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Organ Specific Changes in Markers of Oxidative Stress in Choline, Carnitine and Caffeine Supplemented and Exercised Rats

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Abstract: ***Objective*:** of this study was to determine changes in oxidative indices in tissues of exercised rats fed diet fortified with choline, carnitine and caffeine; and relate to those reported in humans.

***Methods*:** 7-week old male Sprague-Dawley rats were studied for five weeks. Diets were supplemented with choline, carnitine and caffeine and rats were either exercised or not during the last 2 weeks of the dietary regimen. Tissue samples were collected at the end of the period and analyzed for the concentrations of TBARS, hydroperoxides and protein carbonyls.

Results: The changes modulated by the supplement as well as exercise regimens in the oxidative stress markers were tissue specific. The changes in serum TBARS of rats were unlike those in women under similar conditions of treatment we reported earlier.

Conclusions: The effects of choline, carnitine and caffeine supplements on the indices of oxidative stress were tissue specific and were not always correlated with serum TBARS. The changes in serum TBARS in rats were contrary to those reported in humans under similar conditions of treatment.

Keywords: Caffeine, carnitine, choline, exercise, oxidative-indices, TBARS, hydroperoxides, protein-carbonyls, rats.

INTRODUCTION

Mitochondrial machinery that oxidizes energy substrates and carries out respiration also produces significant amounts of reactive oxygen species (ROS), which leak out of mitochondria and cause substantial damage to various cellular components [1]. Thus enhanced mitochondrial activity is expected to enlarge the ROS pool and, in turn, contribute to oxidative stress [2]. Approximately 85-90% of oxygen we breathe is used by the mitochondria [3] and 2% of the total oxygen consumption in the resting state is used to produce H_2O_2 by the mitochondria [4]. Oxidative stress is the phenomenon of the imbalance of prooxidant and antioxidant elements in a living being. An increase in prooxidants or a reduction in antioxidants can shift the equilibrium towards prooxidants and thus enhance oxidative stress on the individual.

Carnitine and acylcarnitines have been suggested to improve mitochondrial function by reducing oxidants and prooxidants load [5-8]. Choline deficiency has been implicated in increasing prooxidants in tissue mitochondria [9-11]. There is some evidence that caffeine protects against radiation damage by decreasing TBARS, hydroperoxides and protein carbonyls and preserving levels of glutathione and superoxide dismutase [12]. However, combined effect of these dietary supplements on oxidative stress has not been studied outside our laboratory [13].

Earlier we have shown that choline promotes carnitine conservation in humans and animals and this conservation is reflected in accumulation of carnitine by all tissues especially the skeletal muscle [14, 15]. A functional consequence of the increased carnitine was the loss of body fat in guinea pigs [14, 16]. The addition of caffeine on top of choline and carnitine in the diets of rats resulted in reduction of regional fat pads and increased production of short-chain acylcarnitines which is indicative of enhanced fatty acid mobilization and oxidation [17, 18]. Fatty acid oxidation is also known to be enhanced by Starvation and exercise besides dietary supplements [19]. Thus it was hypothesized that oxidative stress may be increased in choline, carnitine plus caffeine (CCC) supplemented rats. Our objective was to assess oxidative stress level in the male rats fed CCC-supplemented diets and exercised by determining the concentration of select markers of oxidative stress in their tissues.

MATERIALS AND METHODS

Experimental Design and Treatment of Animals

The study protocol was approved by the animal Care and Use Committee of University of Tennessee. Twenty 7-week old male Sprague-Dawley rats (Harlan Sprague-Dawley, Indianapolis, IN) were studied for four weeks. The rats were individually housed under conditions controlled for light (12-hour light-dark cycle), temperature (20-22 °C) and rela-

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tive humidity (50%). The rats were given free access to diet and water. The endogenous concentrations of choline and carnitine in the commercial diet, Teklad 22/5, were 2.1 g and 30 mg per kg diet, respectively. The chow was nonsupplement (NS) diet. The supplemented (S) diet was commercial chow fortified with caffeine, carnitine, and choline at the rate of 0.1, 5 and 11.5 g per kg non-purified diet, respectively.

For the dietary treatment period of 4 weeks, the 20 rats were randomly divided into two groups and assigned to the S or NS diet (n = 10). During week 3-4, five rats of each dietary group were randomly assigned to exercise (E) routine and the other 5 rats were not exercised (NE). For exercise they were run on a rodent treadmill five days/week at 15% grade for 10 min at the start which was gradually increased to 18 m/min for 25 min/day. The duration of diet and exercise treatment period was 4 and 2 weeks, respectively, and accordingly groups were designated as NS/NE, NS/E, S/NE and S/E. At the end of the 4th week, the rats were anesthetized and killed; blood samples were collected and immediately centrifuged at 2000 x g for 10 min. Following blood collection, liver, heart, kidney, skeletal muscle, testes and brain were excised from the carcasses, rinsed with saline solution, blotted dry and frozen in liquid nitrogen. Serum and tissues were stored at –80 °C until analyzed for various metabolites.

Reagents and Treatment of Samples

Reagents were prepared and stored under controlled temperatures and in dark glass bottles. The samples stored at –80 °C were briefly thawed in water and placed in ice for complete thawing. Samples of one tissue type were always assayed in sets of minimum four (corresponding to each treatment) and repeated if a result of single determination appeared to be a complete outlier. Approximately 300 mg frozen tissue samples were homogenized in 0.9 % normal saline solution and centrifuged at low speed and resulting supernatant re-centrifuged at 10,000 x g at 4 °C for 10 min. The final supernatant was collected into clean micro-centrifuge tubes with minimal air space and frozen at –80 °C until assayed.

Determination of Markers of Oxidative Stress

The protein content of serum and tissue homogenates was determined by the method described by Bradford using a commercial dye reagent [20]. The lipid peroxide molecule, such as malondialdehyde (MDA) in serum and tissue homogenates were determined as thiobarbituric acid reactive substances (TBARS) with minor modifications, using the method originally described by Placer *et al*. [21] and modified by Ohkawa *et al*. [22]. The relationship between absorbance and concentrations was linear from 0.1 to 5 nmoles of MDA (r = 0.999). The FOX (ferrous ion oxidation with xylenol orange) assay, as described by Wolff [23], was used to determine water-soluble lipid hydroperoxides in serum and tissue homogenates. Absorbance and concentrations were linearly related from 0-5 μmoles of hydrogenperoxide (r = 0.997). Protein carbonyls in serum and tissue homogenates were determined according to the method described by Levine and colleagues [24] and later modified by Reznick and Packer [25]. The principle of this assay involves the reaction of protein carbonyls with 2,4-dinitrophenylhydrazine to form protein hydrazones. The assay is sensitive to detect protein carbonyls in samples with 0.5 mg per sample and results are reproducible with protein carbonyl contents of 0.2-1 mg per mg proteins (r = 0.999).

Statistics

All results are presented as group means ± standard error of mean. Data were analyzed using two-way ANOVA. Duncan's post hoc test was applied for multiple comparisons. The correlations between concentrations of TBARS and lipid hydroperoxide or protein carbonyl were calculated. The statistical significance level was set at $p \leq 0.05$.

RESULTS

The effects of exercise (NS/E), supplementation (S/NE) and the supplement cum exercise (S/E) compared to the control group i.e. without supplement and exercise (NS/NE) on TBARS, lipid hydroperoxides and protein carbonyls in serum, liver, muscle, brain, testis and kidney are presented in Tables **1** through **5**.

Serum

The supplemented and exercised groups had significantly higher levels of TBARS ($p \leq 0.01$) compared to the NS/NE group (Table **1**). Protein carbonyl concentrations were significantly higher in both supplemented groups ($p \leq 0.05$), but not in the NS/E group. A significant interaction of supplement and exercise was observed for TBARS ($p \leq 0.01$). TBARS and hydroperoxides were positively correlated, whereas TBARS and protein carbonyls were negatively correlated ($p \leq 0.01$).

Liver

The supplemented rats had significantly higher TBARS ($p \leq 0.05$) but had no significant change in protein carbonyl or hydroperoxide concentrations (Table **2**). No significant change of TBARS concentration was observed with exercise alone. TBARS and protein carbonyls were positively correlated ($p \leq 0.05$).

Skeletal Muscle

Neither the TBARS nor hydroperoxide concentrations were affected by exercise or supplement (Table **3**). However, exercise alone significantly lowered protein carbonyl concentrations when compared to those in the group with the supplement alone. A positive correlation was found between TBARS and hydroperoxides ($p \leq 0.03$)

Brain

The data for TBARS and hydroperoxide concentrations did not differ significantly among the groups (Table **4**). Protein carbonyl concentrations were decreased in both exercised groups but not in the group given the supplement alone ($p \leq 0.01$). A significant interaction of supplement and exercise was observed for protein carbonyls ($p \leq 0.001$).

Table 1. Comparison of Serum TBARS, Hydroperoxide and Protein Carbonyl Concentrations in Rats Fed Diets With or Without Supplement and With or Without Exercise[1]

Groups	TBARS		Hydroperoxides		Protein Carbonyls	
	nmol/ mg Protein					
NS/NE	0.09	± 0.01[a]	0.54	± 0.03	2.32	± 0.04[a]
NS/E	0.20	± 0.02[b]	0.39	± 0.04	2.49	± 0.10[ab]
S/E	0.23	± 0.02[b]	0.42	± 0.03	2.80	±0.10[b]
S/NE	0.23	± 0.01[b]	0.40	± 0.05	2.81	± 0.18[b]
	Statistical significance[2]					
Supplement	< 0.001		NS		0.003	
Exercise	0.003		NS		NS	
S x E[3]	0.004		NS		NS	

NS = no supplement, S = supplement, NE = no exercise, E = exercise
[1] Values are means ± SEM (n = 5) and those bearing different superscript letters within a column are significantly different at $p \leq 0.05$
[2] Two-way ANOVA, $p \leq 0.05$; NS = not significant ($p > 0.05$)
[3] Supplement and exercise interaction

Table 2. Comparison of Hepatic TBARS, Hydroperoxide and Protein Carbonyl Concentrations in Rats Fed Diets With or Without Supplement and With or Without Exercise [1]

	TBARS		Hydroperoxides		Protein Carbonyls	
	nmol/mg Protein					
NS/NE	0.42	± 0.01[a]	3.55	± 0.11	5.33	± 0.24
NS/E	0.40	± 0.04[a]	3.84	± 0.05	5.26	± 0.16
S/E	0.57	± 0.03[b]	3.57	± 0.13	5.93	± 0.20
S/NE	0.49	± 0.03[b]	3.31	± 0.20	5.41	± 0.29
	Statistical significance[2]					
Supplement	< 0.001		NS		NS	
Exercise	NS		NS		NS	
S x E[3]	NS		NS		NS	

NS = no supplement, S = supplement, NE = no exercise, E = exercise
[1] Values are means ± SEM (n = 5) and those bearing different superscript letters within a column are significantly different at $p \leq 0.05$
[2] Two-way ANOVA, $p \leq 0.05$; NS = not significant ($p > 0.05$)
[3] Supplement and exercise interaction

Testes

The supplementation had no effect on the TBARS concentration (Table **5**). However, exercise alone significantly lowered TBARS concentration ($p \leq 0.01$). Hydroperoxides and protein carbonyl concentrations were not affected by exercise and/or supplement.

Kidney

No significant effect of any of the treatments was observed on any of the three markers of oxidative stress in this tissue (data not shown).

DISCUSSION

Since the combination of choline, carnitine and caffeine with or without exercise promotes fat mobilization and oxidation, it was hypothesized that these conditions may tip the balance in favor of oxidative stress [17, 18]. However, in our study in women CCC supplementation reduced oxidation stress on the basis of TBARS measured in serum [13]. So it was logical for us to ask if the combination of CCC mitigated oxidative stress in many other organs of the body and for that we turned to rat model. Unfortunately, like in most studies samples were deep frozen before analysis could be performed which may have affected absolute values [26, 27]

Table 3. Comparison of Skeletal Muscle TBARS, Hydroperoxide and Protein Carbonyl Concentrations in Rats Fed Diets with or without Supplement and With or Without Exercise [1]

	TBARS		Hydroperoxides		Protein carbonyls	
	nmol/ mg protein					
NS/NE	2.52	± 0.26	1.05	± 0.11	8.89	± 0.27[ab]
NS/E	2.01	± 0.14	0.94	± 0.07	7.97	± 0.13[a]
S/E	2.49	± 0.35	1.30	± 0.25	8.11	± 0.28[ab]
S/NE	2.64	± 0.54	0.89	± 0.05	9.15	± 0.56[b]
	Statistical significance[2]					
Supplement	NS		NS		NS	
Exercise	NS		NS		0.012	
S x E[3]	NS		NS		NS	

NS = no supplement, S = supplement, NE = no exercise, E = exercise
[1] Values are means ± SEM (n = 5) and those bearing different superscript letters within a column are significantly different at $p \leq 0.05$
[2] Two-way ANOVA, $p \leq 0.05$; NS = not significant ($p > 0.05$)
[3] Supplement and exercise interaction

Table 4. Comparison of Brain TBARS, Hydroperoxide and Protein Carbonyl Concentrations in Rats Fed Diets With or Without Supplement and with or Without Exercise [1]

	TBARS		Hydroperoxides		Protein Carbonyls	
	nmol/ mg Protein					
NS/NE	2.31	± 0.20	5.52	± 0.24	9.87	± 0.50[b]
NS/E	2.27	± 0.06	5.56	± 0.52	7.91	± 0.48[a]
S/E	2.38	± 0.17	5.88	± 0.30	8.06	± 0.17[a]
S/NS	2.55	± 0.20	4.91	± 0.56	10.83	± 0.52[b]
	Statistical significance[2]					
Supplement	NS		NS		NS	
Exercise	NS		NS		< 0.001	
S x E[3]	NS		NS		0.001	

NS = no supplement, S = supplement, NE = no exercise, E = exercise
[1] Values are means ± SEM (n = 5) and those bearing different superscript letters within a column are significantly different at $p \leq 0.05$
[2] Two-way ANOVA, $p \leq 0.05$; NS = not significant ($p > 0.05$)
[3] Supplement and exercise interaction

but not relative values of our experimental results as the parameters across the treatments were determined under identical conditions [28].

The increases in serum concentrations of TBARS and protein carbonyls, even without changes in lipid hydroperoxides, suggest higher degree of oxidative stress in the supplemented animals. Lipid hydroperoxides are not very stable and get transformed into more stable products such as alkanes and aldehydes, a form of the latter is MDA which is measured as TBARS [29] but these are not the artifacts of the TBA assay system itself [30]. Exercise independently affected serum TBARS in the manner similar to that caused by the supplements (Table **1**). The increase in the concentrations of TBARS is also observed in liver and testes but not in skeletal muscle, brain and kidneys of the supplemented rats. Thus oxidative stress assessed through serum TBARS was not uniformly applicable to other tissues of the rats. Tissues have been shown to respond differently to oxidative stress and the markers may vary accordingly [31]. Further, the serum TBARS response to CCC supplementation in rat model was contrary to that we found in free-living women who showed significant reduction in concentration of serum TBARS after 1-2 weeks of supplementation [13]. Unlike the rats in the current study, caffeine was not a part of the supplement mix given to the women in our earlier study, however, they did consume on an average 240 mg of caffeine per day from a variety of foods and beverages. The difference in response to choline and carnitine in humans vs rodents must

Table 5. Comparison of Testes TBARS, Hydroperoxide and Protein Carbonyl Concentrations in Rats Fed Diets With or Without Supplement and With or Without Exercise [1]

	TBARS		Hydroperoxides		Protein carbonyls	
	nmol/ mg protein					
NS/NE	0.50	± 0.09[b]	7.95	± 0.38	7.17	± 0.77
NS/E	0.28	± 0.02[a]	9.14	± 0.46	7.13	± 0.31
S/E	0.61	± 0.08[b]	8.33	± 0.59	8.10	± 0.44
S/NE	0.64	± 0.02[b]	8.83	± 0.14	8.17	± 0.32
	Statistical significance[2]					
Supplement	0.001		NS		NS	
Exercise	NS		NS		NS	
S x E[3]	NS		NS		NS	

NS = no supplement, S = supplement, NE = no exercise, E = exercise
[1] Values are means ± SEM (n = 5) and those bearing different superscript letters within a column are significantly different at $p \leq 0.05$
[2] Two-way ANOVA, $p \leq 0.05$; NS = not significant ($p > 0.05$)
[3] Supplement and exercise interaction

be attributed to the species specific differences in metabolism e.g. rats metabolize choline 60 times faster than do humans [32].

In skeletal muscle TBARS and hydroperoxides were not affected by treatments. Contrary to the expectations that exercise may have enhanced oxidative stress in skeletal muscles, it actually mitigated any effect as evident from carbonyls data (Table **3**). This may be related to the training, duration and intensity of exerciser regimen. Regular exercise has been shown to enhance concentrations of antioxidants in skeletal muscle and liver [33, 34]. Exercise has been shown to lower concentration of oxidized DNA in skeletal muscle without affecting TBARS and protein carbonyls [35]. These studies may indicate that oxidative stress induces antioxidant systems to cope with ROS [36].

The increase in TBARS concentration in liver of our rats is consistent with the increase of fatty acid oxidation in liver indicated by a rise of serum ß–hydroxybutyrate [18] however, the antioxidative benefit of the supplement was not evident [13]. Perhaps the enhancement of mitochondrial activity under the influence of CCC far exceeded the antioxidation protection the levels of carnitine supplement could offer. Carnitine supplement alone has been shown to significantly improve antioxidation status in old but not in young rats [8, 37, 38]. The protection against peroxidation by carnitine may be related to conservation of tocopherol [8, 13, 38, 39] which in turn stabilizes membranes [6, 7].

It is concluded that effects of supplements and exercise on the indices of oxidative stress were tissue specific and they were not always correlated with serum TBARS. The changes in serum TBARS in rats were contrary to those seen in humans under similar conditions of treatment. This suggests species differences in metabolism and the diversity of tissue response within a species which deserves due consideration.

CONFLICT OF INTEREST

The authors confirm that this article content has no conflicts of interest.

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