

Effects of creatine supplementation on homocysteine levels and lipid peroxidation in rats

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Hyperhomocysteinaemia is an independent risk factor for CVD. Recent data show a relationship between homocysteine (Hcy) and free radical formation. Since creatine synthesis is responsible for most of the methyl group transfers that result in Hcy formation, creatine supplementation might inhibit Hcy production and reduce free radical formation. The present study investigated the effects of creatine supplementation on Hcy levels and lipid peroxidation biomarkers. Thirty rats were divided into three groups: control group; diet with creatine group (DCr; 2% creatine in the diet for 28 d); creatine overload plus diet with creatine group (CrO + D; 5 g creatine/kg by oral administration for 5 d + 2% in the diet for 23 d). Plasma Hcy was significantly lower ($P < 0.05$) in DCr (7.5 (SD 1.2) $\mu\text{mol/l}$) and CrO + D (7.2 (SD 1.7) $\mu\text{mol/l}$) groups compared with the control group (12.4 (SD 2.2) $\mu\text{mol/l}$). Both plasma thiobarbituric acid-reactive species (TBARS) (control, 10 (SD 3.4); DCr, 4.9 (SD 0.7); CrO + D, 2.4 (SD 1) $\mu\text{mol/l}$) and plasma total glutathione (control, 4.3 (SD 1.9); DCr, 2.5 (SD 0.8); CrO + D, 1.8 (SD 0.5) $\mu\text{mol/l}$) were lower in the groups that received creatine ($P < 0.05$). In addition, Hcy showed significant negative correlation ($P < 0.05$) with plasma creatine ($r = -0.61$) and positive correlation with plasma TBARS ($r = 0.74$). Plasma creatine was negatively correlated with plasma TBARS ($r = -0.75$) and total peroxide ($r = -0.40$). We conclude that creatine supplementation reduces plasma Hcy levels and lipid peroxidation biomarkers, suggesting a protective role against oxidative damage. Modulating Hcy formation may, however, influence glutathione synthesis and thereby affect the redox state of the cells.

Creatine supplementation: Methyl balance: Homocysteine: Lipid peroxidation

Over the last few years, increasing evidence has shown that hyperhomocysteinaemia is an independent risk factor for the development of CVD^(1,2) and may be associated with neurodegenerative disease^(3,4), diabetes⁽⁵⁾ and end-stage renal dysfunction^(6,7). Recent *in vitro* and animal studies have indicated an association between elevated homocysteine (Hcy) levels and the formation of reactive oxygen species, especially the superoxide anion (O_2^-) and H_2O_2 ^(8–10), by the auto-oxidation of Hcy and/or cysteine⁽¹¹⁾. Intracellular reactive oxygen species production may cause cellular damage⁽⁹⁾ and disorders of the antioxidant defence system⁽¹²⁾, demonstrating a mechanistic connection between hyperhomocysteinaemia, oxidative stress and disease.

Hcy is an amino acid formed exclusively by demethylation of methionine⁽¹³⁾. In Hcy synthesis, methionine is activated by ATP to form S-adenosylmethionine (SAM). SAM acts primarily as a universal methyl donor in the synthesis of methylated compounds such as neurotransmitters (adrenaline, noradrenaline), DNA, RNA, phosphatidylcholine and creatine⁽¹⁴⁾. A subproduct of these methylation reactions is S-adenosylhomocysteine, which is hydrolysed to adenosine and Hcy⁽¹⁵⁾. Hcy can be remethylated to form methionine by the action of

the enzyme methionine synthase which uses $N^5,10$ -methylene-tetrahydrofolate-reductase as a methyl donor. The catabolism of methionine is performed by trans-sulfuration, with Hcy reacting with serine to form cystathionine in an irreversible reaction catalysed by cystathione- β -synthase. Through this pathway, Hcy is converted to cysteine, an important amino acid in the synthesis of proteins and reduced glutathione (GSH)⁽¹³⁾.

Creatine is a compound naturally found in food, especially meat and fish, although only half of the daily creatine requirement (about 1 g/d) comes from the diet, while the rest is endogenously synthesised in the liver⁽¹⁶⁾. The first step in creatine synthesis involves the reversible transfer of the amidino group of arginine to glycine to form guanidinoacetic acid (GAA) and ornithine in a reaction catalysed by the enzyme L-arginine:glycine amidinotransferase in the kidneys. Next, the irreversible transfer of a methyl group from SAM to GAA is catalysed by the enzyme SAM:guanidinoacetate *N*-methyltransferase⁽¹⁷⁾. Through this reaction, the endogenous synthesis of creatine consumes a considerable number of methyl groups. In humans, creatine synthesis has been reported to account for 70% of Hcy formation^(18,19). Wyss & Kaddurah-Daouk⁽¹⁷⁾ reported that this process utilises

Abbreviations: CrO + D, creatine overload plus diet with creatine; DCr, diet with creatine; GAA, guanidinoacetic acid; GSH, reduced glutathione; Hcy, homocysteine; SAM, S-adenosylmethionine; TBARS, thiobarbituric acid-reactive species.

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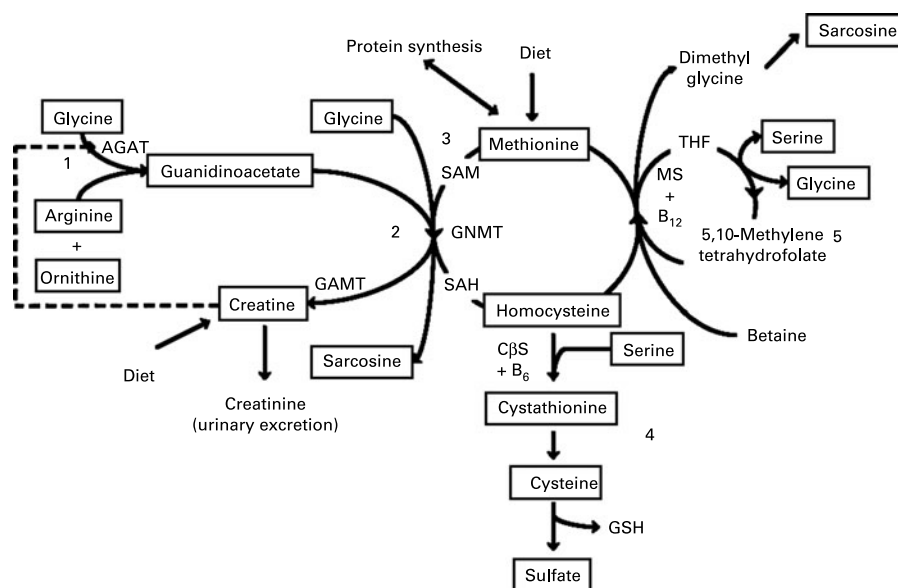


Fig. 1. Schematic presentation of the interaction of homocysteine and creatine metabolism in humans and animals: an increase in creatine intake inhibits the action of the enzyme L-arginine:glycine amidinotransferase (AGAT) required for reaction 1. As consequence, the pathway of S-adenosylmethionine (SAM) to S-adenosylhomocysteine (SAH) is down-regulated and there is a decrease in homocysteine formation (reaction 2). Under normal conditions (normal creatine intake), methionine will interact with guanidinoacetate, forming homocysteine. THF, tetrahydrofolate; MS, methionine synthase; GNMT, glycine N-methyltransferase; GAMT, guanidinoacetate methyl-transferase; C β S, cystathionine- β -synthase; GSH, reduced glutathione; reaction 3, transmethylation; reaction 4, trans-sulfuration; reaction 5, remethylation.

more SAM than all other methyltransferase reactions combined.

Thus, creatine synthesis and Hcy formation are metabolically connected⁽⁶⁾ (Fig. 1). Creatine supplementation has been shown to down-regulate the synthesis of L-arginine:glycine amidinotransferase and consequently reduce endogenous formation of creatine, changing the methylation flux and reducing Hcy synthesis^(6,20). However, little is known about the possible interactions between methyl balance modulation and lipid peroxidation when Hcy production is decreased by creatine supplementation.

The present study investigated the effects of two protocols of creatine supplementation on plasma levels of Hcy and amino acids involved in Hcy synthesis, and on oxidative stress in healthy rats.

Materials and methods

Animals and treatment

Thirty Wistar rats were obtained from the Central Animal House of the Faculty of Medicine of Ribeirão Preto, University of São Paulo, after approval by the institution's Ethics Committee of Animal Experimentation. The animals were kept in individual cages on a 12 h light–12 h dark cycle at a mean temperature of 22°C and were divided at random into three groups of ten rats each: control; diet with creatine (DCr); creatine overload plus diet with creatine (CrO + D). The animals had free access to food and water throughout the experiment.

Creatine supplementation was performed by adding 2% monohydrate creatine (Acros Organics, Fair Lawn, NJ, USA) to the control diet (AIN-93)⁽²¹⁾ for the DCr group for 4 weeks. The DCr + O group received monohydrated creatine

(5 g/kg) by oral administration for the first 5 d, followed by maintenance with the 2% creatine diet until the end of the 4-week period (adapted from Hultman *et al.*⁽²²⁾). The animals received fresh food every 2 d. Food intake and weight were measured weekly throughout the study period.

At the end of 4 weeks, 24 h urine samples were collected from the metabolism cages before the animals were killed by decapitation. Blood was collected and centrifuged and plasma was stored at –70°C. The liver and soleus muscle were removed, weighed, immediately frozen in liquid N₂ (–196°C) and stored at –70°C.

Measurement of metabolites

The determination of plasma Hcy levels involves preliminary pretreatment of the sample with S-adenosylhomocysteine hydrolase and dithiothreitol to release Hcy from the protein-bound form. Plasma Hcy, vitamin B₁₂ and folate were determined by competitive immunoassay (IMMULITE[®] kit; DPC MedLab, Los Angeles, CA, USA). Plasma concentrations of methionine, sarcosine, glycine, serine, ornithine and cystathionine were determined by GC (GC-17A; Shimadzu[®], Kyoto, Japan) with amino acid derivatisation by the EZ:Faast Amino Acid Analysis kit (Phenomenex[®], Torrance, CA, USA).

Total GSH was determined in plasma and liver as described by Sedlack & Lindsay⁽²³⁾. TBARS and total peroxide, as parameters of lipid peroxidation, were determined in plasma and liver by the methods of Mihara & Uchiyama⁽²⁴⁾ and Södergren *et al.*⁽²⁵⁾, respectively. Plasma and liver α -tocopherol was determined by HPLC (LC-20A; Shimadzu[®]) as described by Jordão *et al.*⁽²⁶⁾.

Plasma, urine and muscle creatine was determined by the Jaffe reaction using a method adapted from Taussky⁽²⁷⁾

and De Saedeleer & Marechal⁽²⁸⁾. Samples were heated to convert creatine to creatinine, which reacts with picric acid in an alkaline medium, forming a reddish complex that is measured photometrically. For the assay, 600 μ l of 44.4 mM-picric acid were added to 200 μ l of the sample and homogenised. From each sample, 300 μ l were added to two tubes containing 300 μ l distilled water (A_1 and B_1). After heating one (A_1) to 100°C for 1.5 h in a sealed vial, 1 ml of buffer solution containing 208 mM-sodium hydroxide, 12.7 mM-sodium tetraborate and a surfactant was added to all samples (A_1 and B_1). Readings were obtained with a spectrophotometer at 510 nm after incubation at 37°C for 10 min in a water-bath. Absorbances were defined as A_1 and B_1 . After the addition of 100 μ l of 11.4 mM-acetic acid and incubation at room temperature for 5 min, readings were obtained again and absorbances defined as A_2 and B_2 . Creatine concentration was calculated by the formula $(A_1 - B_1) - (A_2 - B_2)$ using a standard curve of 5 to 1000 μ M-creatine carried out in triplicate. To determine urinary creatine, 200 μ l urine were diluted 1:25 in 4.8 ml distilled water and processed as described above.

For the determination of muscle creatine, the soleus muscle was weighed, macerated in liquid N_2 and homogenised in 1 ml distilled water. The homogenate was diluted to 5 ml by the addition of distilled water (1:5) and free creatine was determined by the procedure described above. Total muscle creatine was determined by phosphocreatine hydrolysis. For this procedure, 300 μ l of muscle homogenate were heated to 65°C for 10 min in 0.4 μ M-HCl and neutralised with 2 μ M-NaOH, followed by the creatine measurement procedure described above. Total creatine corresponded to the sum of free creatine and phosphocreatine. Urinary creatinine was determined using an enzymic kit (LABTEST[®]; Labtest Diagnóstica, Lagoa Santa, Minas Gerais, Brazil).

Statistical analysis

Data are reported as mean values and standard deviations, and groups were compared by ANOVA followed by the Tukey post test. Correlation coefficients were calculated to determine

possible within-group associations between variables. In all analyses, the level of significance was set at $P < 0.05$.

Results

Table 1 presents the general and biochemical characteristics of the animals in the present study. No significant differences in body-weight gain, food intake, urinary creatinine or intramuscular creatine concentrations were detected between groups. Plasma folate was significantly decreased in the CrO + D group, which received dietary and overload creatine, compared with the control group, the no-creatine control. There was a significant increase in plasma and urinary creatine, and in phosphocreatine concentrations for the DCr (dietary creatine only) and CrO + D groups compared with the control group. A significant increase of total creatine was observed only in the CrO + D group.

Figure 2 presents the plasma Hcy and methionine levels of the experimental groups after 4 weeks of creatine supplementation. A significant decrease in plasma Hcy was observed for the DCr (7.5 (SD 1.2) μ mol/l) and CrO + D (7.2 (SD 1.7) μ mol/l) groups compared with the control group (12.4 (SD 2.2) μ mol/l). The reduction detected in the DCr and CrO + D groups compared with the control group was 39 and 41 %, respectively. In contrast, no differences were observed in methionine concentration. Significant negative correlations ($P < 0.05$) were detected between plasma Hcy and both plasma creatine (Fig. 3 (A)) and total intramuscular creatine ($r = 0.44$).

Table 2 presents the plasma concentrations of amino acids related to Hcy metabolism. A significant increase in glycine concentration was detected only in the CrO + D group compared with the others, whereas cystathionine was reduced in all groups supplemented with creatine compared with the control group. No differences in sarcosine, serine or ornithine were detected.

Table 3 presents the concentration of lipid peroxidation biomarkers and antioxidants in plasma and in liver. Plasma TBARS were significantly reduced after creatine supplementation, as was total plasma GSH. This reduction was not detected in the liver for the DCr group, however.

Table 1. Comparison of the general and biochemical characteristics of the control, dietary creatine (DCr) and creatine overload plus diet (CrO + D) groups after 4 weeks of experimentation (Mean values and standard deviations)

	Control		DCr		CrO + D	
	Mean	SD	Mean	SD	Mean	SD
Weight gain (g)	179.3	20.3	173.7	35.5	175.1	29.0
Food intake (g/d)	22.7	2.8	20.3	3.4	20.1	3.2
Plasma folate (nmol/l)	141.2 ^a	22.4	119.4 ^a	29.5	103.7 ^b	24.1
Plasma vitamin B ₁₂ (pmol/l)	2676.8	136.9	2778.8	160.5	2711.2	152.0
Urinary creatinine (mg/24 h)	5.7	1.8	7.7	2.0	6.4	2.0
Plasma creatine (μ mol/l)	80.1 ^a	26.9	191.5 ^b	41.4	204.8 ^b	29.8
Intramuscular creatine (μ mol/g wet weight)	15.7	0.8	14.8	1.6	15.1	1.4
Intramuscular PCr (μ mol/g wet weight)	2.0 ^a	0.7	4.34 ^b	2.3	5.91 ^b	1.12
Intramuscular tCr (μ mol/g wet weight)	17.8 ^a	1.1	19.1 ^a	3.4	21.0 ^b	1.8
Urinary creatine (μ mol/24 h)	0.4 ^a	0.3	2.5 ^b	1.0	1.7 ^b	0.7

PCr, phosphocreatine; tCr, total creatine.

^{a,b} Mean values within a row with unlike letters were significantly different ($P < 0.05$; ANOVA followed by the Tukey post test).

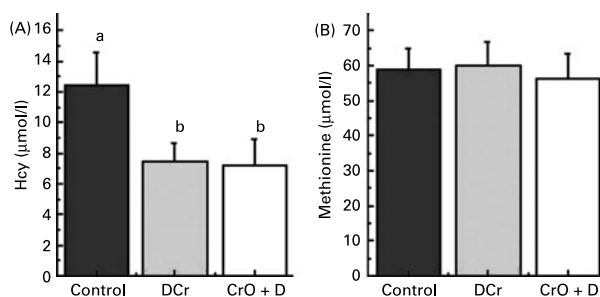


Fig. 2. Effects of 4 weeks of creatine supplementation on plasma homocysteine (Hcy) (A) and methionine (B) levels in the control, dietary creatine (DCr) and creatine overload plus diet (CrO + D) groups. Values are means, with standard deviations represented by vertical bars. ^{a,b} Mean values with unlike letters were significantly different ($P < 0.05$; ANOVA followed by the Tukey post test).

No differences were observed for total peroxide concentration in plasma, or for α -tocopherol in liver or plasma (Table 3).

A significant correlation ($P < 0.05$) was detected between plasma Hcy and plasma TBARS concentration (Fig. 3 (B)) and a significant negative correlation between plasma creatine and plasma TBARS (Fig. 3 (C)). Significant negative correlations ($P < 0.05$) were also found between plasma total peroxide and plasma creatine ($r = -0.40$). In addition, significant correlations were detected between folate and Hcy ($r = 0.57$). The amino acid glycine was correlated with Hcy concentration ($r = -0.45$), plasma total GSH ($r = -0.54$), plasma TBARS ($r = -0.52$) and total peroxides ($r = -0.37$). The amino acid cystathionine was correlated with Hcy concentration ($r = 0.50$), total plasma ($r = 0.38$) and liver ($r = 0.54$) GSH.

Discussion

The present study demonstrated that the Hultman *et al.* (22) adapted protocol with an overload supplementation period led to significant increases in plasma (154%) and total intramuscular creatine (18%) concentrations for the CrO + D group in relation to the control group. In contrast, the addition of 2% of creatine in the diet led to a significant increase just in plasma creatine (139%) concentrations for the DCr group (Table 1). Hultman *et al.* (22), in a pioneering study on human subjects, demonstrated that supplementation with 20 g creatine/d for 6 d increased the intramuscular stores of creatine by 20%. After this period, the concentration gradually decreased to baseline values, but elevated values of intramuscular creatine concentration were maintained for 30 d by ingestion of 2 g/d. The objective of the overload phase is to reach the maximal accumulation in muscular creatine; after this, the levels of muscle creatine can be maintained with relatively small dosages. Therefore, regimens of a loading phase followed by a maintenance phase appear to be effective in increasing total intramuscular creatine levels in rats.

Although there was a significant difference between the supplementation protocols in creatine levels, the same significant reductions in plasma Hcy concentrations were observed in both the DCr and CrO + D groups compared with the control group, with no changes in methionine concentration (Fig. 2 (B)), and significant negative correlations were seen between Hcy and plasma creatine (Fig. 3 (A)). Taken together, these data confirm the hypothesis that creatine

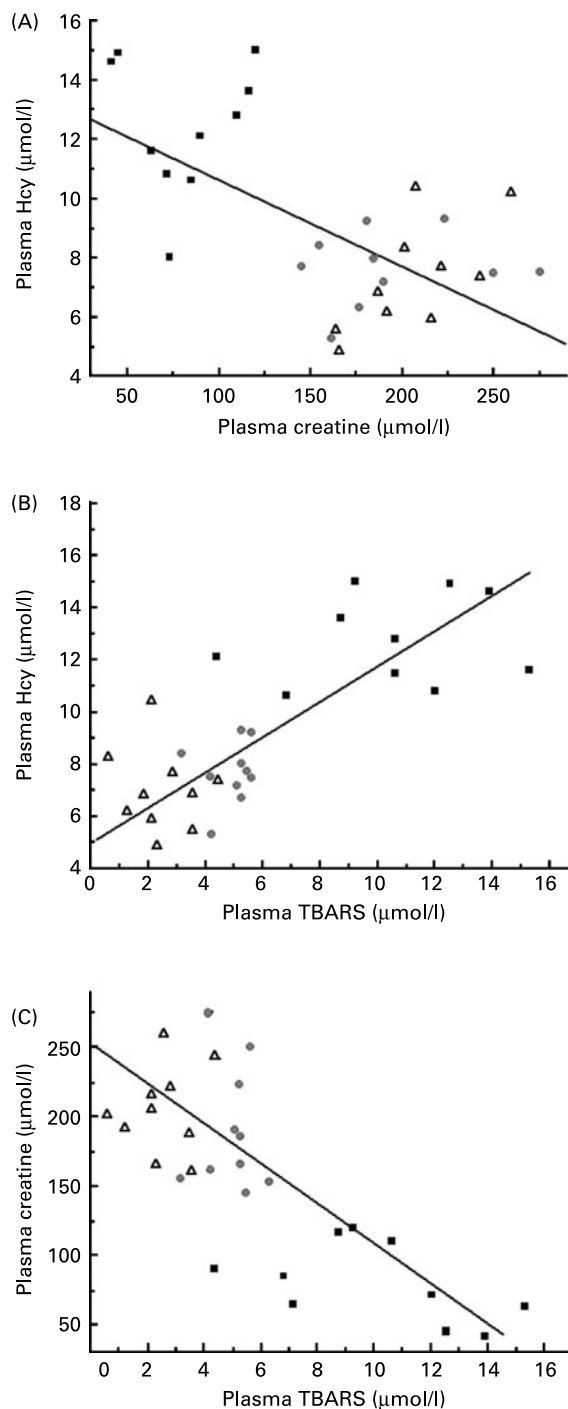


Fig. 3. Linear regression demonstrating a significant negative relationship ($r = -0.61$; $P < 0.01$) between homocysteine (Hcy) and plasma creatine (A), a significant positive relationship ($r = 0.74$; $P < 0.01$) between plasma Hcy and thiobarbituric acid-reactive species (TBARS) (B) and a significant negative relationship ($r = -0.75$; $P < 0.01$) between plasma creatine and plasma TBARS (C). The graphs present the data for all experimental groups: control (■), dietary creatine (●) and creatine overload plus diet (△).

supplementation reduces plasma Hcy levels in rats. Although no difference was detected between DCr and CrO + D animals, this was the first study to compare Hcy levels after applying different protocols of creatine supplementation. Previous animal studies have also detected reduced Hcy

Table 2. Plasma levels of the amino acids involved in the metabolism of homocysteine for the control, dietary creatine (DCr) and creatine overload plus diet (CrO + D) groups after 4 weeks of experimentation (Mean values and standard deviations)

	Control		DCr		CrO + D	
	Mean	SD	Mean	SD	Mean	SD
Sarcosine (μmol/l)	2.4	0.5	4.1	2.0	4.2	1.4
Glycine (μmol/l)	544.8 ^a	121.0	594.4 ^a	89.0	682.3 ^b	118.0
Serine (μmol/l)	410.9	86.4	394.8	77.1	405	67.2
Ornithine (μmol/l)	23.9	4.3	20.2	3.5	23.2	5.6
Cystathionine (μmol/l)	7.0 ^a	5.5	1.7 ^b	0.7	1.2 ^b	0.8

^{a,b} Mean values within a row with unlike letters were significantly different ($P < 0.05$; ANOVA followed by the Tukey post test).

concentrations after creatine supplementation^(6,20). Stead *et al.*⁽²⁰⁾ studied how methylation demand and Hcy formation were modulated by creatine and GAA intake, using rats receiving either 0.4% creatine, 0.36% GAA or a control diet. Hcy levels were reduced 25% by creatine, and increased 50% with GAA, while plasma methionine levels were unchanged. Taes *et al.*⁽⁶⁾ also detected significant reductions in plasma Hcy levels in a group of nephrectomised rats fed 2% creatine for 2 weeks, as well as a significant negative correlation between Hcy and plasma creatine.

In contrast to the animal data, studies conducted in human subjects have been somewhat controversial. Norlund *et al.*⁽²⁹⁾ demonstrated a significant correlation between plasma Hcy and serum creatine, and suggested that creatine turnover may be a factor determining Hcy levels. Korzun⁽³⁰⁾ studied healthy human subjects of both sexes who received creatine for 28 d and detected a small but significant reduction of Hcy levels compared with controls. In contrast, Steenge *et al.*⁽³¹⁾, in a study on women ingesting either a placebo or 20 g creatine/d for 5 d plus 3 g creatine/d for 8 weeks, did not find a significant difference in Hcy concentrations in any of the groups after the intervention. These authors also concluded that 61 d creatine supplementation did not suppress endogenous synthesis to an extent that would affect plasma Hcy levels in healthy women. Taes *et al.*⁽⁷⁾ also found no changes in Hcy levels after creatine supplementation at 2 g/d for 28 d in chronic renal patients on haemodialysis,

since the subjects were also receiving vitamins B₆, B₁₂ and folate. These authors also discussed the possibility that vitamin supplementation can mask the expected fall in Hcy levels in the presence of creatine supplementation. Thus, studies conducted on animals have demonstrated that inhibition of the endogenous methylation demand by creatine supplementation can reduce Hcy levels, but these results have not been reproduced in human subjects.

Creatine supplementation was associated with a decrease in folate concentration, different from Taes *et al.*⁽⁶⁾ who found a folate-saving effect induced by creatine supplementation. The importance of folate in remethylation and for Hcy level is well known^(7,8). House *et al.*⁽³²⁾ related that folate is required in the metabolic pathway for several amino acids, including glycine, serine and methionine and showed a marked elevation in glycine concentration in prolonged folate deficiency. In the present study, the decrease in folate and increase in glycine concentrations in the CrO + D group demonstrate the modulation of the flux through remethylation induced by creatine supplementation. These mechanisms appear to be down-regulated to maintain the normal methionine levels (Fig. 2 (B)) while the repression on L-arginine:glycine amidinotransferase exerted by exogenous creatine permits conservation of the amino acid methionine⁽²⁰⁾. However, further research is required confirming these hypotheses.

Recent studies have also demonstrated the importance of some amino acids on methionine metabolism^(14,33). Fukada

Table 3. Biomarkers of oxidative stress and the antioxidant defence system in the plasma and liver of the control, dietary creatine (DCr) and creatine overload plus diet (CrO + D) groups after 4 weeks of experimentation (Mean values and standard deviations)

	Control		DCr		CrO + D	
	Mean	SD	Mean	SD	Mean	SD
Plasma						
TBARS (μmol/l)	10.0 ^a	3.4	4.9 ^b	0.7	2.4 ^c	1.0
Total peroxide (μmol H ₂ O ₂ equivalents/l)	41.2	6.3	37.8	10.2	34.6	6.9
Total GSH (μmol/l)	4.3 ^a	1.9	2.5 ^b	0.8	1.8 ^b	0.5
α-Tocopherol (μmol/l)	26.2	6.1	20.5	5.1	18.9	3.7
Liver						
TBARS (nmol/mg protein)	0.13 ^a	0.01	0.18 ^b	0.01	0.12 ^a	0.01
Total GSH (mmol/mg protein)	44.0 ^a	10.0	34.1 ^a	14.2	29.4 ^b	10.0
α-Tocopherol (μmol/l)	76.8	12.0	82.8	15.7	87.6	27.4

TBARS, thiobarbituric acid-reactive species; GSH, reduced glutathione.

^{a,b,c} Mean values within a row with unlike letters were significantly different ($P < 0.05$; ANOVA followed by the Tukey post test).

et al.⁽³³⁾ showed the hypohomocysteinaemic effect of glycine and serine in animals loaded with an excess of methionine. In the present study, the glycine sparing effect in the CrO + D group compared with the control group (Table 2), along with the negative correlations between glycine and Hcy, suggests that the action of methyl-group acceptor of glycine is not required when Hcy formation is inhibited (Fig. 1, reaction 2). In addition, this sparing effect can also indicate an inhibition of the one-carbon unit-providing action of glycine to the $N^{5,10}$ -methylene-tetrahydrofolate-dependent remethylation of Hcy (Fig. 1, reaction 5). Thus, it seems reasonable to consider the important role of glycine on Hcy-modulated metabolism induced by creatine supplementation.

Although some authors have demonstrated an association between hyperhomocysteinaemia and free radical formation^(8–10), the present study is the first to demonstrate that the reduction of plasma Hcy levels by creatine supplementation is associated with a significant reduction of plasma lipid peroxidation biomarkers (Table 3). Also, the significant negative correlations between plasma creatine and the plasma lipid peroxidation biomarkers (TBARS and total peroxide) (Fig. 3 (C)) are *in vivo* confirmation of *in vitro* findings on the potential of creatine to remove reactive oxygen species^(34,35) and act as an antioxidant in a dose-dependent response. Lawler *et al.*⁽³⁴⁾ demonstrated a direct dose–response relationship of creatine concentration with superoxide anion (O_2^-), peroxyxynitrite ($OONO^-$) and 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) as determined by the rate of renewal of the $ABTS^+$ radical, and concluded that creatine has a significant role as an antioxidant. The authors state, however, that this potential appears to be lower than the normal physiological levels of GSH. Sestili *et al.*⁽³⁵⁾ also demonstrated that creatine has a direct antioxidant activity on cultured cells exposed to different oxidative agents. These authors verified that creatine, at doses similar to those detected in plasma after supplementation, had an antioxidant cytoprotective activity on three cell lines against three different oxidative agents: H_2O_2 , $OONO^-$ and *tert*-butylhydroperoxide (tB-OOH). In addition, the cytoprotection was independent of the antioxidant status of the catalase and glutathione peroxidase enzymes, suggesting a direct interaction between creatine and oxidant and/or free radicals. Souza *et al.*⁽³⁶⁾ assayed the chemiluminescence of excited carbonyls in urine, as a measure of the extent of tissue lipid peroxidation. In a study on the effects of different types of exercise, they found that lipid peroxidation in individuals who performed force/hypertrophy training with creatine supplementation was significantly lower than in a placebo group, suggesting protective antioxidant effects of creatine. However, in the present study, the antioxidant effect found in the plasma was not observed in the liver, suggesting that the antioxidant effects of creatine can be different in these tissues. Several investigators have suggested, however, that the antioxidant effects of creatine can be attributed to indirect antioxidant mechanisms such as hydration, membrane stabilisation and improved energy capacity of the cell^(1,16). The potential of creatine supplementation for the reduction of Hcy levels and consequently of cell toxicity that is demonstrated in the present study may also be an indirect form of the antioxidant action of this substance.

Hcy is the key intermediate in the coordination of the trans-sulfuration and remethylation pathways and its production

may influence the synthesis and function of methionine and GSH⁽³⁷⁾. GSH is the most abundant thiol in the antioxidant defence system, directly acting to neutralise reactive oxygen species⁽³⁸⁾. In the present study, the significant reductions of liver and plasma GSH (Table 3) and cystathionine concentrations (Table 2), in addition to the significant correlation between these two compounds, suggested that modulation of the methyl balance and the consequent reduction of Hcy can down-regulate the trans-sulfuration pathway in a dose-dependent response. This might decrease GSH production, affecting the redox status of the cells. Martinov *et al.*⁽³⁹⁾, in a mathematical modelling study of SAM metabolism in the liver, stated that when SAM levels are low, priority for this substrate goes to transmethylation reactions, promoting reduction of the trans-sulfuration pathway. These findings demonstrate the important relationship between the processes of methylation, Hcy formation and GSH formation.

In conclusion, creatine supplementation by the two protocols studied reduced plasma Hcy concentrations, possibly by modulating the methyl balance. This reduction of Hcy was associated with reduced lipid peroxidation biomarkers, suggesting the *in vivo* antioxidant cytoprotective effect of creatine. Alternatively, creatine supplementation might modulate the bioavailability of Hcy for the remethylation and trans-sulfuration processes, in turn influencing GSH synthesis, and affecting the redox status of the cells. Future studies should include a larger number of oxidative stress biomarkers and antioxidants, to determine the specific link with Hcy metabolism and changes induced by creatine supplementation.

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All the authors declare that there are no potential conflicts of interest.

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