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ORIGINAL RESEARCH ARTICLE

Carnitine modulates rat crucial myocardial adenosine triphosphatases and acetylcholinesterase enzyme activities in choline-deprived rats

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Abbreviations: AChE, acetylcholinesterase; ATP, adenosine triphosphate; ATPase(s), adenosine triphosphatase(s); Mg, magnesium; Na, sodium; K, potassium; Ca, calcium; [Mg]i, intracellular magnesium concentration; ANOVA, analysis of variance; LPC, lysophoshatidylcholine; [Na]i, intracellular sodium concentration; ROS, reactive oxygen species; SERCA, sarco(endo)plasmic reticulum (SR) calcium transport ATPase (Ca^{2+}-ATPase); i.e, Latin words id est (that is); SD, standard deviation; SEM, standard error of means.

Abstract

Choline is considered as an essential nutrient and its deficiency has been associated with cardiovascular morbidity. It is also precursor of acetylcholine (important cholinergic component of the heart autonomic nervous system) whose levels are regulated by acetylcholinesterase (AChE). Cardiac contraction-relaxation cycle depends on ion gradients established by active channels and pumps like the adenosine triphosphatases (ATPases) Na^+/K^+-ATPase and Mg^{2+}-ATPase. Carnitine, structurally relevant to choline, is used as adjunct in the management of cardiac diseases. The study aimed to investigate the impact of dietary choline deprivation on rat crucial myocardial acetylcholinesterase (AChE, cholinergic marker), Na^+/K^+-ATPase and Mg^{2+}-ATPase activities and the possible modifications after carnitine supplementation. Male Wistar Albino adult rats were divided into four groups and received for one month standard or choline deficient diet with or without carnitine co-administartion in drinking water 0.15% w/v. The enzyme activities were determined spectrophotometrically in the myocardium homogenate. Under conditions of both choline dietary deprivation and carnitine supplementation myocardial Na^+/K^+-ATPase showed elevated activity along with a concomitant decrease in Mg^{2+}-ATPase and AChE. The results suggest that the inhibited AChE activity in the choline-deprived myocardium might modulate the cholinergic myocardial

neurotransmission whereas the altered ATPases' activities could interfere with the heart contractile response.

Key words: choline deficiency; carnitine; myocardial; ATPases; acetylcholinesterase

Introduction

Choline is an essential nutrient involved in cellular membrane integrity, metabolic reactions (as methyl-donor) and biosynthesis of different macromolecules among which phospholipids and acetylcholine (Zeisel 2009).

Choline deficiency state has been mainly associated with the development of liver steatosis (Zeisel 2009). However, in regards to its effects on the heart, the data are limited (Kesten et al. 1945; Wilgram et al. 1954; Williams 1960).

Carnitine, a chemical analogue of choline, mediates the transport of long-chain fatty acids into mitochondria for energy production (Longo et al. 2006). Cardiac and skeletal muscle, although they require carnitine for normal fuel metabolism, they cannot synthesize it (Dayanand et al. 2011). L-carnitine (the biologically active stereoisomer) has been shown to possess important antioxidant capacity (Mingorance et al. 2011) and its therapeutic potential has been demonstrated in the setting of cardiomyopathy, heart failure and ischemic heart disease (Paulson et al. 1984; Matsuishi et al. 1985; Whitmer 1987; Fernandez and Proto 1992; Kobayashi et al. 1992; Gürlek et al. 2000) with promising results.

The metabolic and electrophysiological properties of the myocardium depend on specific ion channels and transporters within distinct membrane domains (Balse et al. 2012), which are also regulated by heart autonomous nervous system (Bois et al. 2007).

Acetylcholinesterase (AChE), responsible for acetylcholine degradation, terminates cholinergic neurotransmission and plays a key role in the regulation of the parasympathetic tone of the heart (Abramochkin et al. 2012). Moreover, its local activity doesn't only reflect autonomic neuronal status (Jo et al. 1992), but it also influences cardiomyocytes' metabolic state (Ito et al. 1989). Decreased myocardial AChE activity has been observed in myocardial infarction (Menache et al. 1982) and heart failure (Dunlap et al. 2003).

Na^+/K^+-ATPase is a crucial ion pump for the myocardiocyte repolarizing process as well as for the sodium [Na^+] and potassium [K^+] transmembrane electrochemical gradient maintenance (McNamara et al. 1974). Furthermore, it is considered a reliable marker for cellular energy production due to its high affinity for ATP; intracellular concentration directly affects the pump's activity (Apell et al. 1986). However, the Na^+/K^+ pump's functional potential is altered or adjusted depending upon animal species, methodology and experimental conditions (Karli et al. 1979; Blanco and Merker 1998).

Free intracellular Mg^{2+} ([Mg]i), primarily regulated by ATP and creatine phosphate (Michailova and McCulloch 2001), contributes to heart's electrical homeostasis; its presence is necessary for electrolyte transcellular transport and ATP homeostasis (Seelig 1972). Myocardial infarction, hypertension and heart failure have been related with hypomagnesaemia (Ueshima 2005; Adamopoulos et al. 2009). The functional significance of Mg^{2+}-dependent ATPase in heart is not yet clear, but it seems to reflect free magnesium intracellular concentration (Saks et al. 1984).

The present study was designed to evaluate the changes of acetylcholinesterase (AChE), Na^+/K^+-ATPase and Mg^{2+}-ATPase enzyme activities in adult rats under the state of choline deficiency and determine the possible modifications after L-carnitine treatment.

Materials and Methods

Animals

Male Albino Wistar rats (n=24) 10-12 weeks of age (350±30gr body weight), purchased from the National Center of Scientific Research ‘‘Demokritos” were used. On arrival, the animals were maintained on a rodent standard diet, provided food and water *ad libitum* and housed at a constant room temperature (22 ±1^{o}C) under a 12 h light: 12 h dark (light 08:00–20:00 h) cycle. They were acclimatized to laboratory conditions for a minimum of one week before the start of the experiment. Temperature and humidity were controlled for the entire period of experimentation. Animals were cared for in accordance with the Guide to the Care and Use of Experimental Animals as set by the Canadian Council (www.ccac.ca). All animals procedures were carried out under the authority of the relevant project license obtained from the Prefecture of Athens and were approved by the Institutional Animal Care and Use Committee of the University of Athens for Medical Sciences. The number and suffering of the animals were kept to as minimum as possible.

Induction of choline deprivation and L-carnitine supplementation

After acclimatization for one week to constant environmental conditions, rats were randomly assigned in four groups of 6 animals, each fed with: a) standard diet (Control-CA), b) choline deficient diet (CDD), c) standard diet and L-carnitine in drinking water 0.15% w/v (CARN), d) choline deficient diet and L-carnitine in drinking water 0.15%w/v (CDD+CARN). The mean daily dose of L-carnitine was approximately 200mg/kg body weight. Diets were purchased from AnaLab Ltd, Athens, Greece and L-carnitine was obtained from Vianex SA, Athens, Greece. The analytical composition (g/kg) of the choline deficient diet was: sugar 413, starch 110, dextrin 110, hydrogenated vegetable oil 100, pea protein 90, soya protein isolate 60, corn oil 50, mineral mix 35, vitamin mix 10, cellulose 10, vitamin free casein 10, L-cystine 2. The standard diet was enriched by choline (1.1g/kg) at the expense of

sucrose. Rats were handled according to the above dietary pattern for four weeks, when they were sacrificed by decapitation.

Tissue preparation

After decapitation hearts were rapidly removed and stored at -80^{o}C till preparation. Hearts were homogenized in 10 vol. ice-cold (0–4^{o}C) medium containing 50 mM Tris (hydroxymethyl) aminomethane–HCl (Tris–HCl), pH 7.4 and 300 mM sucrose, using an ice-chilled glass homogenizing vessel at 900 rpm (4–5 strokes). Then, the homogenate was centrifuged at 1,000 x g for 10 min to remove nuclei and debris (Tsakiris 2001). In the resulting supernatant, the protein content was determined according to the method of Lowry et al. 1951 and consequently the enzyme activities were measured.

Determination of myocardial AChE activity

Acetylcholinesterase activity was determined by following the hydrolysis of acetylthiocholine according to the method of Ellman et al. (1961) as described by Tsakiris (2001). The incubation mixture (1 ml) contained 50 mM Tris–HCl, pH 8, 240 mM sucrose and 120 mM NaCl. The protein concentration of the incubation mixture was 80–100 μg/ml. The reaction was initiated after addition of 0.03 ml of 5,5′-dithionitrobenzoic acid (DTNB) and 0.05 ml of acetylthiocholine iodide, which was used as substrate. The final concentration of DTNB and substrate were 0.125 and 0.5 mM, respectively. The reaction followed spectrophotometrically by the increase of absorbance (DOD) at 412 nm.

Determination of Na^{+}/K^{+}-ATPase and Mg^{2+}-ATPase activities

Na^+/K^+-ATPase activity was calculated from the difference between total ATPase activity (Na^+,K^+,Mg^{2+}-dependent ATPase) and Mg^{2+}-dependent ATPase activity. Total ATPase activity was assayed in an incubation medium consisting of 50 mM Tris–HCl, pH 7.4, 120 mM NaCl, 20 mM KCl, 4 mM MgCl2, 240 mM sucrose, 1 mM ethylenediamino-tetraacetic acid K_2-salt (K^+-EDTA), 3 mM disodium ATP and 80–100 μg protein of the homogenate in a final volume of 1 ml. Ouabain (1 mM) was added in order to determine the activity of Mg^{2+}-ATPase. The reaction was started by adding ATP and stopped after an incubation period of 20 min by addition of 2 ml mixture of 1% lubrol and 1% ammonium molybdate in 0.9 M H_2SO_4 (Tsakiris 2001; Bowler and Tirri 1974). The yellow color which developed was read at 390 nm.

Biochemical Assays for determination of Choline and Carnitine

Choline was quantified in serum using a colorimetric EnzyChrom™ Choline Assay Kit (ECHO-100, BioAssay Systems, USA); free choline is oxidized by choline oxidase to betaine and H_2O_2 which reacts with a specific dye to form a pink colored product. The color intensity at 570nm is directly proportional to the choline concentration in the sample. Linear detection range: 1 to 100μM. On the other hand, carnitine was determined in serum by a coupled enzyme assay (Catalog Number MAK063, Sigma-Aldrich, USA), which results in a colorimetric (570 nm) product, proportional to the L-carnitine present. Typical detection range for this is 2–10 nmoles.

Statistical analysis

Statistical analysis has been performed by the Statistical package SPSS 19 (Academic license). Prior to any statistical test, the normality of the studied variables was evaluated by the Kolmogorov–Smirnov (KS) normality test and the variances among the groups by the Levene's test.

ONE WAY ANOVA test and Kruskal Wallis test were performed for all variables with normal and non-normal distribution respectively. Statistical significance was considered for p values of <0.05. Whatever the p value of these tests was lower than 0.05 the statistical significance between the groups was checked, one by one, by performing Student's t-test for independent samples and non-parametric Mann Whitney test respectively.

Results

Enzyme Activities

The effect of choline deficiency, with or without carnitine co-administration, on the adult rat myocardial Na^+/K^+-ATPase activity (μmol Pi/h x mg protein) is presented in Figure 1. A statistical significant change was noted only in the CDD+CARN group compared to control (+60% in the CCD+CARN group vs CA, $p<0.05$).

Mg^{2+}-ATPase activity (μmol Pi/h x mg protein) results are depicted in Figure 2. The enzyme activity showed statistically significant decrease in the CDD+CARN group when compared to control (-18.14% in the CDD+CARN group vs CA, $p<0.05$) along with a trend ($p=0.06$) towards the establishment of reduced Mg^{2+}ATPase activity in the CDD group; no further significant differences were found between the other groups.

Myocardial AChE activity (ΔOD/min x mg protein) (Figure 3), was found significantly decreased in the CDD+CARN group when compared to all other groups (-22% in the CDD+CARN group vs CA, $p<0.05$, -22.35% vs CARN group, $p<0.01$ and -19.5% vs CDD, $p<0.05$).

Choline and carnitine concentrations in serum

Choline serum concentration was significantly decreased in both groups that received choline deficient diet (CDD, CDD+CARN) compared to control ($p<0.01$ and $p<0.004$ respectively) whereas

no change was noted in the CARN group. However, choline concentration was significantly increased in the CDD+CARN group compared to CDD ($p<0.009$) (Table 1).

Carnitine serum concentration showed statistical significant changes in all groups compared to control with adverse variations. A significant decrease was observed in the CDD group ($p<0.009$) while a significant increase in the CARN ($p<0.004$) and CDD+CARN group ($p<0.004$) compared to control (CA). Statistical significant differences were also detected between the non-control groups: CDD+CARN group demonstrated higher carnitine levels compared to choline deficient diet (CDD, $p<0.01$) or carnitine (CARN, $p<0.04$) groups (Table 1).

Discussion:

The present study explored the role of carnitine on the myocardium activities of Na^+/K^+-ATPase, Mg^{2+}ATPase and AChE in a choline deficiency state. The results suggest that carnitine administration might relieve the possible adverse effects of choline dietary deprivation on the heart performance.

Choline and carnitine concentrations in serum confirmed the successful establishment of the experimental setting. The carnitine deficiency observed in the state of choline deficiency, is in accordance to previous studies (Zeisel and Niculescu 2005) and could also be attributed to: a) the lack of exogenous carnitine administration b) the low methionine content in the choline deficient diet (Mato et al. 2008) (methionine is necessary for carnitine biosynthesis) (Dayanand et al. 2011) and c) the potentially impaired intestinal absorption capacity of carnitine because of the choline deficit (Takahashi et al. 1982; Gudjonsson et al. 1986). On the contrary, in the rats receiving choline deficient diet and carnitine, the higher levels of carnitine could easily be explained by the exogenous carnitine administration, which managed not only to prevent the deficiency that could be established by the choline dietary depletion, but to overcome the levels of the control group as well. Last but not

least, the partially restored choline levels noted in the CDD+CARN group could be possibly due to carnitine metabolism into methionine, which promotes endogenous choline biosynthesis.

This is the first study to examine the electrophysiological properties of myocardium under the above conditions. The number of existing studies involving Na^+/K^+-ATPase and choline in rat heart are limited and refer to the pump's activity under pathological conditions only, such as myocardial infarction; in this setting the impact of betaine (oxidized derivative of choline) administration (Ganesan et al.) or the effects of the released lysophoshatidylcholine (LPC) have been investigated (Prielipp et al.). In contrast to the above studies, we tried to identify the choline deficiency-mediated alterations of the membrane-bound ATPases in the absence of any underlying disease.

Choline deficiency leads to mitochondrial dysfunction (Marcinek 2004) and impaired permeability of cell membranes due to diminished concentrations of phosphatidylcholine (da Costa et al. 2004), to increased oxidative stress (Repetto et al. 2010) and to reduced availability of acetyl-CoA derivatives (Kakar et al. 1987). These alterations in membrane structural intergrity could allow an influx of sodium and efflux of potassium following their electrochemical gradient. Therefore, the stimulated Na^+/K^+ pump in the CDD group compared to control might be targeted to restore ion balance. However, the fact that it didn't reach statistical significance is not surprising, because choline dietary restriction has been associated with energy deficit (Hensley et al. 2000) due to diminished nucleotide synthesis in the presence of methyl donor deficiency (James et al. 1992). Increased intracellular sodium concentration ([Na]i) might impair the heart performance (it has been associated with heart failure) even without changes in the Na/K pump function (Despa et al. 2002), while it could also activate the reverse mode of Na/Ca exchanger leading to calcium influx. It is well known that cellular Ca overload is responsible for disturbed myocyte relaxation and diastolic dysfunction (Zile and Brutsaert 2002) along with cardiac impaired contractile response (Tsuji et al. 2001). These observations are in accordance with our recent study regarding the evaluation of the heart mechanical

properties under the same experimental conditions where we clearly demonstrated the adverse effects mediated by choline dietary restriction on the heart function (Strilakou et al. 2013).

Mg^{2+}-ATPase exhibited a trend towards compromised activity in the CDD group which is plausible, since ATP, one of its main substrates, has reduced availability in this case. The consequent decreased [Mg]i has been associated with cardiomyocyte dysfunction and impaired handling of calcium homeostasis resulting in abnormalities in heart contractility (Griffiths 2000) and decreased force production (Jia et al. 2004).

According to Martinez et al. (2013) a state of methyl donor deficiency, such as choline dietary deprivation, provokes oxidative stress and alters the cardiac cell function and morphology, particularly at the mitochondrial level. Carnitine treatment offers antioxidant protection in cardiomyocytes under conditions of increased ROS (Dayanand et al. 2011), prevents age-related decline in mitochondrial function (Hagen et al. 2002; Kumaran et al. 2004) and increases mitochondrial ATP production as well (Vaz and Wanders 2002).

Conceivably, the significant elevation of Na^+/K^+-ATPase's activity observed in the CDD+CARN group compared to control could be justified by a) the increased fragility and membrane permeability in the choline deficiency setting leading to sodium influx and b) the ATP augmented production due to carnitine treatment (Bartlett and Eaton 2004) that could facilitate the pump's functional potential. This finding is consistent with the study of Lee at al. (2009) which suggests that activation of Na^+/K^+ ATPase improves Ca handling and might have positive inotropic effect on rat heart cells. Moreover, one could also assume that the provided energy and availability of ATP via carnitine, could potentially stimulate SERCA pump, which might compensate for the expected Ca overload triggered in the CDD group, since it would lead to reuptake of Ca in the sarcoplasmatic reticulum stores and reinforce the heart contractile response (Müller et al. 2003). This concept was validated by our recent

study in which carnitine administration in a choline deficiency setting restored myocardium contractile performance at levels similar to those of the control group (Strilakou et al. 2013).

Furthermore, in the CDD+CARN group the significant decline in the Mg^{2+} ATPase activity compared to control, is not surprising and could be explained by the increased availability of ATP due to carnitine treatment (ATP acts as a main Mg^{2+}chelator, while low concentrations of free intracellular Mg^{2+} stimulate Na^{+}/K^{+}- ATPase's activity) (Bara et al. 1993); compatible with our data is the study of Beeler et al. (1983) in which an inactivation of Mg^{2+} ATPase has been observed in vitro in the presence of high ATP levels in rat myocytes.

One of the main compensatory mechanisms in the setting of heart failure is the shift of the sympathetic/parasympathetic equilibrium in favor of the sympathetic nervous system (Bibevski and Dunlop 2011). Older studies suggest that choline deficiency leads to hypersensitivity to catecholamines due to decreased levels of acetylcholine (Maheshwari et al. 1971) while the presence of sympathetic tone has been also associated with depletion of magnesium in rats' hearts and stimulation of the Na^{+}/K^{+} pump (Wester 1992) that are in agreement with our findings. In our experimental protocol, neither choline deprivation nor carnitine supplementation alone had a significant effect on AChE activity. On the contrary, under a choline deficiency setting where acetylcholine is diminished (Nagler et al. 1968), the co-administration of carnitine led to a significant decline of acetylcholinesterase's enzymatic activity when compared to control. The decreased activity of AChE that prevents the hydrolysis of the remaining acetylcholine along with the carnitine treatment that facilitates cholinergic neurotransmission, might eventually increase the beneficial parasympathetic drive on the heart (Levine 1997). The changes in AChE are consistent with the other enzymatic activities demonstrated and in agreement to previous studies suggesting that high levels of ATP diminish Mg^{2+}-ATPase, especially under low concentrations of acetylcholine (Maheshwari et al.

1971; Nery da Matta et al. 1996) and that modulation of Na^+/K^+ pump takes place under conditions in which both sympathetic and parasympathetic tone are effected (Gao et al. 1997).

In conclusion, the intriguing possibility that arises is that choline dietary withdrawal could make the myocardium more easily susceptible to cell damage under forthcoming stressful conditions (Dhalla et al. 1999), while carnitine treatment manages to preserve the energy reserves of the heart reflecting a possible compensatory potential to the choline deficiency.

Clinical Perspectives

Taking into account that in certain clinical conditions, i.e. patients under parentenal nutrition or with kidney or renal failure, a state of choline deficiency could be established, the administration of carnitine could be beneficial for the management of co-existing or potentially arising in these cases cardiac diseases, since most of the related drugs used (b-blockers, digoxin) act through modulation of the heart electrophysiological properties and excitation/contraction coupling. Future studies are necessary to elucidate the underlying mechanisms of carnitine in heart function under choline deficiency conditions.

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Table 1 Choline and carnitine serum concentrations (mean±SD) in rats fed with standard or choline deficient diet for one month with or without carnitine administration.

Group	**Choline (µg/dl)**	**Carnitine (ng/µl)**
Control (CA)	84.68±4.60[a]	2.80 ±0.73[a]
CARN	84.62±2.99[b]	27.25±1.13[b]
CDD	15.00±2.00[c]	1.53 ±0.05[c]
CDD+CARN	26.92±1.39[d]	35.83±1.35[d]

Statistical significance: *Choline*: a/c $p<0.01$, a/b $p>0.05$ (ns), a/d $p<0.004$, c/d $p<0.009$, b/c $p<0.009$, b/d $p<0.004$; *Carnitine*: a/c $p<0.009$, a/b $p<0.004$, a/d $p<0.004$, c/d $p<0.01$, b/c $p<0.009$, b/d $p<0.04$. Control (CA): rats fed with standard diet, CARN: rats fed with standard diet and receiving carnitine in drinking water, CDD: choline deprived rats, CDD+CARN: choline deprived rats receiving carnitine in drinking water.

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Figure 1

Effects of choline deficient diet on the myocardium Na^+/K^+-ATPase activity of adult rats and modulation by carnitine co-administration after one month of intervention. Each value indicates the mean ± SEM of six independent experiments (six rats per group). The average of each experiment aroused from three evaluations in the homogenized myocardium of each animal. The asterisk represents the statistical significant difference between the indicated group (*$p<0.05$). CA: rats receiving standard diet, CDD: rats fed with choline deficient diet, CARN: rats receiving standard diet and carnitine in drinking water, CDD+CARN: rats receiving choline deficient diet and carnitine in drinking water.

Figure 2

Effects of choline deficient diet on the myocardium Mg^{2+}-ATPase activity of adult rats and modulation by carnitine co-administration after one month of intervention. Each value indicates the mean ± SEM of six independent experiments (six rats per group). The average of each experiment aroused from three evaluations in the homogenized myocardium of each animal. The asterisk represents the statistical significant difference between the indicated group (*$p<0.05$). CA: rats receiving standard diet, CDD: rats fed with choline deficient diet, CARN: rats receiving standard diet and carnitine in drinking water, CDD+CARN: rats receiving choline deficient diet and carnitine in drinking water.

Figure 3

Effects of choline deficient diet on the myocardium acetylcholinesterase (AChE) activity of adult rats and modulation by carnitine co-administration after one month of intervention. Each value indicates the mean ± SEM of six independent experiments (six rats per group). The average of each experiment aroused from three evaluations in the homogenized myocardium of each animal. Asterisks represent the statistical significant differences between the indicated groups (*$p<0.05$, **$p<0.01$). CA: rats receiving standard diet, CDD: rats fed with choline deficient diet, CARN: rats receiving standard diet and carnitine in drinking water, CDD+CARN: rats receiving choline deficient diet and carnitine in drinking water.

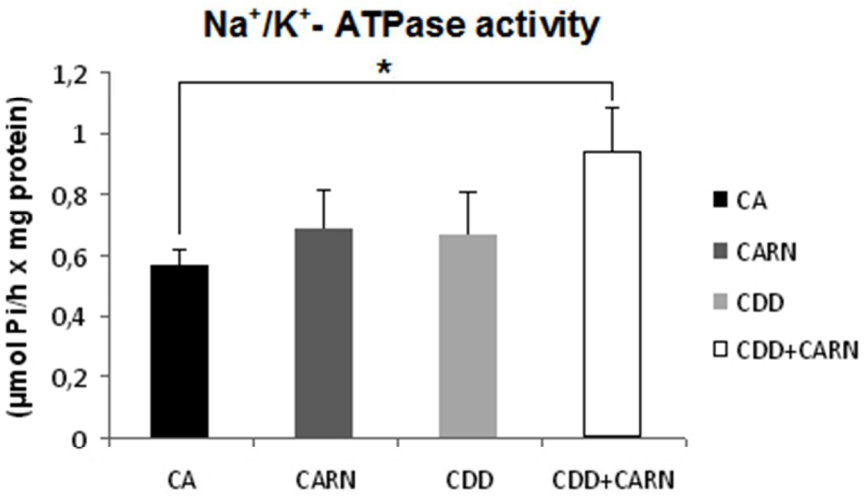

Figure 1

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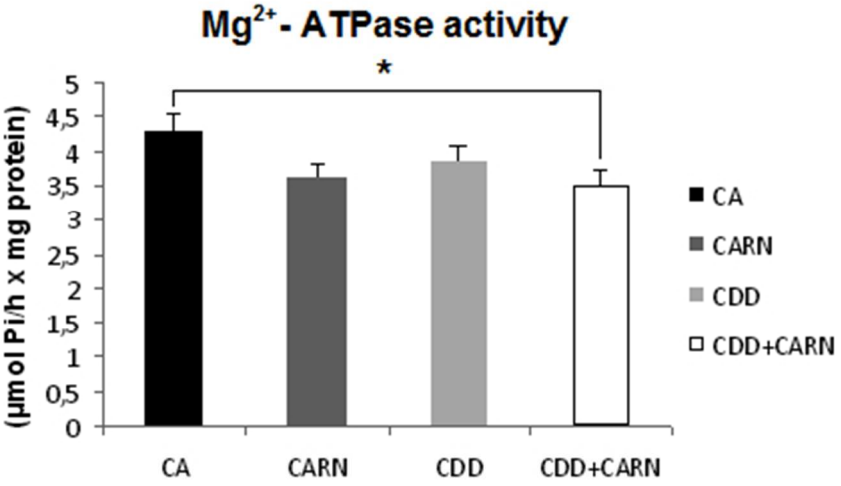

Figure 2

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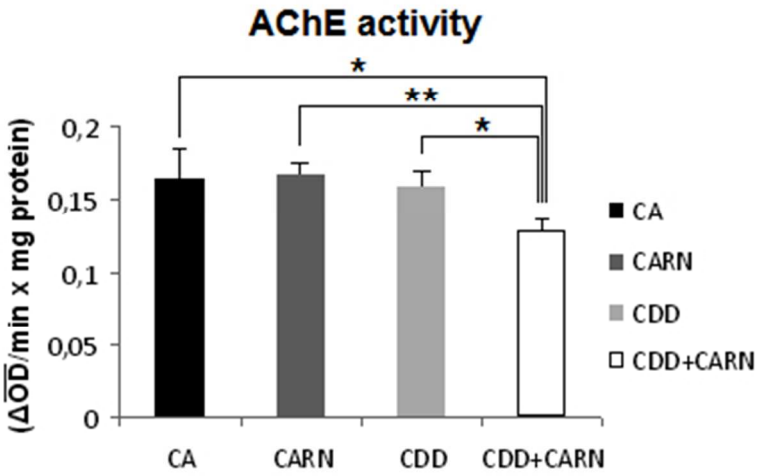

Figure 3

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