

Application of DPPH assay for the evaluation of the antiradical activity of Creatine Lysinate

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ABSTRACT

The aim of the current study was the estimation of the radical-scavenging activity of Creatine lysinate against 0.05 mM methanol solution of DPPH radical by measuring the decrease in the absorbance at $\lambda = 516$ in methanol. The antiradical effect of the compound examined is presented with the following parameters calculated: radical scavenging activity in [%], IC₅₀ value; antioxidant power 1/IC₅₀, and Trolox equivalent activity. relative radical scavenging activity (RRSA, [%]), and a relative decrease in radical scavenging activity (RDRSA, [%]). From the experimental results, it was observed that the DPPH binding ability of Creatine lysinate (IC₅₀ = 73.75 mM) is lower compared to the standard Trolox (IC₅₀ = 0.001154 mM), which antioxidant power (1/IC₅₀ = 8.67) is higher in comparison with Creatine lysinate (1/IC₅₀ = 0.014). The experimental data show that Creatine lysinate (IC₅₀ = 73.75 mM) is more active compared to Creatine monohydrate (IC₅₀ = 102.48 mM) due to lower IC₅₀ and higher scavenging activity.

In comparison with mono application of alone antioxidant, the beneficial effect of the combination of Creatine monohydrate and Creatine lysinate with other antioxidants in form of food supplements could be an important strategy for a synergistic effect in the reduction of free radicals in the treatment of disorders resulted from oxidative stress.

Keywords: Creatine lysinate, DPPH, Radical-scavenging activity, Reactive oxygen species, Trolox

Introduction

Reactive oxygen species include charged and neutral species such as superoxide anion (O₂^{•-}), singlet oxygen (¹O₂), hydroxyl (HO[•]), alkoxyl (RO[•]), and peroxy (ROO[•]) radicals [1]. Oxidative stress is as a result of the overrun of free radicals and the decreased activity of endogenous antioxidant protective enzymes [2]. The increased generation of reactive oxygen species

leads to the disruption of multiple metabolic processes which results at the beginning of the development of the earlier pathological processes in neurodegenerative diseases such as Alzheimer, Parkinson, Huntington's [3], amyotrophic lateral sclerosis (Lou Gehrig disease) [4], cancer [5], rheumatic diseases [6], and aging [7].

Creatine as an antioxidant is important for human health and can provide benefits for different diseases [8]. Pre-exercise and post-exercise Creatine supplementation prevents bone mineral content and density [9] in the aging population [10], and supports bone health in older women [11]. Creatine exerts a protective effect in pathological conditions of the brain and muscle [12]. Creatine supplementation shows effectiveness on aging muscle [13, 14] as on muscle function in childhood myositis [14], muscle function in cancer [15], muscular atrophy [16], sarcopenia [17], myopathy [18], and in spinal and bulbar muscular atrophy [19].

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Creatine or vitamin D supplementation can help in individuals with a spinal cord injury [20].

Creatine supplementation can provide a beneficial effect on the cognitive function of healthy individuals [21], traumatic brain injury [22], and the mental-associated decrease in visuomotor skills [23]. Dietary supplementation with Creatine is important for bipolar depression [24], pregnancy [25], vascular health [26], and cancer therapy [27-29].

Antioxidants from natural sources and foods [30] are important for protection against age-related diseases [31, 32]. The investigation of antioxidant nutraceuticals [33] is an important therapeutic approach for the effective decrease of reactive oxygen species [34].

The aim of the current study was the comparative evaluation of the radical-scavenging activity (RSA) of Creatine lysinate against 0.05 mM methanol solution of DPPH (2,2-diphenyl-1-picrylhydrazyl) radical by measuring the decrease in the absorbance at $\lambda = 516$ nm. Based on the eventually experimentally confirmed antiradical properties of Creatine lysinate, the study would help to justify the advantages of choosing both antiradical compounds in the more active combination over monotherapy, which would contribute to increasing the effectiveness against oxidative stress-related diseases.

Materials and Methods

Materials

I. Test compounds: Creatine lysinate (synthesized from L. Vezenkov)

II. Reagents with pharmacopoeial purity

1. 1,1'-diphenyl-2-picrylhydrazyl (DPPH) (99 %), (Sigma Aldrich, N: STBD 4145 V)
2. 6-hydroxy-2,5,7,8-tetramethyl-chroman-2-carboxylic acid (Trolox) (Sigma-Aldrich, N:51796 PMV 291913)
3. methanol (99.9 %) (Sigma-Aldrich, N: SZBD 063 AV UN 1230)
4. distilled water.

Methods

I. Determination of radical scavenging activity by DPPH assay.

1. Preparation of 0.1 mM methanol solution of DPPH

An accurately measured quantity of 0.0039 g DPPH ($M = 394.32$) was dissolved with methanol and diluted in a volumetric flask of 100.0 ml with methanol to obtain 0.1 mM DPPH solution.

2. Preparation of stock solution of Trolox

An accurately measured quantity of 0.0125 g 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) ($M = 252.294$) was dissolved in distilled water and was diluted in a volumetric flask of 50.0 ml with distilled water to obtain a concentration of 1 mM Trolox (1000 μM). An aliquot part of 1.0 ml was diluted in a volumetric flask of 10.0 ml with distilled water to obtain the stock solution of Trolox with a concentration of 0.1 mM (100 μM).

3. Preparation of working solutions of Trolox

From the stock solution of 0.1 mM Trolox and aliquot parts respectively of 10.0 ml, 20.0 ml, 30.0 ml, and 40.0 ml were diluted with distilled water separately in volumetric flasks of 50.0 ml to obtain final concentrations of working solutions of Trolox: 0.02 mM (20 μM), 0.04 mM (40 μM), 0.06 mM (60 μM), 0.08 mM (80 μM).

An aliquot part of 1.0 ml of 0.1 mM was diluted in a volumetric flask of 10.0 ml with distilled water to obtain the stock solution of Trolox with a concentration of 0.01 mM (10 μM). An aliquot part of 2.0 ml of 0.01 mM was diluted in a volumetric flask of 10.0 ml with distilled water to obtain the stock solution of Trolox with a concentration of 0.002 mM (2 μM).

4. Preparation of phosphate buffer solution pH = 7

For the preparation of phosphate buffer solution with pH = 7, accurate quantities of 0.1 g KH_2PO_4 , 0.2 g K_2HPO_4 , and 0.85 g NaCl were dissolved in distilled water and diluted in a volumetric flask of 100.0 ml with distilled water.

5. Preparation of stock solution Creatine lysinate

An accurately measured quantity of 1.3866 g Creatine lysinate ($M = 277.32$) was dissolved in phosphate buffer pH = 7 and was diluted in a volumetric flask of 50.0 ml with phosphate buffer pH = 7 to obtain the stock solution with a concentration of 100 mM (0.1 M).

6. Preparation of working solutions of Creatine lysinate

From the stock solution of 100 mM, Creatine lysinate and aliquot parts respectively of 10.0 ml, 20.0 ml, 30.0 ml, and 40.0 ml were diluted with phosphate buffer pH = 7 separately in volumetric flasks of 50.0 ml to obtain final concentrations of working solutions of 20 mM, 40 mM, 60 mM, 80 mM.

7. DPPH assay procedure

DPPH assay was performed according to the following procedure: 5 ml 0.1 mM methanol solution DPPH was mixed separately with 5 ml of Creatine lysinate in concentrations 20 mM, 40 mM, 60 mM, and 80 mM to obtain final concentrations respectively of 0.05 mM methanol solution DPPH and 10 mM, 20 mM, 30 mM, 40 mM of Creatine lysinate. As control recorded a mixture of 5 ml 0.1 mM methanolic DPPH solution and 5 ml of methanol. The mixtures were shaken vigorously and allowed to stand for incubation in dark for 1 h at temperature: $25^\circ\text{C} \div 27^\circ\text{C}$ After incubation, the absorbances were measured

against blank methanol at $\lambda = 516$ nm using a UV-VIS spectrophotometer Hultett Packard N: 8452 A. All the tests were performed in triplicates and the results were averaged.

II. Calculation methods

1. Calculation of radical scavenging activity (RSA, [%])

The results of DPPH-radical scavenging activity (RSA), and for not-scavenged radical (R, [%]), for a period of 1 h reaction of 0.05 mM methanol solution of DPPH with solutions of standard Trolox, and 10 mM ÷ 40 mM Creatine lysinate, were calculated by the equation:

$$\text{RSA} [\%] = \frac{ADPPH_{\text{control}} - \text{sample}}{ADPPH_{\text{control}}} \times 100 \quad (1)$$

$$R [\%] = \frac{A_{\text{sample}}}{ADPPH_{\text{control}}} \times 100 \quad (2)$$

A DPPH control – absorbance of the solution of DPPH-radical before interaction with the compound investigated

A_{sample} – absorbance of the solution of DPPH-radical after reacting with the compound investigated

the absorbance of the DPPH solution in control is measured against methanol.

2. Calculation of IC₅₀ value (inhibitory concentration)

The IC₅₀ value is the concentration of the test samples at which the inhibition percentage reaches 50%. A lower IC₅₀ value corresponds to a higher antiradical activity of the tested sample. IC₅₀ values (mM), were calculated from the DPPH radical–scavenging curve of Creatine lysinate and standard Trolox at $\lambda = 516$ nm according to the following procedure: inhibition ratios (y) were plotted against the sample concentrations (x), and the respective regression line ($y = a \cdot x + b$) was drawn. The sample concentration (x), was calculated by substituting the value of (y) with 50 in the regression equation.

3. Calculation of antioxidant power: 1/IC₅₀

4. Calculation of Trolox equivalent antioxidant capacity

The DPPH radical scavenging activity of the sample was expressed as Trolox equivalent antioxidant capacity (TEAC) calculated as follows:

$$\text{TEAC} = \frac{IC_{50\text{Trolox}}}{IC_{50\text{sample}}} \quad (3)$$

The higher TEAC value means a higher DPPH radical scavenging activity.

5. Calculation of relative radical scavenging activity (RRSA, [%]) and a relative decrease in radical scavenging activity (RDRSA, [%])

The relative radical scavenging activity (RRSA, [%]) and the relative decrease in radical scavenging activity (RDRSA, [%]) for Creatine lysinate with concentrations 10 ÷ 40 mM, were compared to the activity of standard Trolox with the same concentrations, and were calculated by the following equations:

$$\text{RRSA} [\%] = \frac{RSAsample}{RSATrolox} \times 100 \quad (4)$$

$$\text{RDRSA} [\%] = \frac{RSATrolox - RSAsample}{RSATrolox} \times 100 \quad (5)$$

RSA_{sample} - radical binding activity of Creatine lysinate

RSA_{Trolox} - radical binding activity of the standard Trolox.

Results and Discussion

Antioxidant methods are classified as hydrogen atom transfer (HAT)-based, and electron transfer (ET)-based assays. In spectrophotometric ET-based methods is measured the capacity of a redox-potential compound in the reduction of a colored oxidizing agent. The degree of color change in the reduction of an oxidant (either an increase or decrease of absorbance at a specific wavelength) is correlated to the concentration of the antiradical compound. In electron transfer-based methods, the increase in the radical-scavenging effect of the test compounds is directly proportional to the decrease of the absorbance of a solution of:

- 1,1-diphenyl-2-(picrylhydrazyl) ($\lambda = 516$ nm): DPPH free radical scavenging assay
- 2,2-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid ($\lambda = 734$ nm): ABTS method [35].

The DPPH (1,1-diphenyl-2-picrylhydrazyl; α, α -diphenyl- β -picrylhydrazyl) is a stable free organic radical with dark purple color. The DPPH radical scavenging method is a decolorization assay, which mechanism is based on the reaction between the DPPH solution and compounds by measuring its capacity to directly scavenge the DPPH radicals. In this interaction, DPPH is reduced to its nonradical form 1,1'-diphenyl-2-(2,4,6-trinitrophenol) hydrazine (DPPHH), and antioxidants are oxidized from DPPH. The determination of the capacity of compounds to directly scavenge the DPPH radicals is based on the monitoring of the decrease of absorbance of the DPPH radical most commonly at $\lambda = 517$ nm [36, 37].

The method is applicable for the study of the radical scavenging activity of the test compounds because they do not possess a measurable absorbance at the absorption maximum $\lambda = 517$ nm, where the DPPH-test is carried out.

DPPH method has been applied for the investigation of the antioxidant activity and of free radical scavenging effect of extracts from different plants such as *Piper longum* L. [38], *Zingiber officinale* var. *rubrum* [39], milk thistle [40].

Marinova *et al.* [41] have reported that the literature review described different modifications of the original methods of Blois [36] and Brand-Williams *et al.* [37] for the determination of the DPPH free radical scavenging activity. Following Blois for the DPPH method, are mixed 1 ml 0.1 mM methanol solution of DPPH solution is added with 3 ml of various concentrations of compounds in methanol or with 3 ml of reference standard Butylhydroxytoluene. After 30 min. at 25 °C in dark, the absorbance is measured at $\lambda = 517$ nm [36].

The modification methods include the differences in the following parameters [41]:

- wavelength of absorbance measurements: between 492 nm and 540 nm: 515 nm, 516 nm, 517 nm, 518 nm, 520 nm, and 525 nm, with the most utilized at $\lambda = 517$ nm
- concentration of DPPH solutions in the range from 0.05 mM to 1.5 M: 0.05 mM, 0.06 mM, 0.09 mM, 0.10 mM
- the most applied solvents: methanol and ethanol
- ratio between volumes of sample/DPPH solution: 1:1, 1:7, 5, 1:600, 3:1
- duration of reaction: varies from 1 min. to 240 min.: 5 min., 10 min., 15 min., 20 min., 30 min., 60 min., 120 min
- standard solutions used for expression of the results. Vitamin C, Vitamin E, BHT, BHA, Trolox
- temperature (from 25 °C to 27 °C).

The reducing ability of Creatine lysinate and standard Trolox presented as a percentage of antiradical activity (RSA %), was assessed by DPPH free radical scavenging assay. For the investigation of the radical scavenging activity was applied methodology described by the original DPPH methods of Blois [36] and Brand-Williams [37] with the following parameters modified in our previous work [42]:

- 0.05 mM DPPH
- Trolox solution as standard
- methanol as solvent

- temperature: 25 °C \div 27 °C
- ratio sample/DPPH solution: 1:1
- duration of reaction: 1 h
- measurement of the decrease of DPPH absorbance at $\lambda = 516$ nm wavelength.

The reaction mixture consisted of 5 ml of 0.1 mM methanol solution DPPH and 5 ml respectively of 20 mM, 40 mM, 60 mM, and 80 mM of Creatine lysinate, to obtain final concentrations correspondingly of 0.05 mM methanol solution DPPH and 10 mM, 20 mM, 30 mM, 40 mM of Creatine lysinate.

The changes in color from deep violet to light yellow were observed. The decrease of the absorbance was registered and spectra of 0.05 mM DPPH methanol solution at $\lambda = 516$ nm after 1 h reaction with solutions of Creatine lysinate is illustrated in **Figure 1**.

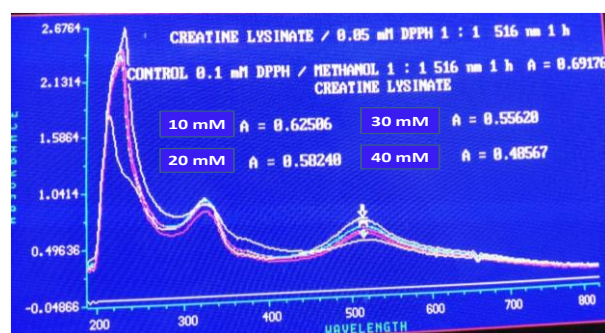


Figure 1. Spectra of 0.05 mM methanol solution of DPPH at $\lambda = 516$ nm after 1 h reaction with solutions of Creatine lysinate.

Table 1 are presented experimental results of absorbances of 0.05 mM methanol solution of DPPH after 1 h reaction with Creatine lysinate and Trolox. **Table 2** are summarized data for radical scavenging activity RSA (%), and for not scavenged radical (R).

Table 1. Absorbance at $\lambda = 516$ nm of 0.05 mM methanol solution of DPPH after 1 h reaction with solutions of Creatine lysinate and standard Trolox.

N:	Creatine lysinate		Trolox	
	C [mM]	A [AU]	C [mM]	A [AU]
1.	10	0.62506	0.001	0.48924
2.	20	0.58240	0.01	0.33066
3.	30	0.55620	0.02	0.23593
4.	40	0.48567	0.03	0.10475

Table 2. Radical-scavenging activity RSA [%] of Creatine lysinate and standard Trolox and not scavenged radical (R) at $\lambda = 516$ nm after 1 h reaction with 0.05 mM methanol solution of DPPH.

	Creatine lysinate	Creatine lysinate	Trolox	Trolox
	C [mM]	RSA [%]	C [mM]	RSA [%]
1.	10	9.64	0.001	28.02
2.	20	15.81	0.01	51.35
3.	30	19.60	0.02	65.29

4.	40	29.79	0.03	84.59
			0.04	90.62
	Creatine lysinate	Creatine lysinate	Trolox	Trolox
	C [mM]	R [%]	C [mM]	R [%]
N:	10	90.36	0.001	71.98
2.	20	84.20	0.01	48.65
3.	30	80.40	0.02	34.71
4.	40	70.21	0.03	15.41

In our previous work, the following results for standard Trolox have been obtained:

1. parameters of regression equations for absorbances: $y = -10.95 \cdot x + 0.466$ ($R^2 = 0.963$)
 radical-scavenging activity: $y = 1611 \cdot x + 31.41$ ($R^2 = 0.964$)
 unscavenged DPPH-radical: $y = -1611 \cdot x + 68.58$ ($R^2 = 0.964$)

2. the concentration at which the inhibition of radicals reaches 50 %: $IC_{50} = 0.01154$ mM

3. antioxidant power : $1/IC_{50} = 8.67$ [42]

The results for the absorbance values of 0.05 mM DPPH methanol solution at $\lambda = 516$ nm after 1 h reaction with solutions of Creatine lysinate were put against the corresponding concentrations into linear regression analysis and the linear dependence between the absorbances and concentration in the tested range was observed. The calibration curve for Creatine lysinate is shown in **Figure 2**. Linearity is characterized by the coefficient of linear regression, which is $R^2 > 0.963$.

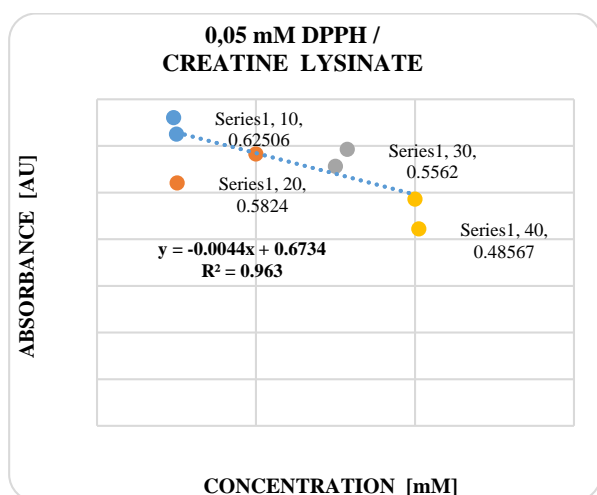


Figure 2. The absorbance of 0.05 mM DPPH methanol solution at $\lambda = 516$ nm after 1 h reaction with solutions of Creatine lysinate.

The scavenging activity percentages are subjected to a linear regression analysis against the respective concentrations. **Figure 3.** is illustrated the calibration curve which presents the linear relationship between the enhanced radical binding activity with the increase of concentration from 10 mM to 40 mM. **Figure 4.** presents results for not-scavenged DPPH-radical at $\lambda = 516$ nm after 1 h reaction with solutions of Creatine lysinate.

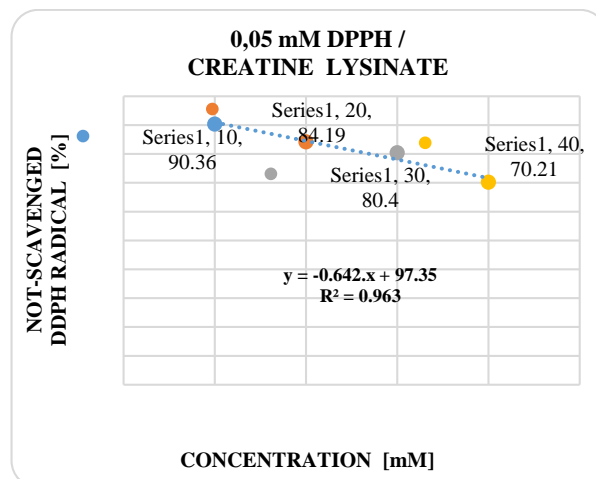


Figure 3. Radical-scavenging activity of 0.05 mM methanol solution of DPPH at $\lambda = 516$ nm after 1 h reaction with solutions of Creatine lysinate.

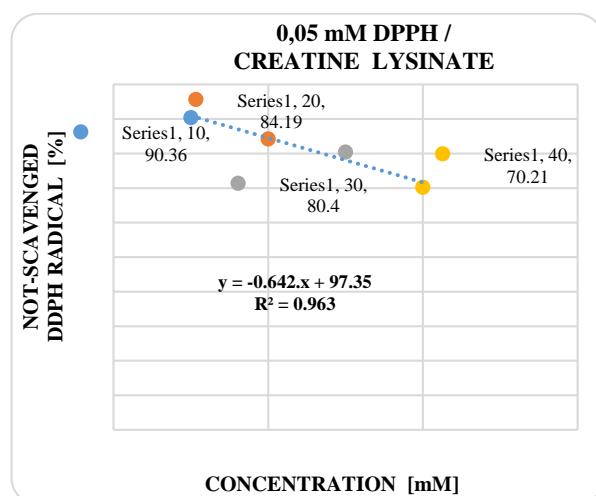


Figure 4. Not-scavenged DPPH-radical at $\lambda = 516$ nm after 1 h reaction with solutions of Creatine lysinate.

Calculation of IC_{50} value (inhibitory concentration) and antioxidant power $1/IC_{50}$

The results are expressed as IC_{50} values which determine the number of antioxidants needed for decreasing the radical concentration by 50 % and antioxidant power: $1/IC_{50}$. The regression analysis method was employed and the obtained regression equations for standard Trolox and Creatine lysinate were used to calculate the IC_{50} values that provide 50 % inhibition of the DPPH radical.

A lower IC_{50} value defines that at less concentration the compounds exert higher scavenging activity and antiradical

effect. Due to the lower $IC_{50} = 0.001154$ mM, standard Trolox possesses a higher antioxidant power $1/IC_{50} = 8.67$ than Creatine lysinate ($1/IC_{50} = 0.014$). The experimental results show that Creatine lysinate ($IC_{50} = 73.75$ mM) is more active compared to Creatine monohydrate ($IC_{50} = 102.48$ mM) due to lower IC_{50} .

Calculation of Trolox equivalent antioxidant capacity

The DPPH radical scavenging activity of Creatine lysinate expressed as Trolox equivalent antioxidant capacity is: TEAC = 0.00016.

Calculation of relative radical scavenging activity (RRSA, [%]) and a relative decrease in radical scavenging activity (RDRSA, [%])

From the regression equations for the radical-binding effect, the values for the radical-scavenging activity of 0.01 mM, 0.02 mM, 0.03 mM, and 0.04 mM solutions of Creatine lysinate were calculated.

The obtained data were used for the calculation of relative radical scavenging activity (RRSA, [%]) and a relative decrease in radical scavenging activity (RDRSA, [%]) shown in **Table 3**.

Table 3. Relative radical scavenging activity (RRSA, [%]) and a relative decrease in radical scavenging activity (RDRSA, [%]).

	C [mM]	RSA [%]	RRSA [%]	RDRSA [%]
1.	0.01	2.6564	5.17	94.83
2.	0.02	2.6628	4.08	95.92
3.	0.03	2.6693	3.16	96.84
4.	0.04	2.6757	2.95	97.05

Conclusion

From the experimental results, it was observed that the DPPH binding ability of Creatine lysinate ($IC_{50} = 73.75$ mM) is lower compared to the standard Trolox ($IC_{50} = 0.001154$ mM), which antioxidant power ($1/IC_{50} = 8.67$) is higher in comparison with Creatine lysinate ($1/IC_{50} = 0.014$). The experimental data show that Creatine lysinate ($IC_{50} = 73.75$ mM) is more active compared to Creatine monohydrate ($IC_{50} = 102.48$ mM) due to lower IC_{50} and higher scavenging activity.

The beneficial effect of combinations in the reduction of free radicals could be more effective in comparison with the mono application. Due to radical-scavenging activity, the application of Creatine monohydrate and Creatine lysinate in combination with other kinds of antioxidants such as Vitamin C or Coenzyme Q in form of food supplements could be an important strategy for obtaining of synergistic effect in an additive treatment of disorders resulting from oxidative stress-related diseases.

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Conflict of interest: None

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Ethics statement: None

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