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Short communication

Effect of D-aspartate uptake on uncoupling protein-3 and α-tubulin expressions in rat Harderian gland☆

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ABSTRACT

Although D-aspartate (D-Asp) has been recognized as having an important physiological role within different organs, high concentrations could elicit detrimental effects on those same organs. In this study, we evaluated the oxidative stress response to D-Asp treatment in rat Harderian gland (HG) by measuring total cellular hydroperoxide levels. Further, we examined the effect of D-Asp uptake on the expression of the mitochondrial uncoupling protein-3 (UCP3), β-actin, and α-tubulin. In rat HG, elevated levels of D-Asp significantly increased hydroperoxide production. This phenomenon was probably due to D-Asp uptake as well as lipid and porphyrin increased levels. Higher UCP3 levels and lower α-tubulin expression were also observed after D-Asp treatment. On the contrary, β-actin expression was unchanged. Given the possible role of UCP3 in lipid handling, the higher expression of mitochondria UCP3 protein in D-Asp-treated HG may reflect a major need to export excessive amounts of hydroperoxides deriving from a greater fatty acid flux across these organelles and higher mitochondrial porphyrin levels. Moreover, abundance of hydroperoxides in D-Asp treated rat HG could determine the decrease of α-tubulin expression. Thus, our findings indicate that a high concentration of D-Asp is critical in initiating a cascade of events determined by oxidative stress.

1. Introduction

D-Aspartate (D-Asp) is an endogenous amino acid present in a wide variety of tissues, particularly in the central nervous system (CNS) and glandular tissues [1]. Important biological roles have been postulated for D-Asp. For instance, besides its well known neuroexcitatory activity, D-Asp is crucial for neurotransmission and neurosecretion in the CNS, for the biosynthesis and/or secretion of hormones in the endocrine gland [2–8], as well as for the secretory activity in some exocrine glands [9,10]. Numerous studies have demonstrated that D-Asp administered intraperitoneally or intravenously accumulates in the rat pineal gland as well as in the testis, pituitary, Harderian and adrenal glands [4,10,11]. A lot of vegetables, marine food, and beverages contain exceptionally high quantities of D-Asp, and their consumption is likely to result in an increased accumulation of D-Asp in various tissues [12]. The possible physiological consequences of excessive uptake of D-Asp in the tissues are still unknown. A recent study suggests that the administration of high doses of D-Asp elevates the levels of reactive oxygen species (ROS), malondialdehyde, and hydroperoxide in cytosol and mitochondria of rat testis, events that by being accompanied by enhanced glutathione levels, elevated activities of glutathione-dependent enzymes and catalase are indicative of a state of oxidative stress [13,14]. Recently we have demonstrated that exogenous administration of D-Asp to adult rat induces a massive increase of secretion in the Harderian gland (HG), whereas other D- and L-amino acids did not show any effects [10]. In rodents, this gland is a tubulo-alveolar orbital gland [15] that produces large quantities of lipids and porphyrins [16], both of which increase after D-Asp administration [10].

Thus, the aim of the present study was to investigate whether D-Asp may induce oxidative stress in rat Harderian gland following repeated exposure. We evaluated the oxidative stress response to D-Asp treatment by determining total cellular hydroperoxide levels. Further, we studied the effect of D-Asp uptake on mitochondrial oxidative stress by determining the expression of the uncoupling protein-3 (UCP3). In effect, being a member of the subfamily of mitochondrial anion carriers present in the HG [17,18],

Abbreviations: D-Asp, D-aspartic acid; HG, Harderian gland; ROS, reactive oxygen species; UCP3, uncoupling protein-3.

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this protein may play an important role in exporting fatty acid peroxides accumulated within the mitochondria under conditions of increased lipid handling [19]. Finally, we assessed cell damage induced by D-Asp accumulation by determining alterations in the expression of β-actin and α-tubulin, since it has well know that oxidative stress causes microtubule and microfilament depolimerizations with consequence loss of cell integrity.

2. Materials and methods

2.1. Animals

Male Wistar rats, *Rattus norvegicus albinus*, weighing 300–350 g, purchased from Charles River Laboratory (Italy), were kept under regulated conditions of temperature (28 ± 2 °C) and lighting (12 h light, 12 h dark cycles). They received commercial food pellets ad libitum.

2.1.1. Experiments

The rats (n = 18) were divided in two groups: the first group was allowed to drink a solution consisting of 20 mM D-aspartate for 15 days; rats of the second group (control) were given to drink fresh water for 15 days. At the end of the treatment, rats were first anesthetized by an i.p. injection of chloral hydrate (40 mg/100 g body mass) and then decapitated. The HGs were dissected out, weighed and rapidly immersed in liquid nitrogen for biochemical analyses. The experimental protocol, as well as the housing conditions, was in accordance with the Italian guidelines (D.Lvo 116/92) and authorized by the local Animal Care Committee (Servizio veterinario ASL 44, Prot. Vet. 22/95).

2.2. Tissue homogenization

Harderian glands from each animal were first homogenized (Ultra-Turrax T25 homogenizer) with 0.2 M Tris–HCl, pH 8.2, in a ratio of 1:20. Tissue homogenate (100 μl) was supplemented with 20 μl of 0.5 M trichloroacetic acid (TCA) and centrifuged at 15,000 × g for 10 min. The supernatants were neutralized (to pH 6–8) using 1 M NaOH and the resulting sample was used for the D-aspartic acid analysis, as below.

2.2.1. Specific determination of D-Asp in rat HG

The method used was based on the separation of D-Asp from other amino acids by means of an appropriate derivatization of amino acids [20]. 10 μl of sample (prepared as above) was mixed with 100 μl of 0.5 M sodium pyrophosphate, pH 9.5, and 20 μl of OPA-NAC reagent (*o*-phthaldialdehyde-N-acetyl-L-cysteine), which was prepared by mixing 20 mg of OPA with 10 mg of N-acetyl-cysteine in 2 ml 50% methanol. After 2 min, distilled H_2O was added to make a final volume of 1000 μl. After mixing, 50 μl of the reaction mixture was injected onto a C_{18} Supelcosil column (0.45 × 25 cm, Supelco, Inc., Belafonte, PA, USA) connected to a Beckman-Gold HPLC system. The column was eluted at 1.2 ml/min with a gradient consisting of solution A [which was prepared by mixing 920 ml of bidistilled H_2O, 30 ml of 1 M citrate-phosphate buffer, pH 5.3, and 50 ml acetonitrile (the final pH must be adjusted to 5.6 with 1 M citric acid or 1 M NaOH)] and solution B, which consisted of 90% acetonitrile and 10% distilled H_2O. The program gradient was: 0–5% B for 10 min; 5–30% B for 30 min, 30–100% B for 10 min, staying at 100% B for 5 min and returning to 0% B for 1 min. The fluorescence was monitored using an excitation wavelength of 330 nm and an emission wavelength of 450 nm. D-Asp was eluted at about 6 min (determined by using a standard of D- and L-amino acids) followed by L-Asp 0.5 min later, and was well separated from other amino acid enantiomers. To verify that the peak eluted at about 6 min really was D-Asp, 20 μl of the sample was mixed with 20 μl of 0.5 M pyrophosphate buffer, pH 8.2, and 2 μl of purified D-aspartate oxidase (D-AspO), then incubated for 20 min at 37 °C. After incubation, the sample was mixed with 5 μl of 0.2 M NaOH, 100 μl of pyrophosphate, pH 9.5, and subjected to HPLC as before. The absence of the D-Asp peak, or its reduction, confirmed the presence of D-Asp in the original sample.

2.2.2. D-Asp content determination

A standard curve was obtained using 10 μl of the mixture of standard amino acids plus all other components as used for the sample, under the same conditions as before. The standard mixture was obtained by mixing stock solutions of the following 22 amino acids plus ammonia: L-Ser, L-Asp, L-Met, L-Glu, L-Asn, L-Thr, L-Leu, L-Gln, L-Ile, Gly, Taurine, L-Arg, L-Phe, L-His, L-Ala, L-Trp, L-Val, L-Tyr, GABA, L-Lys, L-Cys, L-Pro, and NH_4SO_4, such that each amino acid had the concentration of 0.4 μmol/ml (total 8.8 μmol/ml) in 0.1 M HCl. The areas of the peaks of amino acid standards were used to calculate the amounts of D-Asp contained in the HG from control and D-Asp-treated rats.

2.3. Measurement of hydroperoxides

A specific assay was used for determination of hydroperoxides [21]. Total hydroperoxides react with glutathione (GSH) by the enzyme GSH peroxidase to produce oxidized glutathione (GSSG) and the alcohol. GSSG is rapidly reduced by NADPH *via* GSH reductase. Thus, total amount of NADPH oxidized is a measure of the hydroperoxides. For this assay, HGs were homogenized 1:5 with 0.9% NaCl in 5 mM Tris–HCl buffer, pH 7.0. The homogenate is centrifuged for 10 min at 3000 g and the supernatant is then centrifuged for 60 min at 100,000 × g. This supernatant is made 50% with acetone a −20 °C. The pellet formed by immediate centrifugation at 15,000 × g for 10 min is resolubilized in about 200 μl of 5 mM Tris–HCl buffer, pH 7.0 and then centrifuged at 17,000 × g for 20 min to remove the nonresuspended material. 500 μl of 0.124 M Tris–HCl, pH 7.6, and 0.2 mM ethylenediaminetetraacetic acid, tetrasodium salt, are added to 200 μl of test solution. Then, 25 μl of 4 mM NADPH, 10 μl of purified GSH peroxidase (2500 nmol of NADPH oxidized/min/ml), and 0.1 ml of 4.25 mM GSH are added. The solution is incubated at 33 °C for 15 min. At the end, the absorbance of NADPH in the test solution is read at 340 nm.

2.4. Protein-extract preparation

Harderian glands were homogenized in an isolation medium consisting of 220 mM mannitol, 70 mM sucrose, 20 mM Tris–HCl, 1 mM EDTA, 5 mM EGTA, and 5 mM $MgCl_2$, pH 7.4 (all from Sigma–Aldrich Corp., St Louis, MO, USA), supplemented with a protease inhibitor cocktail (Roche Applied Science, Mannheim, Germany). After homogenization, samples were centrifuged at 700 g for 10 min. The supernatants were collected and transferred into new tubes for subsequent centrifugation at 7000 g. The obtained mitochondrial pellets were washed twice and then resuspended in a minimal volume of isolation medium and kept on ice. The supernatant contained the cytosolic fraction. Protein concentrations of mitochondrial and cytosolic fractions were estimated using a modified Bradford assay (Bio-Rad, Melville, NY, USA).

2.5. Western immunoblot analysis

Thirty-microgram protein aliquots from the mitochondrial and cytosolic fractions were resuspended in 1 × SDS loading buffer, then heated for 3 min at 95 °C. Afterwards, the samples were subjected to SDS–PAGE (13% polyacrylamide). Analysis of mitochondrial and cytosolic samples was performed on two separate gels, using glands from three D-Asp-treated rats and three control rats each one. After

Table 1
D-Asp and hydroperoxides concentrations in Harderian gland from D-Asp treated and control rats.

	Control	D-Asp-treated
D-Asp levels (μmol/g tissue)	0.11 ± 0.02	0.75 ± 0.06*
Hydroperoxides (nmol/g tissue)	4.9 ± 0.03	13.2 ± 0.01*

The results represent the mean ± SD obtained from three individual determinations.
* $P < 0.001$ vs control.

electrophoresis, proteins were transferred to a nitrocellulose membrane. Each membrane was treated for 1 h with blocking solution (5% non-fat powdered milk in 1× TBS/Tween) and then it was incubated overnight at 4 °C with a polyclonal primary antibody as following: a rabbit anti-human UCP3 (Chemicon International, Temecula, CA) diluted 1:1000 on the mitochondrial fractions; a mouse anti-human α-tubulin (Santa Cruz Biotechnology, St. Cruz, CA) diluted 1:2000 or a mouse anti-human β-actin (Santa Cruz Biotechnology, St. Cruz, CA) diluted 1:2000 on the cytosolic fractions. After washing with TBS/Tween, membranes were incubated with the horseradish-peroxidase conjugated secondary antibody (1:4000) for 1 h at room temperature. The reactions were detected using an enhanced chemiluminescence (ECL) system (Amersham Life Science, UK). Bands were scanned and then quantified by a Scan program which converts optical density into numerical values. Equal loading of the protein samples was checked by Ponseau staining.

2.6. Statistical analysis

The values obtained were compared by Student's *t*-test for between-group comparisons. Values for $P < 0.01$ were considered statistically significant. All data were expressed as the mean ± S.D. from at least three independent experiments.

3. Results and discussion

3.1. D-Asp and hydroperoxide concentrations in the rat HG after D-Asp treatment

To prove the efficiency of our experimental conditions, we measured the accumulation of D-Asp in D-Asp-treated rat HG by a specific and sensitive HPLC method, entailing the diasteromeric separation of D-Asp from other amino acids by means of a D-aspartate oxidase (Fig. 1).

In accordance with our previous study [10], we found that D-Asp levels in D-Asp-treated rat HG (0.75 ± 0.06 μmol/g tissue) were significantly ($P < 0.001$) higher than those of basal values (0.11 ± 0.02 μmol/g tissue) (Fig. 1; Table 1). Experimental evidence suggests that the uptake of D-Asp occurs *via* NMDA receptors and putative specific binding sites present in rat HG [22].

The amount of total hydroperoxides in D-Asp-treated rat HG (13.2 ± 0.01 nmol/g tissue) was significantly ($P < 0.001$) higher than that in controls (4.9 ± 0.03 nmol/g tissue) (Table 1). Consistently, a marked elevation of oxidative stress markers (i.e. reactive oxygen species, malondialdehyde, hydroperoxide, glutathione, glutathione-dependent enzymes and catalase) has been demonstrated in testis of prepuberal rats following D-Asp administration [13,14]. Since we recently demonstrated that D-Asp acute treatment markedly increases both lipid and porphyrin content in rat HG [10], it is likely that the elevated hydroperoxide levels in D-Asp-treated rat HG were produced by the accumulation of these molecules. Further, since D-AspO (EC 1.4.3.1) is a peroxisomal flavoprotein [23] that specifically metabolizes D-Asp into oxaloacetate, ammonia, and hydrogen peroxide [24,25], it is possible that induction of oxidative stress following D-Asp exposure could also be related to its metabolism by inducible D-AspO.

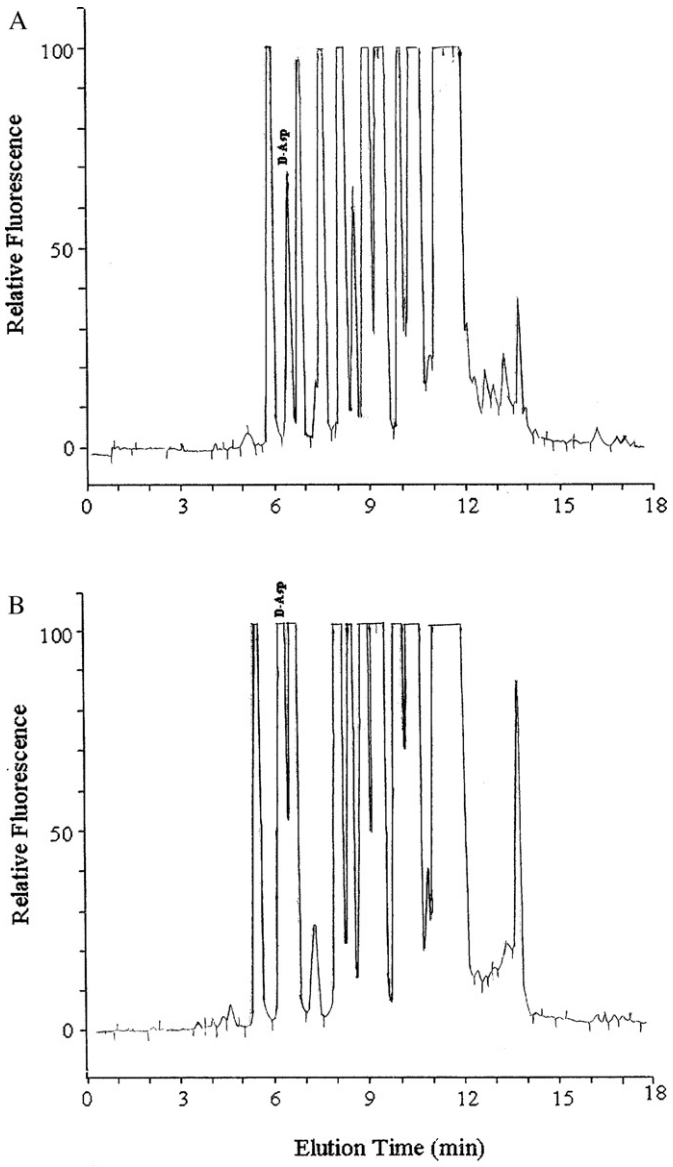

Fig. 1. Typical HPLC determination of D-Asp by the OPA-NAC method in HGs from control (A) and D-Asp-treated rats (B). D-Asp was eluted at about 6 min (determined by using a standard of D- and L-amino acids).

3.2. UCP3 expression in rat HG after D-Asp treatment

Total protein content in mitochondrial fractions, isolated from rat HG, was analyzed by Western blot analysis (Fig. 2). UCP3 expression in D-Asp-treated rat HG was significantly higher than that of control HG (Fig. 2). In view of the previous studies concerning the role played by UCP3 in lipid handling [19], we speculate that the higher expression of mitochondrial UCP3 protein in D-Asp-treated HG (showing elevated lipid content) could reflect a greater fatty acid flux in mitochondria and, thus, the need to export the fatty acid peroxides out of these organelles to counteract their damaging effects. Consistently, in a previous paper we found increased UCP3 level in hyperthyroid rat HG showing higher lipid content than in euthyroid HG [17]. Furthermore, since D-Asp-treated HGs show enhanced porphyrin content [10], we cannot exclude that the increased levels of UCP3 in D-Asp-treated rat HG

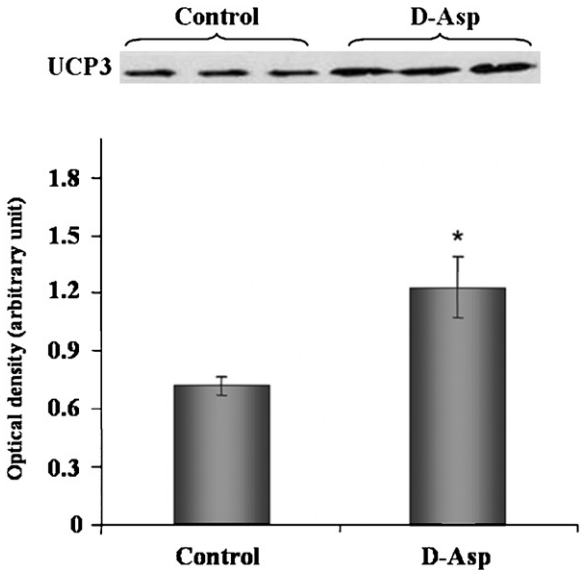

Fig. 2. Upper panel: Western blot detection of UCP3 protein in HGs from D-Asp-treated rats and controls. A specific band of 33 kDa was observed (by comparison with co-migrating size markers; Bio-Rad, Melville, NY). The bands are representative of three D-Asp-treated glands and three control glands. Lower panel: the amount of UCP3 was quantified using the Scan program (see upper panel). Values shown represent the means ± S.D. of three determinations. $^{*}P < 0.01$ vs controls.

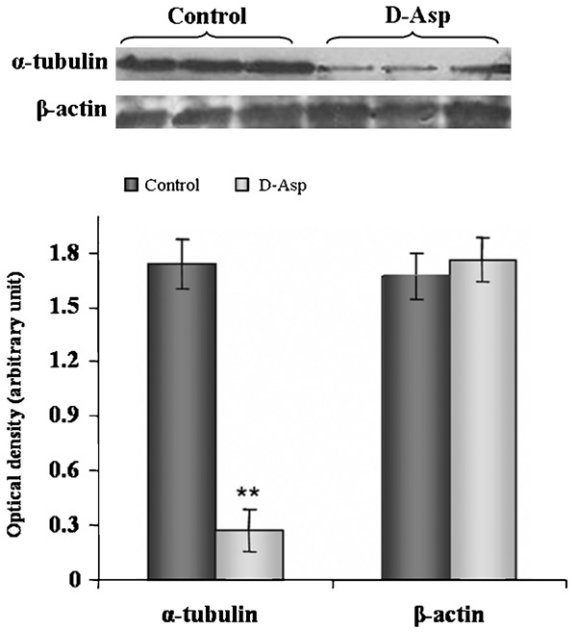

Fig. 3. Upper panel: Western blot detection of α-tubulin and β-actin proteins in HGs from D-Asp-treated rats and controls. Specific bands of 50 kDa and 42 kDa were observed, respectively (by comparison with co-migrating size markers; Bio-Rad, Melville, NY). The bands for each experimental group are representative of three glands. Lower panel: the amount of α-tubulin and β-actin were quantified using the Scan program (see upper panel). Values shown represent the means ± S.D. of three determinations. $^{**}P < 0.001$ vs controls.

could also be useful to counteract also the ROS generated by the porphyrin accumulation. Indeed, it is well known that the accumulation of porphyrins in the mitochondrial membrane damages these organelles by provoking alteration in the cellular respiratory chain and the production of ROS, which, in turn, contribute to producing lipid peroxides.

3.3. Expression of α-tubulin and β-actin in rat HG after D-Asp treatment

Western blot analysis revealed that α-tubulin level in D-Asp-treated rat HG was nearly 5-fold lower than that of control HG (Fig. 3). By contrast, D-Asp treatment did not alter β-actin expression in rat HG (Fig. 3). It has been demonstrated that lipid peroxides are a potential target of tubulin. For example, hydroperoxides disrupt the formation of microtubules in neurons [26]. In fact, evidence from in vitro studies indicates that even very low concentrations of peroxides are sufficient to interfere with tubulin and, therefore, microtubule function [27]. Further, oxidation of cellular proteins causes a loss of microtubule function, culminating in microtubule depolymerization and proteosome-dependent degradation of α-tubulin [28]. Therefore, D-Asp-treated rat HG, with its abundance of hydroperoxides, is at risk of microtubule polymerization.

4. Conclusions

Our findings indicate that in rat HG elevated levels of D-Asp induce significant increase in hydroperoxide production, which is probably elicited by repeated exposure to D-Asp uptake as well as lipid and porphyrin increased levels. Under such conditions, we also detected higher UCP3 levels and a lower α-tubulin expression. In view of the possible role played by UCP3 in lipid handling [19], the higher expression of UCP3 protein in D-Asp-treated HG could be interpreted as a molecular response to counteract the detrimental effects of hydroperoxide accumulation triggered by a greater fatty acid flux across these organelles and by higher mitochondrial porphyrin levels within the mitochondrial matrix. Finally, the increased oxidative status well correlates with the decrease in α-tubulin expression most likely resulting from microtubule depolymerization and α-tubulin degradation induced by hydroperoxide accumulation. Unexpectedly, D-Asp treatment did not alter β-actin expression in rat HG, suggesting that the oxidative status did not affect the microfilaments.

Taken together, our findings seem to suggest that an excessive uptake of D-Asp could significantly trigger a cascade of molecular events determined by states of oxidative stress.

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References

[1] A. D'Aniello, Brain. Res. Rev. 53 (2007) 215.
[2] M.J. Schell, O.B. Cooper, S.H. Snyder, Proc. Natl. Acad. Sci. U.S.A. 94 (1997) 2013.
[3] Y. Nagata, H. Homma, J.A. Lee, K. Imai, FEBS Lett. 444 (1999) 160.
[4] A. D'Aniello, M.M. Di Fiore, G.H. Fisher, A. Milone, A. Seleni, S. D'Aniello, A.F. Perna, D. Ingrosso, FASEB J. 14 (2000) 699.
[5] G. D'Aniello, A. Tolino, A. D'Aniello, F. Errico, G.H. Fisher, M.M. Di Fiore, Endocrinology 141 (2000) 3862.
[6] H. Wang, H. Wolosker, J. Pevsner, S.H. Snyder, D.J. Selkoe, J. Endocrinol. 167 (2000) 247.
[7] H. Wolosker, A. D'Aniello, S.H. Snyder, Neuroscience 100 (2000) 183.
[8] A. D'Aniello, P. Spinelli, A. De Simone, S. D'Aniello, M. Branno, F. Aniello, G.H. Fisher, M.M. Di Fiore, R.K. Rastogi, FEBS Lett. 552 (2003) 193.
[9] W. Masuda, C. Nouso, C. Kitamura, M. Terashita, T. Noguchi, Arch. Biochem. Biophys. 420 (2003) 46.
[10] R. Monteforte, A. Santillo, M. Di Giovanni, A. D'Aniello, A. Di Maro, G. Chieffi Baccari, Amino Acids 37 (2009) 653.
[11] K. Imai, T. Fukushima, K. Hagiwara, T. Santa, Biomed. Chromatogr. 9 (1995) 106.
[12] M. Friedman, J. Agric. Food. Chem. 47 (1999) 3457.
[13] K.N. Chandrashekar, Muralidhara, Theriogenology 70 (2008) 97.
[14] K.N. Chandrashekar, Muralidhara, Amino Acids 38 (2010) 817.
[15] G. Chieffi, C. Chieffi Baccari, L. Di Matteo, M. d'Istria, S. Minucci, B. Varriale, Int. Rev. Cytol. 168 (1996) 1.
[16] A. Gorchein, Biomed. Chromatogr. 17 (2003) 526.

[17] G. Chieffi Baccari, R. Monteforte, P. de Lange, F. Raucci, P. Farina, A. Lanni, Endocrinology 145 (2004) 3338.
[18] A. Santillo, R. Monteforte, P. De Lange, A. Lanni, P. Farina, C. Chieffi Baccari, J. Cell. Physiol. 215 (2008) 481.
[19] A. Lombardi, R.A. Busiello, L. Napolitano, F. Cioffi, M. Moreno, P. de Lange, E. Silvestri, A. Lanni, F. Goglia, J. Biol. Chem. 285 (2010) 16599.
[20] A. D'Aniello, M.M. Di Fiore, G.H. Fisher, Trends Comp. Biochem. Physiol. 4 (1998) 1.
[21] R.L. Heath, A.L. Tappel, Anal. Biochem. 76 (1976) 184.
[22] M. Di Giovanni, E. Topo, A. Santillo, A. D'Aniello, G. Chieffi Baccari, Amino Acids 38 (2010) 229.
[23] P.P. Van Veldhoven, C. Brees, G.P. Mannaerts, Biochim. Biophys. Acta 1073 (1991) 203.
[24] A. D'Aniello, G. D'Onofrio, M. Pischetola, G. D'Aniello, A. Vetere, L. Petrucelli, G.H. Fisher, J. Biol. Chem. 268 (1993) 26941.
[25] A. D'Aniello, A. Vetere, L. Petrucelli, Comp. Biochem. Physiol. B 105 (1993) 731.
[26] Y. Yamanaka, S. Yoshida, H. Doi, Mech. Ageing Dev. 129 (2008) 215.
[27] M. Kawakami, L. Ward, H. Doi, Lipids 35 (2000) 205.
[28] D. Bernhard, A. Csordas, B. Henderson, A. Rossmann, M. Kind, G. Wick, FASEB J. 19 (2005) 1096.