided daily by triglycerides for 8 weeks)[9] to enhance DHA content in cardiac membrane phospholipids. This strong DHA increase in cardiac membranes is consistent with the few papers published in humans reporting an average 50% increase in DHA in cardiac biopsies after supplementation with fish oil providing 1g or 6 g of EPA+DHA per day for 6 months or 21 days, respectively.[17,22] Furthermore, the supplementation with DHA as phospholipids, at the daily dose of 60 mg DHA kg^{-1} BW is as efficient as a supplementation with DHA in form of ethyl ester at the daily dose of 360 mg DHA kg^{-1} BW for increasing DHA at the same level in rat cardiac membranes.[15] All these data suggest that phospholipids represent an interesting dietary vector for bio-accretion of DHA in heart, as shown for other organs, by comparison with triglyceride form or ethyl ester form, probably due to a different metabolic behaviour.[23]

Interestingly, the supplementation with GPL-DHA is associated with a much higher increase in cardiac phosphocreatine level than reported previously with fish oil, at the same amount of DHA (60 mg DHA kg^{-1} BW per d), and even for a lower dose (35 mg kg^{-1} BW per d) while ATP values were quite similar[9], leading thus to a much higher basal myocardial phosphocreatine-to-ATP ratio in DHA-treated rats than in control rats. This is of peculiar importance since according to the creatine kinase-phosphocreatine energy-shuttle hypothesis, phosphocreatine transfers the high-energy phosphate bond from the site of ATP production (mitochondria) to the site of ATP utilisation (myofibrils), and is essential for maintaining high cardiac performance state.[24]

Ouabain, a digitalis-like factor extracted from *Acokanthera Oblongifolia*, inhibits Na^+/K^+-ATPase activity resulting in an increase in intracellular Na^+ and promoting Ca^{2+} entry in cardiac cells via the Na^+/Ca^{2+} exchanger.[25] This leads to an increase in myocardial contraction helping the heart of patients with congestive heart failure or heart rhythm problem, but with potential toxic effect due to an extremely narrow therapeutic index. Indeed, if too much Na-K-ATPase is inhibited, toxicity ensues from intracellular calcium overload.[9]. We have demonstrated previously that a 2-month fish oil supplementation in rats promoted positive inotropy of ouabain without toxicity in isolated perfused hearts.[9] Here we demonstrate that supplementing rats for only one month with exactly the same amount of DHA, or even at a lower dose (35 mg kg^{-1} BW per d), as phospholipids, and without EPA, was as efficient in promoting inotropy without toxicity. We previously found that fish oil n-3 PUFA (EPA+DHA) incorporation into cardiac membranes has a direct influence on ouabain affinity of Na^+/K^+-ATPase leading to lower inhibition of Na^+/K^+-ATPase by ouabain together with higher inotropic effect, thus such a mechanism may be envisaged herein with GPL-DHA.[9] Considering that ouabain toxicity results mainly from excessive levels of cytosolic calcium and subsequent energy metabolism alteration, additional mechanisms could explain the prevention of ouabain toxicity by n-3 PUFA. DHA incorporation within the cardiac cell membranes could also affect other cardiac ion channels directly involved in Ca^{2+} transport and prevent partially ouabain-induced calcium increase via a finer regulation of calcium concentration within the cardiac cells. [26,27] Indeed, a dual modulatory action on calcium channels by DHA incorporation into membrane phospholipids has been shown to prevent excessive or deficient influx of calcium from compromising the contractility of cardiomyocytes.[28] Finally, n-3 PUFA can optimise energy metabolism by promoting mitochondrial efficacy. Indeed not only phosphocreatine levels are increased in hearts of DHA supplemented rats, but also PCr depletion with high dose ouabain is partially and significantly prevented by our DHA source with a positive impact over time when compared to fish oil.[9] The positive impact of DHA on cardiac

energy metabolism can be explained by two main mechanisms. First, as a natural ligand for PPARs, DHA intervenes in the regulation of many genes involved in cardiac energy metabolism leading to increased activity of glycogen phosphorylase, mitochondrial succinate, β-hydroxybutyrate dehydrogenase and cell membrane 5'-nucleotidase activities.[29] Secondly, in rat models, incorporation of DHA into mitochondrial phospholipids, in contrast to EPA, has been shown to delay the Ca^{2+}-induced opening of the permeability transition pore involved in cardiac pathologies.[30]

In summary, avian glycerophospholipids enriched in DHA appear as a promising dietary supplement or a novel ingredient for functional foods to improve cardiac bioenergetics in normal situation and could contribute to promote cardiac health. In another hand, GPL-DHA could be used as a complementary treatment to digitalis in congestive heart failure to improve the efficacy of lower drug doses, and reducing their side effects. To note, the effective doses of DHA from phospholipids in rats, 35 and 60 mg kg^{-1} BW per d, could be equivalent to a daily intake of 2.45 and 4.2 g DHA in humans considering a body weight of 70 kg, amounts that are in accordance with current dietary recommendations for cardioprotection.[4,17,22] Further studies are required to elucidate more deeply the mechanisms underlying the cardiac beneficial effect of this source of DHA.

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Conflicts of interest

G. Pieroni has created a start-up, ASL, to commercialize avian phospholipids enriched in DHA, and T. C. Coste has acquired some shares of ASL.

M. Bernard, J-M. Maixent, A. Gerbi, C. Lan, P. J. Cozzone and M. Armand have no conflict of interest to disclose.

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Figure legends

Fig. 1 Contractile values (dP/dt) of isolated hearts at baseline (A) and changes from baseline values with increasing ouabain doses (B), in control rats C and rats fed a diet supplemented with different doses of DHA (DHA 10, 35 or 60 mg kg^{-1} BW per d) for 4 weeks (n = 7 of 13 animals for each condition per group). Values are medians with first and third quartiles, and minimal and maximal values are represented as box plot. Between the groups, medians not sharing a common superscript letter at a given dose of ouabain are significantly different at $p< 0.05$ (Dunn's Multiple Comparison Test). dP/dt, first pressure derivative.

Fig. 2 Effect of sequential additions of increasing concentrations of ouabain (10^{-7} to 10^{-4} M) on intracellular phosphocreatine (PCr) levels in hearts of control rats C and rats fed a diet supplemented with different doses of DHA (DHA 10, 35 or 60 mg kg^{-1} BW per d) for 4 weeks (n = 7 of 13 animals, for each condition per group). Values are medians with first and third quartiles. Between the groups, medians not sharing a common superscript letter at a given dose of ouabain are significantly different at $p< 0.05$ (Dunn's Multiple Comparison Test).

Fig. 3 Time course of changes in function and energy metabolism during ouabain intoxication (3 x 10^{-4} M ouabain) displayed as percentage above dP obtained at 10^{-4} M (A) and phosphocreatine levels (B), in control rats C and in rats fed a diet supplemented with different doses of DHA (DHA 10, 35 and 60 mg kg^{-1} BW per d) for 4 weeks (n = 7 of 13 animals for each condition per group). Values are medians with first and third quartiles, and minimal and maximal values are represented as box plots.

Between the groups, medians not sharing a common superscript letter at a given time are significantly different at $p < 0.05$ (Dunn's Multiple Comparison Test).

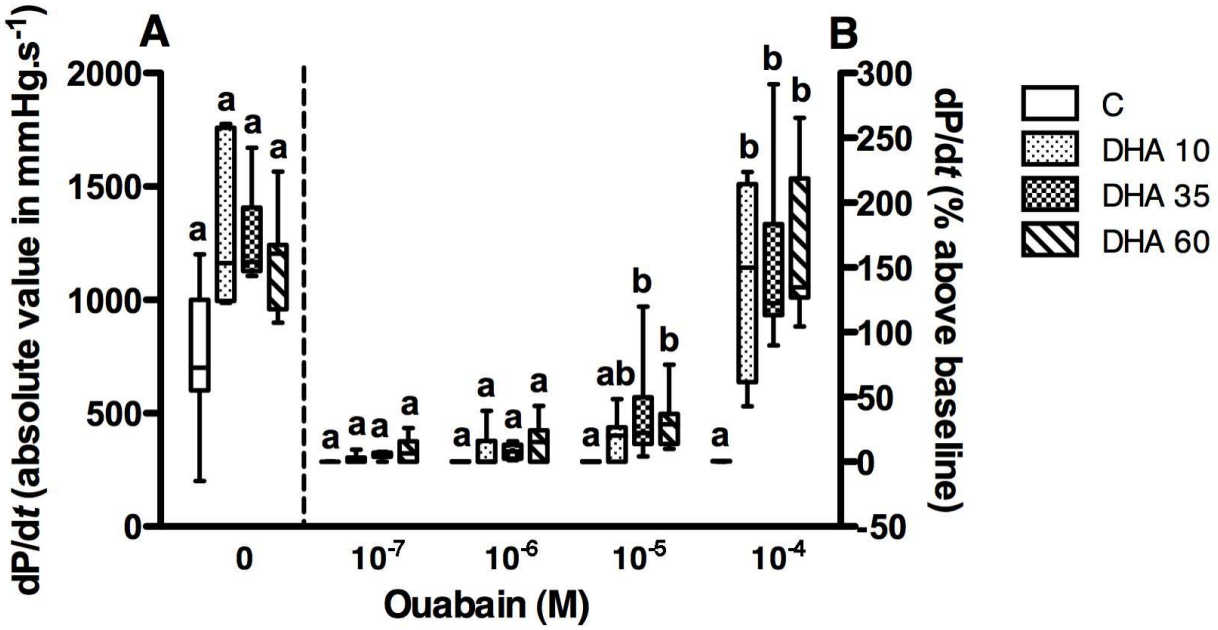

Figure 1

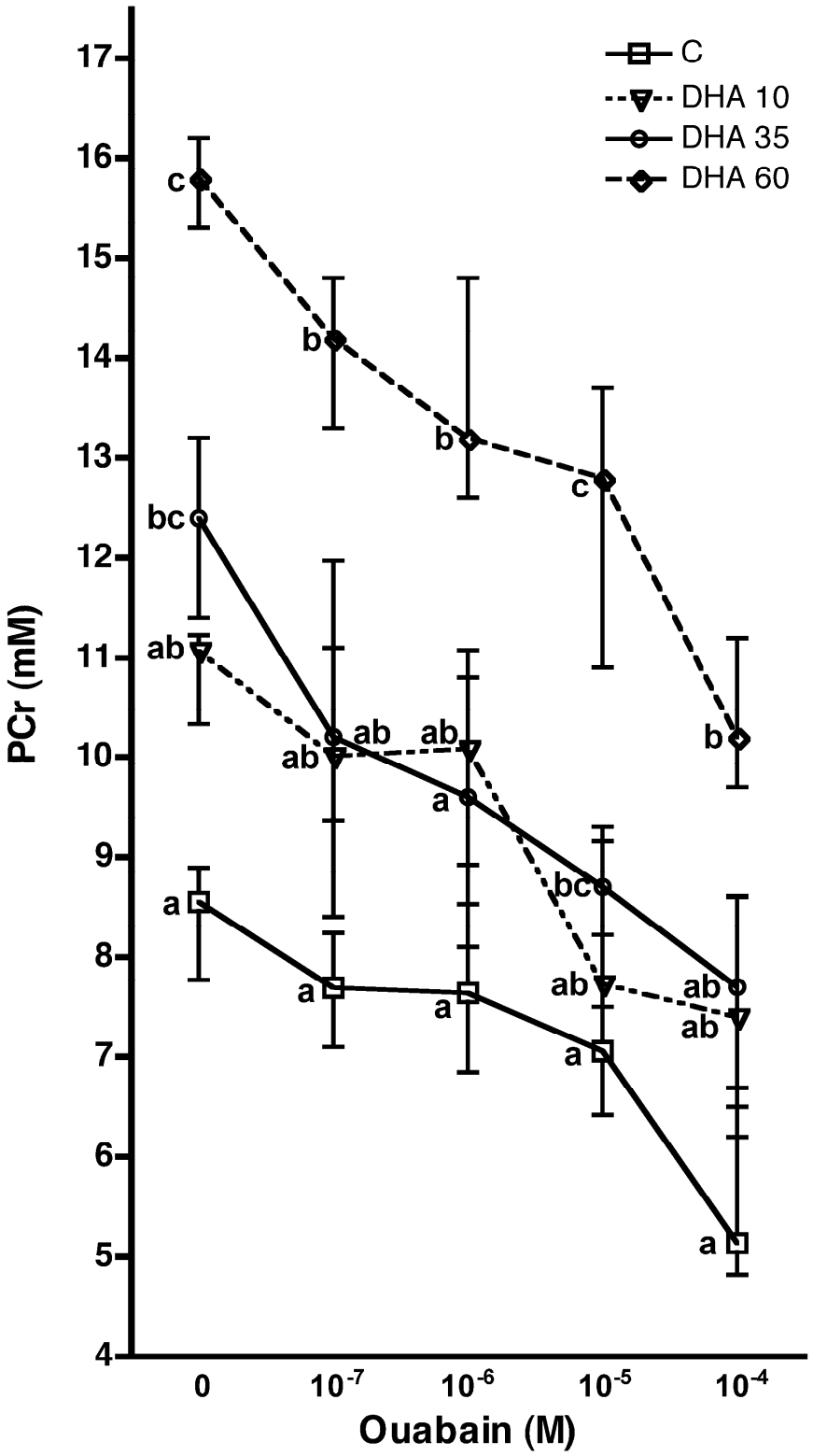

Figure 2

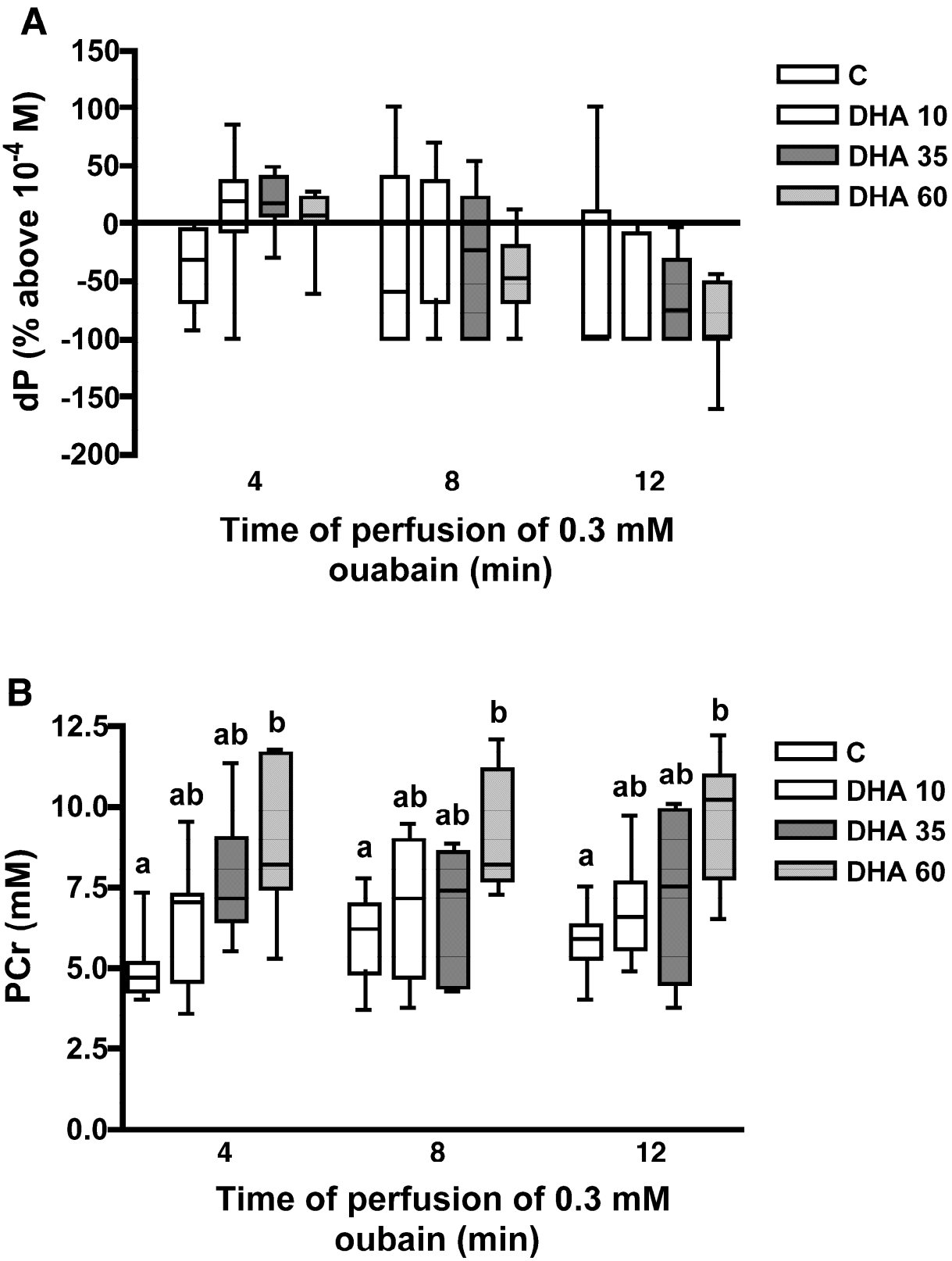

Figure 3

Table 1 Fatty acid composition of egg yolk powders

Fatty acids	Regular	DHA-enriched
	g per 100g total fatty acids	
14:0	0.32	0.29
16:0	26.36	23.08
16:1 n-7	3.15	2.86
18:0	8.49	6.64
18:1 n-9	38.26	38.79
18:2 n-6	16.89	12.96
18:3 n-3	0.45	0.29
20:4 n-6	2.17	0.75
20:5 n-3	0	0.18
22:5 n-3	0.08	0.22
22:4 n-6	0.18	0.43
22:6 n-3	0.61	3.70
n-6/n-3	16.88	3.39
AA/DHA	3.56	0.20

Table 2 Effect of DHA supplementation on phospholipid fatty acid composition of myocardial membranes[1]

Fatty acids	C	DHA10	DHA35	DHA60
		g per 100g total fatty acids		
C16:0	13.69 (5.09)	13.95 (2.15)	12.44 (1.29)	12.59 (0.45)
C16:1 n-7	0.93 (0.39)	0.91 (1.35)	0.78 (0.37)	0.33 (0.72)
C18:0	19.04 (4.21)	20.23 (3.84)	19.54 (2.93)	19.31 (1.11)
C18:1 n-9	10.45 (1.69)	10.55 (5.43)	9.91 (0.63)	9.95 (0.86)
C18:2 n-6	20.44 (1.82)	18.67 (2.07)	19.73 (3.40)	19.01 (2.40)
C20:4 n-6	16.25 (3.32)	15.98 (2.81)	16.11 (1.17)	15.76 (2.46)
C22:5 n-3	1.88 (0.44)	2.02 (0.58)	2.34 (0.59)	1.07 (2.29)
C22:6 n-3	12.09 (2.56)^{a}	13.73 (2.40)^{ab}	14.43 (3.24)^{ab}	16.17 (2.36)^{b}
SFA	32.34 (9.15)	33.62 (2.10)	31.60 (4.02)	31.59 (1.28)
MUFA	11.44 (2.00)	11.45 (6.77)	10.50 (0.55)	10.34 (1.58)
PUFA	52.37 (8.07)	50.59 (6.51)	53.53 (2.94)	52.92 (3.88)
Total n-6	38.08 (5.45)	34.56 (3.67)	35.39 (2.91)	35.45 (1.68)
Total n-3	14.02 (2.86)	15.75 (2.98)	17.14 (3.41)	18.31 (2.20)
n-6/n-3	2.76 (0.41)^{a}	2.38 (0.32)^{ab}	2.10 (0.52)^{b}	1.89 (0.23)^{b}
DHA/AA	0.74 (0.09)^{a}	0.84 (0.05)^{ab}	0.92 (0.15)^{b}	1.03 (0.29)^{b}

[1] Results are expressed as median values and (interquartile range) for each group of rats (n = 6 of 13

animals for each condition per group). C, control diet; DHA10, diet supplemented with DHA 10 mg kg^{-1} BW per d; DHA35, diet supplemented with DHA 35 mg kg^{-1} BW per d; DHA60, diet supplemented with DHA 60 mg kg^{-1} BW per d.

Between groups, medians not sharing a common superscript letter in a given row are significantly different at $p< 0.05$ (Dunn's Multiple Comparison Test).

Table 3 Effect of DHA supplementation and of ouabain administration on ATP levels in rat heart[1]

Conditions	C	DHA10	DHA35	DHA60
		mM		
Baseline	7.88 (1.12)	8.85 (1.82)	8.70 (1.5)	9.50 (1.80)
Ouabain 10^{-7} M	7.43 (1.23)	7.75 (2.05)	8.00 (0.60)	8.00 (1.50)
Ouabain 10^{-6} M	7.01 (0.80)	8.12 (2.03)	8.10 (1.70)	8.20 (1.70)
Ouabain 10^{-5} M	6.84 (2.59)	6.63 (3.59)	6.80 (1.70)	6.70 (1.90)
Ouabain 10^{-4} M	5.90 (1.34)	5.47 (4.52)	6.20 (1.80)	6.00 (2.70)
Ouabain intoxication test (3×10^{-4} M)				
4 min	5.51 (2.61)	5.89 (3.81)	6.89 (0.80)	6.05 (1.73)
8 min	5.26 (1.50)	5.51 (2.63)	6.92 (2.79)	5.02 (2.46)
12 min	3.94 (3.60)	5.63 (0.70)	6.72 (4.35)	4.89 (1.91)

[1]Results are expressed as median values and (interquartile range) for each group of rats (n = 7 of 13 animals for each condition per group). C, control diet; DHA10, diet supplemented with DHA 10 mg kg^{-1} BW per d; DHA35, diet supplemented with DHA 35 mg kg^{-1} BW per d; DHA60, diet supplemented with DHA 60 mg kg^{-1} BW per d.

Table 4 Effects of DHA supplementation on PCr/ATP ratio in rat heart[1]

Conditions	C	DHA10	DHA35	DHA60
Baseline	1.12 (0.19)^{a}	1.26 (0.17)^{ab}	1.52 (0.26)^{b}	1.65 (0.26)^{b}
Ouabain 10^{-7} M	0.96 (0.41)^{a}	1.30 (0.19)^{ab}	1.31 (0.50)^{ab}	1.84 (0.54)^{b}
Ouabain 10^{-6} M	1.18 (0.24)^{a}	1.29 (0.52)^{ab}	1.27 (0.45)^{ab}	1.75 (0.43)^{b}
Ouabain 10^{-5} M	1.10 (0.14)^{a}	1.35 (0.74)^{ab}	1.21 (0.57)^{ab}	1.86 (0.43)^{b}
Ouabain 10^{-4} M	0.98 (0.68)^{a}	1.22 (1.79)^{a}	1.22 (0.30)^{a}	1.60 (0.52)^{a}
Ouabain intoxication test (3×10^{-4} M)				
4 min	0.79 (0.64)	1.61 (1.35)	1.12 (0.36)	1.40 (0.30)
8 min	1.22 (0.37)	0.99 (1.27)	1.06 (0.39)	1.66 (0.94)
12 min	1.58 (0.87)	1.10 (0.43)	1.38 (0.51)	2.24 (1.50)

[1] Results are expressed as median values and (interquartile range) for each group of rats (n = 7 of 13 animals for each condition per group). C, control diet; DHA10, diet supplemented with DHA 10 mg kg^{-1} BW per d; DHA35, diet supplemented with DHA 35 mg kg^{-1} BW per d; DHA60, diet supplemented with DHA 60 mg kg^{-1} BW per d.

Between groups, medians not sharing a common superscript letter in a given row are significantly different at $p < 0.05$ (Dunn's Multiple Comparison Test).