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OXIDANT/ANTIOXIDANT TISSUE BALANCE IN CARNITINE SUPPLEMENTED RATS EXPOSED TO CHRONIC HYPOTHERMIC AND ANAKINETIC STRESS

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ABSTRACT. *Background.* Hypothermia and immobilization are commonly used methods in laboratory stress testing. *Aims*. The study evaluated the effects of chronic hypothermic (5° C) and restraint stress in liver and muscle oxidant/ antioxidant balance, on rats with and without carnitine supplementation*. Material and methods*. The study was made on four groups of male rats, adult (n = 10 animals / group) Wistar breed, for 15 days): group I- exposed to hypothermic stress (5° C), group II-exposed to anakinetic stress, group III- exposed to combined stress (hypothermic - 5° C - and anakinetic stress), group IV- supplemented with carnitine and exposed to combined stress. Tissues samples (liver and muscle) were used to determine the level and activity of the oxidative stress (OS) indicators - malondialdehyde (MDA), carbonilated proteins (CP) and antioxidant (AO) system –hydrogen donors capacity (HD), thiol groups (SH), reduced gluthatione (GSH). *Results.* The statistical analysis, performed on the 4 groups, revealed that chronic combined stress induced significant increases for liver and muscle MDA, and decreases for liver SH and muscle GSH, as compared to chronic hypothermic stress. Regarding chronic combined stress, there were significant increases for MDA, and decreases for GSH, in liver and muscle, as compared to chronic anakinetic stress. Carnitine supplementation in chronic combined stress conditions (group IV) induced significant changes by diminishing the liver and muscle MDA levels; liver PC levels, and increasing the DH and SH liver and muscle groups, muscle GSH groups as compared to combined stress group (III). *Conclusions*. Our experimental results show that chronic combined stress (hypothermic and anakinetic stress) increases the oxidative stress (OS) indicators and decreases those of antioxidant (AO) defense in the studied tissues, as compared to chronic hypothermic and anakinetic stress) Carnitine supplementation in chronic combined stress conditions had benefic effects by diminishing the OS indicators and by increasing the AO defense, in liver and muscle tissue.

Keywords: *chronic combined stress, hypothermic stress, anakinetic stress, carnitine, muscle, liver, oxidants/antioxidants balance*

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REZUMAT. ***Balanţa tisulară oxidanţi/antioxidanţi la şobolanii suplimentaţi cu carnitină, expuşi stresului cronic hipotermic şi anakinetic.*** *Premize.* Hipotermia şi imobilizarea sunt metode frecvent utilizate în provocarea stresului experimental. *Obiective*. S-au urmărit efectele stresului combinat cronic (hipotermic şi anakinetic) asupra balanţei oxidanţi/antioxidanţi la nivel tisular (hepatic şi muscluar), la animale cu şi fără suplimentare de carnitină. *Material şi metodă*. Cercetările au fost efectuate pe patru loturi de şobolani masculi, adulţi (n=10 animale /lot) rasa Wistar, timp de 15 zile: lotul I- supus stresului hipotermic (5° C), lotul II -supus stresului de imobilizare (anakinetic), lotul III – supus stresului combinat (hipotermic - şi stresului anakinetic), lotul IV – suplimentat cu carnitină şi supus stresului combinat. Indicatorii balanţei O/AO au fost evaluaţi prin metode biochimice de dozare din ţesuturile hepatic şi muscular. *Rezultate*. Stresul combinat cronic determină, faţă de stresul hipotermic cronic, la nivel hepatic şi muscular, creşteri semnificative statistic ale MDA, scăderi semnificative statistic ale SH la nivel hepatic şi GSH la nivel muscular, iar faţă de stresul anakinetic cronic, determină creşteri semnificative la nivel hepatic şi muscular ale MDA, şi scăderi semnificative ale GSH. Suplimentarea cu carnitină şi stresul combinat cronic determină, faţă de stresul combinat cronic, scăderi semnificative ale MDA în muşchi, ale PC în ficat, şi creşteri semnificative statistic ale DH şi SH, în ficat şi muşchi, şi GSH în muşchi. *Concluzii*. Stresul cronic combinat – anakinetic şi hipotermic – determină creşteri semnificative ale SO şi scăderi semnificative ale apărării AO, faţă de stresul cronic anakinetic şi stresul cronic hipotermic, în ţesuturile studiate (hepatic şi muscular). Suplimentarea cu carnitină, la lotul supus stresului combinat cronic, a avut efecte protectoare, manifestate la nivel tisular prin scăderea SO şi creşterea indicatorilor AO.

Cuvinte cheie*: stres cronic combinat, stres hipotermic şi anakinetic, ţesut muscular, ţesut hepatic, balanţa oxidanţi/antioxidanţi, carnitină.*

Introduction

Reactive oxygen species (ROS) and reactive nitrogen species (RNS) are produced by cellular metabolism. They play important roles in regulation of cell survival. ROS and RNS may have both beneficial and harmful effects on living systems. The beneficial effects of ROS occur at low/ moderate concentrations and involve physiological cellular response to injury (eg defense mechanism against infectious agents) or mitogenic response. Severe increase of ROS/RNS can induce cell death.

Reactive species (RS) side effects', with possible biological damage, is called oxidative (OS) or nitrosative (NS) stress.

The effect can be seen when, on one hand there is a hyperproduction of oxygen and nitrogen species (RONS) and, on the other hand, there is a lack of enzymatic and non- enzyme antioxidant (AO) system. ROS, adversely, alter lipids, proteins, and DNA and trigger a number of human diseases (Burta et al, 2003; Ji et al, 1998).

Thus, the OS is involved in a variety of pathological conditions, as well as physiological processes (stress, pregnancy, aging).

Under physiologic conditions, the balance between generation and elimination of ROS/RNS maintains the proper function of redox-sensitive signaling proteins. Normally, the redox homeostasis ensures that cells respond properly to endogenous and exogenous stimuli. However, when the redox homeostasis is disturbed, oxidative stress may lead to aberrant cell death and contribute to disease development. (Trachootham et al, 2008; Valko et al, 2007; Dröge, 2002).

Carnitine is a quaternary ammonium compound, biosynthesised mainly in the liver and kidney, from the amino acids lysine and methionine. The total level of carnitine in the body was estimated to be around 20 g, or 120 mmol.

Most studies, developed on humans, failed to demonstrate the increase in muscle carnitine levels, either by oral administration (because of low systemic bioavailability of the pharmaceutical product, 5-15%) or intravenously (carnitine transpoters being considered the limiting factors).

Thus, due to its' low oral bioavailability and significant urinary loss after administration, high doses should be administered for longer periods of time, in order to influence muscle carnitine storage in healthy subjects..

There is scientific evidence, that exogenous carnitine can influence muscle total carnitine content, but without well established functional consequences (Brass, 1995; Brass, 2000; Scholte, 2003; Stephens et al, 2007).

However, carnitine supplementation in animals turned out to have beneficial effects on the muscles carnitine content, by intragastric administration, both in sedentary and trained rats, as well as in horses (Bacurau et al, 2003).

Hypothesis

The study evaluated the effects of cronic hypothermic (5° C) and restraint stress in tissue (liver and muscle) oxidant/antioxidant balance, on rats with and without carnitine supplementation.

Materials and methods

The sudy was performed on adult male rats, Wistar breed, at the Department of Physiology from UMF "Iuliu Haţieganu", Cluj-Napoca, in the Laboratory of Experimental Physiology. The animal tests and experiments were allowed by the Bioethical Board of the UMF "Iuliu Haţieganu", Cluj-Napoca. The animals were caged in polycarbonate cages, at controlled temperature of 21-22ºC, humidity (40-60%) and 12/12h light/dark cycle. Standard lab chow, and water were freely available.

Rats were divided randomly into four groups of ten rats each: I - hypothermic stress (5° C), II- anakinetic stress, III - exposed to combined stress (hypothermic - 5° C - and anakinetic stress), IV- supplemented with carnitine and exposed to combined stress.

Chronic cold stress was applied to the animals for 3 hours daily, 15 days long. The rats were placed in a cold room (ambient temperature 5°C), according to the literature data.

Chronic immobilization stress was applied to the animals for 3 hours daily, for 15 days long, according to the literature data. Immobilization stress was applied to the animals by using cylindrical tubes with dimensions of 15 cms long and 8 cms in diameter, containing numerous perforations which served as breathing holes.

The animals were daily supplemented with L-Carnitine by oropharyngeal gavage, before exposure to stress, (Carnil 100 mg/ml, provided by Anfarm Hellas S.A. Pharmaceutical Industry Factory, Athens, Greece). Each animal received 100 mg/kgc L-Carnitine, calculated according to daily dosage for humans.

a) *Methods*

At the end of the experimental period, blood was collected from the retro orbitary sinus, Euthanasia was induced according to the recommendation of the Bioethical Board of the University.

Tissues were minced and homogenized and the supernatant was used to determine the level and activity of the oxidative stress (OS) indicators – MDA (Conti, 1991), PC (Reznick *and* Packer, 1994) and antioxidant (AO) system – DH (Janaszewska *and* Bartosz, 2002), SH (Hu, 1994), GSH (Hu, 1994).

b) *Statistical analysis.*

All data are reported as the mean ± SD. Statistical analyses were performed by one-way analysis of variance ANOVA, followed by post hoc Tukey's range test procedure, for pair-wise comparisons. Pearson's correlation was the

test of choice, in order to asses the correlation between normally distributed variables. Statistical significance was at $p<0.05$. Statistical values were obtained using GraphPad Prism 5.0 software and Microsoft EXCEL.

Results

a) Statistical indicators and comparative analysis for OS/AO tissue indicators are presented in table I and II.

Table I

Statistical indicators for centrality and dispersion in liver

Group	MDA (nmol/mg prot)		PC (nmol/mg prot)		DH (%inhibiţie)		SH (µmol/mg prot)		GSH (nmol/mg prot)	
	MA	±DS	MA	±DS	MA	±DS	MA	±DS	MA	±DS
I	0,141	0,010	5,27	0,58	4,42	0,50	0,035	0,0033	3,85	0,29
II	0,145	0,010	5,39	0,52	3,81	0,30	0,025	0,0015	3,95	0,38
III	0,172 [a,b]	0,016	4,93	0,51	4,21	0,33	0,020 [a]	0,0055	3,40 [b]	0,30
IV	0,15 [c]	0,015	3,96 [c]	0,37	5,05 [c]	0,48	0,041 [c]	0,0050	3,54	0,33

Note: (I) hypothermic stress, (II) anakinetic stress, (III) exposed to combined stress (hypothermic and anakinetic stress), (IV) supplemented with carnitine and exposed to combined stress.
ANOVA test, $p<0.05$. a= III vs I; b=III vs II; c=IV vs III;

Table II

Statistical indicators for centrality and dispersion in muscle

Group	MDA (nmol/mg prot)		PC (nmol/mg prot)		DH (%inhibiţie)		SH (µmol/mg prot)		GSH (nmol/mg prot)	
	MA	±DS	MA	±DS	MA	±DS	MA	±DS	MA	±DS
I	0,89	0,088	5,62	0,30	11,31	0,86	0,018	0,0039	1,51	0,13
II	0,70	0,034	4,66	0,56	12,34	1,38	0,016	0,0024	1,85	0,17
III	1,072 [a,b]	0,098	5,26	0,48	10,87	1,11	0,013	0,0044	1,13 [a,b]	0,10
IV	0,74 [c]	0,097	5,16	0,69	30,55 [c]	3,10	0,025 [c]	0,0022	2,09 [c]	0,20

Note: (I) hypothermic stress, (II) anakinetic stress, (III) exposed to combined stress (hypothermic and anakinetic stress), (IV) supplemented with carnitine and exposed to combined stress.
ANOVA test, $p<0.05$. a= III vs I; b=III vs II; c=IV vs III;

Liver and muscle tissue MDA increased significantly in group III (exposed to combined stress) as compared to group I (exposed to hypothermic stress) In group IV (supplemented with carnitine and subjected to combined stress) MDA levels were statistically reduced as compared to group III (Table I, II).

In group IV (supplemented with carnitine and exposed to combined stress) there was a significant decrease of liver CP as compared to group III (Table I).

The statistical analysis, performed on the 4 groups, revealed that chronic combined stress (group III) induced no significant changes for liver and muscle HD as compared to hypothermic stress (group I) and anakinetic stress (group II). (Table I, II).

In group IV (supplemented with carnitine and subjected to combined stress) liver and muscle HD levels were statistically increased as compared to group III) (Table I, II).

Liver SH decreased significantly in group III (combined stress) as compared to group I (hypothermic stress). In group IV (supplemented with carnitine and exposed to combined stress), liver and muscle SH levels increased statistically significant as compared to group III (Table I, II).

Liver GSH decreased statistically significant in group III (combined stress) as compared to group II (stress anakinetic). In group III, muscle GSH levels were statistically reduced as compared to group I and II. (Table I, II).

In group IV (supplemented with carnitine and exposed to combined stress) muscle GSH levels were statistically significant elevated as compared to group III (Table II).

b) Correlation indicators for OS/AO balance in tissues, of the studied groups are presented in table III and IV

Table III

Correlation indicators for OS/AO balance in liver, at the end of the experiment

Parameters		Pearson Correlation Coefficient	p		Parameters		Pearson Correlation Coefficient	p
MDA	CP	-0,06*	0,75		CP	HD	-0,59***	**0,001**
MDA	HD	0,03*	0,85		CP	SH	-0,47**	**0,01**
MDA	SH	-0,33**	0,10		CP	GSH	0,32**	0,12
MDA	GSH	-0,43**	**0,03**		HD	SH	0,59***	**0,02**
SH	GSH	0,07*	0,74		HD	GSH	-0,20*	0,33

* weak correlation,** acceptable correlation,***good correlation,
**** very good correlation (Colton Scale)

There were negative significant correlations between liver OS and liver AO defense indicators (MDA/GSH; CP/HD,SH). The liver AO defense indicators (HD and SH) showed a positive correlation (Table III).

Table IV

Correlation indicators for OS/AO balance in muscle, at the end of the experiment

Parameters		Pearson Correlation Coefficient	p		Parameters		Pearson Correlation Coefficient	p
MDA	CP	0,30**	0,14		PC	DH	0,004*	0,98
MDA	HD	-0,39**	0,055		PC	SH	0,16*	0,44
MDA	SH	-0,40**	0,052		PC	GSH	-0,32**	0,12
MDA	GSH	-0,75***	**<0,0001**		DH	SH	0,72***	**<0,0001**
SH	GSH	0,61***	**<0,001**		DH	GSH	0,68***	**<0,0001**

* weak correlation,** acceptable correlation,***good correlation,
**** very good correlation (Colton Scale)

There were negative correlations between the muscle OS and AO defense indicators (MDA/GSH),but positive ones between AO defense indicators (DH/SH,GSH) (Table IV).

Discussions

Stressors may induce different effects on liver tissue (Zlatković and Filipović, 2011).

Some authors revealed enhanced lipid peroxidation in liver, muscle (Ates et al, 2006; Venditti et al, 2004) and striatum (Méndez-Cuesta, 2011), but diminished tissue AO defense activity (Saggu and Kumar, 2008; Ates et al, 2006) under conditions of acute exposure to combined stress – hypothermic and anakinetic, whereas other authors showed intense AO enzymatic activity in hepatic tissue (Popovic et al, 2009).

Other studies evidenced no changes in liver AO enzyme activity (CAT, SOD, GPx) in animals exposed to acute hypothermic stress (Alva et al,2009). Acute hypothermia, followed by severe hypoxia in rats, induced favorable effect on OS parameters, which values were closed to the control groups' (Alva et al, 2010).

Acute anakinetic or hypothermic stress, followed by social isolation chronic stress, induced different changes on liver CuZnSOD activity, either by increasing the nuclear fraction or the cytosolic one.

Combined chronic stress induces in rats, increased levels of kidney and heart CP, liver, kidney and heart TBARS, liver and kidney CuZnSOD enzyme, heart and kidney CAT, liver and heart GSH-PX Se and decreased levels of heart GSH and liver CAT (Şahin, 2007).

Chronic stress and high levels of glucocorticoids increase ROS and influence the processes, these are involved in.

Thus, some authors showed similar effects of both immobilization stress and corticoids administration in rats, such as increased lipid peroxidation and decreased AO activity in brain, liver and heart (Zafir and Banu, 2009).

Immobilization and intense physical training, experimentally induced on animals, have shown increased oxidation in muscle proteins, a process that could be alleviated by E vitamin administration (Bar-Shaia et al, 2008).

Carnitine supplementation in rats exposed to acute combined stress , reduced lipid peroxidation and increased CAT activity in gastric mucosa (İzgüt-Uysalet and Deri, 2001; Izgüt-Uysal et al, 2001).

Seven days of carnitine oropharyngeal gavage administration improved AO non-enzymatic activity (increased GSH) and attenuated the increase of renal tissue MDA, in rats exposed to intense exercise (Bucioli et al, 2012).

The results of our research study are similar to those presented by other authors Şahin, 2007; Venditti et al, 2004; Zafir and Banu, 2009).

Conclusions

1. Chronic combined stress induces significant increases in liver and muscle MDA, but significant decreases in liver SH and muscle GSH, as compared to hypothermic stress.

2. Chronic combined stress induces significant increases in MDA and significant decreases in GSH both in liver and muscle,as compared to anakinetic stress.

3.Carnitine supplementation and chronic combined stress induces significant decreases in liver and muscle MDA, liver CP, but significant increases in liver and muscle DH and SH, muscle GSH.

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