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ORIGINAL ARTICLE

D-Aspartic acid induced oxidative stress and mitochondrial dysfunctions in testis of prepubertal rats

K. N. Chandrashekar · Muralidhara

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Abstract Previously we demonstrated the potential of D-aspartic acid (D-Asp), an acidic amino acid to induce oxidative response in prepubertal rat testis in vitro. In the present study, we determined the extent of oxidative stress in the testis of prepubertal rats that were administered D-Asp (100 and 500 mg/kg bw/d, i.p. 7 days). D-Asp treatment significantly elevated the levels of reactive oxygen species, malondialdehyde and hydroperoxide in cytosol and mitochondria of testis, which were accompanied by enhanced glutathione levels, elevated activities of glutathione-dependent enzymes and catalase suggesting a state of oxidative stress. Further, the activities of D-aspartate oxidase and 3β-hydroxy steroid dehydrogenase were elevated in the testis. The testis mitochondria of D-Asp-treated rats showed altered citric acid and complex enzyme activities, reduction in membrane potential, increased permeability and intracellular Ca^{2+} levels. Collectively, these findings suggest the potential of D-Asp to induce oxidative perturbations in the testis of prepubertal rats and this mechanism may in part be responsible for the observed physiological effects.

Keywords D-Aspartic acid · Oxidative stress · Testis · Mitochondrial dysfunctions · Steroidogenesis · Prepubertal rats

Abbreviations

D-AspO	D-Aspartate oxidase
DCF	Dichlorofluorescein
4-HNE	4-hydroxy nonenal
HEPES	N-2hydroxyethylpiperazin-N2-ethanesulfonic acid
MDA	Malondialdehyde
MPT	Membrane permeability transition
NO	Nitric oxide
NMDA	N-Methyl-D-aspartate

Introduction

D-Amino acids, the stereoisomers of widely observed L-forms, were earlier believed to be unnatural isoforms with no specific physiological functions. However, in recent times a regulatory role in growth and development has been ascribed to D-amino acids (Homma 2002, 2007; D'Aniello 2007). Further, they are also being recognized as candidates of novel pharmacological significance owing to their altered levels either in serum or tissues under various conditions such as Alzheimer's disease (Hamase et al. 2002), Schizophrenia (Hashimoto et al. 2003; Verrall et al. 2007), renal disease (Krug et al. 2007) and epilepsy (Meldrum et al. 1999). Although, D-amino acids have been known to be synthesized only by microorganisms, plants and invertebrates (Homma 2007), their organ specific distribution in mammals suggest that they are derived endogenously either from intestinal microflora or by

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K. N. Chandrashekar · Muralidhara (✉)
Department of Biochemistry & Nutrition,
Central Food Technological Research Institute (Council of Scientific and Industrial Research), Mysore 570020, India
e-mail: mura16@yahoo.com

spontaneous racemization of aged proteins of long-lived tissues (Shapira and Chou 1987). In addition, dietary D-amino acids formed during food processing also represent a principal source for humans.

D-Aspartic acid (D-Asp) is the most common D-amino acid occurring in relatively large amounts in vegetables, coffee, eggs, marine animals, alcoholic beverages etc. During cooking, food commodities such as milk, meat, grain proteins etc., are often exposed to conditions that could cause racemization subsequently increasing the D-Asp levels (Friedman 1999). D-Asp content in milk has been shown to increase following pasteurization from 1.5% (untreated raw milk) to 3% (condensed and evaporated milk), suggesting that various factors such as pH, temperature etc., interplay during racemization process. Other commercial food products reported to be extremely rich in D-Asp include soy protein (9%), soy based infant formula (10.8%), simulated bacon (13%), nondairy creamer (17%), Casein (31%) and Zein protein (40%) (Man and Bada 1987). Since vast majority of humans consume processed food items on a regular basis, it is likely that they are exposed to high levels of dietary D-Asp. Accordingly, studies on the physiological implications of D-Asp in vivo under chronic exposures assume greater relevance.

In mammals, D-Asp is widely distributed in brain, peripheral tissues and especially in the endocrine glands of adults. Testis is a major neuroendocrine organ where active absorption and storage of D-Asp have been demonstrated (Fuchs et al. 2005). While the precise function of D-Asp in testis is not clear, its levels are shown to increase after birth and reach maximum during tissue maturation (Dunlop et al. 1986). In adult testis, D-Asp is synthesized in seminiferous tubules, secreted into the venous blood and preferentially localized in Leydig cells, the site of testosterone synthesis (D'Aniello et al. 1998). Further, D-Asp is also a precursor for the synthesis of N-methyl-D-aspartic acid (NMDA), a powerful agonist of L-glutamate mediated excitotoxicity (D'Aniello 2007). Experimental evidences suggest the uptake of D-Asp *via* NMDA receptors present in both Leydig and Sertoli cells of testis (Storto et al. 2001). Upon internalization, it has been shown to interact with DNA or nuclear protein(s) involved in gene transcription of steroidogenic acute regulatory (StAR) protein, whose expression facilitates the translocation of cholesterol from outer to inner mitochondrial membrane for testosterone biosynthesis (Nagata et al. 1999). The potential of exogenous D-Asp to accumulate in neuroendocrine organs and induce the synthesis and or release of hypothalamo-pituitary hormones is adequately demonstrated (D'Aniello et al. 1996, 2000; Fukushima et al. 1998). Further, D-Asp is shown to be specifically metabolized by D-aspartate oxidase (D-AspO, a peroxisomal flavoprotein present almost in all tissues) into oxaloacetate, ammonia and hydrogen peroxide (D'Aniello et al. 1993a).

Oxidative stress represents an important pathway for the destruction of cells. An imbalance in prooxidant–antioxidant ratio can lead to cellular damage through membrane lipid destruction and DNA strand breakage (Halliwell 2006). Free radicals thus generated can inhibit mitochondrial complex enzyme activities resulting in the blockade of respiration. Evidences suggest that oxidative stress mechanisms may play a vital role in the expression of toxicity of D-amino acids (Maekawa et al. 2005). The regulatory activity of D-serine on inflammatory responses, signal transduction events, energy metabolism and oxidative stress responses in kidney of adult rats was recently reported (Soto et al. 2008). Recently, we demonstrated the prooxidant potential of D-Asp in testis of prepubertal rats under in vitro conditions (Chandrashekar and Muralidhara 2008). However, the propensity of exogenous D-Asp to induce testicular oxidative stress in vivo has not been investigated. Such an understanding is relevant, since testis and neuroendocrine organs can accumulate exogenous D-Asp owing to the presence of glutamate transporters and NMDA receptors (Fukushima et al. 1998; Hu et al. 2004).

Accordingly, based on our recent findings, we hypothesize that D-Asp at higher doses may induce oxidative imbalances in the testis of prepubertal rats following repeated exposure. We tested the hypothesis by examining the potential of D-Asp to induce oxidative stress in rat testis following repeated exposures. The oxidative stress response was ascertained by determination of oxidative markers such as generation of reactive oxygen species (ROS), hydroperoxide levels, malondialdehyde (MDA), alterations in the activities of antioxidant enzymes and redox status in testis cytosol and mitochondria. Further, we have assessed the mitochondrial dysfunctions by determining the perturbations in the activities of complex enzymes, membrane potential, opening of membrane permeability pore and intracellular Ca^{2+} levels.

Materials and methods

Materials

D-Aspartic acid (CAS No 1783-96-6), 2,7-dicholorofluorescein diacetate (DCF-DA), 2-thio barbituric acid (TBA), 1,1′,3,3′-tetramethoxypropane (TMP), bovine serum albumin (BSA), rhodamine 123 (Rh123), 5-Pregnenolone, Cytochrome C, Fura-AM and 3-(4,5-dimethylthiazolyl)-2,5-diphenyltetrazoliumbromide (MTT) were purchased from M/s Sigma Chemicals, St Louis, MO, USA. All other chemicals used were of analytical grade.

Animals

Prepubertal male rats (CFT-Wistar strain, 4-week old) drawn from the stock colony of our 'Institute animal house facility' were fed commercial pellet diet (M/s Saidurga, Sangli, Maharashtra, India) ad libitum and provided free access to tap water throughout the study. Animals were acclimatized for a week before starting the treatment regimen. All experiments inclusive of animal handling and killing were conducted strictly as per the guidelines of 'Institutional Animal Ethics Committee'.

Experimental design

Prepubertal rats were randomly assigned to three groups and D-aspartic acid (dissolved in 1% aqueous sodium bicarbonate, conc 250 mg/ml) was administered intraperitoneally at dosages of 100 and 500 mg/kg bw/d for 7 consecutive days. Rats which received only the vehicle served as the negative controls. The criteria of dose selection were based on our preliminary findings in which D-Asp (50 mg/kg bw/d for 7 days) caused no significant effect on the oxidative markers in testis. During the experimental period, food intake was monitored daily and body weights were recorded every alternate day and prior to killing. Terminally animals were killed under light ether anesthesia. The testes and liver were excised, washed with ice-cold saline and processed for biochemical investigations. The induction of oxidative stress in cytosol and mitochondria and mitochondrial dysfunctions were determined in testis.

Preparation of cytosol and mitochondria

Mitochondria were prepared as described earlier with minor modifications (Trounce et al. 1996). The testis was homogenized in ice-cold buffer B (2 mM Tris–HCl, pH 7.4, 0.25 M sucrose) and subjected to differential centrifugation to separate cytosol and mitochondria. The mitochondrial pellet obtained was washed and resuspended in buffer A (200 mM mannitol, 70 mM sucrose, 0.1 mM EDTA, 10 mM HEPES/l, pH 7.4). All operations were performed at 4°C. The protein content was estimated using BSA as the standard (Lowry et al. 1951).

Susceptibility of testis cytosol and mitochondria to exogenous D-Asp in vitro

The susceptibility of prepubertal testis cytosol and mitochondria to exogenous D-Asp was assessed by the measurement of lipid peroxidation. An aliquot of sample (0.5 mg protein) was incubated with various concentrations of D-Asp (cytosol, 0–500 μM; mitochondria, 0–1000 μM) for 30 min at 37°C. Terminally, the degree of lipid peroxidation was quantified and expressed as malondialdehyde (MDA) equivalents (Ohakawa et al. 1979).

Markers of oxidative stress induction—in vivo

Reactive oxygen species (ROS) generation in testis cytosol and mitochondria was quantified using dihydrodichlorofluorescein diacetate (DCF-DA), a non-polar compound which after hydrolysis by intracellular esterases, reacts with ROS to form a highly fluorescent dichlorofluorescein (DCF) (Driver et al. 2000). Briefly, testis cytosol or mitochondria (0.2 mg protein) were incubated with Locke's solution (NaCl 154 mM, KCl 5.6 mM, $NaHCO_3$ 3.6 mM, HEPES 5.0 mM, $CaCl_2$ 2.0 mM, glucose 10 mM/l, pH 7.4) containing DCF-DA(5 μM/ml) for 30 min at 37°C. The fluorescence was measured with excitation and emission wavelengths at 480 and 530 nm. ROS levels were quantified from a dichlorofluorescein standard curve and expressed as ρmol DCF formed/min/mg protein.

Lipid peroxidation status was assessed in testis cytosol and mitochondria by thiobarbituric acid reaction (Ohakawa et al. 1979). An aliquot of the sample (1.0 mg protein) was added to tubes containing 1.5 ml of acetic acid (pH 3.5, 20% v/v), SDS (8% w/v, 0.2 ml) and thiobarbituric acid (0.8% w/v, 1.5 ml). The mixture was heated in a boiling water bath for 45 min. Adducts formed were extracted into 1-butanol (3 ml) and the absorbance was read at 532 nm and quantified using TMP as the standard. Precautions were taken to avoid overestimation of MDA formation by carrying out incubation in the presence of 0.17% butylated hydroxyl toluene.

Water soluble hydroperoxide levels were determined following ferrous iron oxidation with xylenol orange (FOX1) as described earlier (Wolf 1994). An aliquot of testis cytosol or mitochondria (0.2 mg protein) was added to 0.95 ml of FOX1 reagent and incubated at room temperature for 30 min. Following centrifugation at $800\times g$ for 10 min, the absorbance of the supernatant was measured at 560 nm (€-1.5×10^4 mol/cm). Nitric oxide levels were measured in testis cytosol and mitochondria as per the specifications employing commercially available Griess reagent (M/s Sigma Chemicals, St.Louis, USA) and quantified from a sodium nitrite standard curve.

Determination of GSH and thiol status

Reduced glutathione (GSH) levels were quantified fluorimetrically as described previously (Mokrasch and Teschke 1984). Briefly, testis cytosol or mitochondria (0.1 mg protein) was added to 1 ml formic acid (0.1 M) and centrifuged at $10{,}000\times g$ for 10 min. An aliquot of the supernatant was added to sodium phosphate buffer (0.1 M, pH 8.0, EDTA 5 mM) containing buffered formaldehyde (1:4 v/v, 0.1 M formaldehyde: 0.1 M Na_2HPO_4) and

0.1 ml of *o*-phthaldehyde. Following incubation at 37°C for 45 min, the fluorescence was measured at excitation and emission wavelengths of 345 and 425 nm, respectively, and quantified from GSH standard curve.

For total thiol estimation, an aliquot of sample (0.05 mg protein) was added to 0.375 ml of Tris–HCl buffer (0.2 M, pH 8.2) containing di-thiobis-nitrobenzoic acid (DTNB, 10 mM) and 1.975 ml of methanol. Following incubation for 30 min at room temperature, the tubes were centrifuged at 3,000×*g* for 10 min. The absorbance of the supernatant was measured at 412 nm and expressed as *η*mol DTNB oxidized/mg protein (€-13.6/mM/cm) (Ellman 1959).

Activities of antioxidant enzymes

Superoxide dismutase activity was measured by monitoring the inhibition of quercetin autoxidation (Vladimir et al. 1989). Testis cytosol or mitochondria (0.01 mg protein) was added to phosphate buffer (0.016 M, pH 7.8) containing TEMED–EDTA (8 mM/0.08 mM) and quercetin (0.15% v/v). The decrease in absorbance was monitored for 3 min at 406 nm and the amount of protein that inhibits quercetin autoxidation by 50% was defined as one unit. Catalase activity was determined by measuring the rate of hydrolysis of H_2O_2 at 240 nm (Aebi 1984). To a reaction mixture (1 ml) containing sodium phosphate buffer (0.1 M, pH 7.4), H_2O_2 (8.8 mM), an aliquot of testis cytosol (0.05 mg protein) was added. The decrease in absorbance was monitored for 3 min and the activity was expressed as μmol H_2O_2 decomposed/min/mg protein (€-43.6/mM/cm).

Glutathione peroxidase activity was measured by monitoring the oxidation of NADPH at 340 nm (Flohe and Gunzler, 1984). Briefly, testis cytosol or mitochondria (0.05 mg protein) were added to phosphate EDTA-buffer (0.1 M, pH 7.0) containing glutathione reductase (0.24U/ml), GSH (1 mM) and NADPH (0.15 mM). Initiating the reaction by the addition of tert-butyl hydroperoxide (0.12 mM) the absorbance was monitored for 3 min and activity expressed as *η*mol NADPH oxidized/min/mg protein (€-6.22/mM/cm). Glutathione-S-transferase activity was measured by monitoring the enzyme catalyzed conjugation of GSH with 1-chloro-2,4-dinitro benzene (CDNB) at 340 nm. An aliquot of the sample (0.05 mg protein) was added to a reaction mixture containing phosphate buffer (0.1 M, pH 6.5, 0.5 mM EDTA), CDNB (1.5 mM) and GSH (1 mM). The increase in absorbance was monitored for 3 min and the activity was expressed as μmol conjugate formed/min/mg protein (€-9.6/mM/cm) (Guthenberg et al. 1985).

Functional enzyme activities

Aldehyde dehydrogenase activity was determined in a reaction mixture containing sodium phosphate buffer (60 mM, pH 7.8, 1 mM EDTA), NAD^+ (1 mM) and propionaldehyde (1 mM). The reaction was started by the addition of mitochondria (0.1 mg protein) and the increase in absorbance was monitored at 340 nm. The activity was expressed as μmol NADH formed/min/mg protein (€-6.22/mM/cm) (Canuto et al. 1983). Malate dehydrogenase activity was measured by the addition of an aliquot of testis cytosol or mitochondria (0.1 mg protein) to potassium phosphate buffer (0.1 M, pH 7.5) containing NADH (14.3 mM) and oxaloacetate (20 mM). The enzyme activity was expressed as μmol NADH oxidized/min/mg protein (€-6.22 mmol/cm) (Kitto 1969). The activity of lactate dehydrogenase (LDH-X) was measured in a reaction mixture containing Tris–HCl buffer (0.1 M, pH 7.0), α-ketovaleric acid (5 mM) and NADH (10 mM). The reaction was started by the addition of cytosol (0.1 mg protein) and the change in absorbance was monitored at 340 nm. The activity was expressed as μmol NADH oxidized/min/mg protein (€-6.22/mM/cm) (Goldberg and Hawtrey 1967).

The activity of 3*β*-hydroxysteroid dehydrogenase in testis cytosol was determined by measuring the rate of conversion of pregnenolone into progesterone. The enzyme activity was assayed in a reaction mixture (3.2 ml) containing Tris–HCl buffer (0.15 M, pH 7.2), pregnenolone (200 μM), NAD^+ (400 μM) and nitroblue tetrazolium (953 μM). The reaction was started by the addition of cytosol (0.25 mg protein) and incubated at 37°C for 60 min. Terminally, the reaction was stopped by the addition of phthalate buffer (pH 4.25), centrifuged and the supernatant was read at 570 nm. The enzyme activity was calculated from a NADH standard curve and expressed as *η*mol NADH formed/h/mg protein (Qujeq 2002).

Determination of ATPase activity

Mitochondrial Mg^{2+} ATPase activity was measured in a medium containing Tris–HCl (50 mM, pH 7.4) containing ATP (5 mM), KCl (20 mM), $MgCl_2$ (5 mM) and Ouabain (0.1 mM). Following addition of mitochondrial protein (0.05 mg), the reaction mixture was incubated for 15 min at 37°C. Terminally, the reaction was stopped by the addition of 20% TCA (Desaiah et al. 1979). After brief centrifugation, the inorganic phosphate formed in the protein free supernatant was determined and the enzyme activity was expressed as μg P_i liberated/mg protein (Fiske and Subbarrow 1925).

Activity of D-aspartate oxidase (D-AspO)

D-AspO activity was measured by the determination of the α-ketoacid formed from the reaction between D-Asp and D-AspO (D'Aniello et al. 1993a). Testis homogenate was prepared in Tris–HCl (0.1 M, pH 8.2) and centrifuged at 10,000×*g* for 30 min. An aliquot of the supernatant was

incubated with D-aspartate (0.1 M) for 30 min at 37°C followed by the addition of 20% TCA. Following centrifugation at 5,000×*g* for 5 min, an aliquot of the supernatant was incubated with 2,4-dinitrophenylhydrazine (1 mM) at 37°C for 10 min. The absorbance was read at 445 nm after the addition of 0.8 ml of sodium hydroxide (1.5 M) and the enzyme activity was expressed as µmol D-Asp oxidized/min/mg protein.

Mitochondrial enzyme activities

The activity of citrate synthase was determined by monitoring the oxidation of DTNB (Srere 1969). Mitochondrial protein (0.05 mg) was added to Tris–HCl buffer (0.1 M, pH 8.1, 0.1% Triton X-100) containing DTNB (0.2 mM) and acetyl CoA (0.1 mM). The reaction was started by the addition of oxaloacetate (10 mM), absorbance monitored at 412 nm for 3 min and the activity was expressed as *η*mol thiol oxidized/min/mg protein (€-13.6/mM/cm). Succinate dehydrogenase activity was determined by incubating testis mitochondria (0.025 mg protein) with phosphate buffer (50 mM, pH 7.4) containing sodium succinate (0.01 mol/l) and *p*-iodonitro tetrazolium violet (2.5 µg/ml) for 10 min. Following addition of 10% TCA, color was extracted with ethyl acetate:ethanol:trichloroacetic acid (5:5:1, v:v:w), absorbance was read at 490 nm. The activity was expressed as mmol substrate oxidized/mg protein (€-2000/mM/cm) (Pennington 1961).

The activities of NADH-cytochrome C reductase and succinate-cytochrome C reductase were measured in testis mitochondria as described previously (Navarro et al. 2002, 2004). The mitochondria (0.1 mg protein) were added to phosphate buffer (0.1 M, pH 7.4) containing NADH (0.2 mM), potassium cyanide (1 mM) and the reaction was initiated by the addition of cytochrome C (0.1 mM). The decrease in absorbance was monitored for 3 min at 550 nm and the activity was expressed as µmol cytochrome C reduced/min/mg protein (€-19.6 mM/cm). To determine the activity of succinate-cytochrome C reductase, the substrate succinate (20 mM) was added instead of NADH to the reaction mixture.

Mitochondrial permeability transition pore opening (MPT)

Mitochondria (0.25 mg protein) was suspended in a swelling medium (mannitol 215 mM, sucrose 71 mM, HEPES 3 mM, pH 7.4) containing 5 mM succinate. The absorbance was monitored at 540 nm immediately following the addition of $CaCl_2$ (100 µM) (Rigobello et al. 2005).

Mitochondrial membrane potential

Mitochondrial membrane potential was determined by measuring the uptake of Rh123 (Andersson et al. 1987). Mitochondria (0.05 mg protein) were added to phosphate-buffered saline (0.1 M, pH 7.4) containing Rh123 (1.5 µM/ml) and incubated at 37°C for 30 min. Following centrifugation at 10,000×*g* for 10 min, the fluorescence was measured at excitation and emission wavelengths of 490 and 520 nm, respectively.

Intracellular Ca^{2+} levels

An aliquot of testis cytosol was added to phosphate-buffered saline (0.1 M, pH 7.4) containing Fura-AM (1 µM/ml) and incubated at 37°C for 30 min. The fluorescence intensity was measured at excitation and emission wavelengths of 490 and 520 nm, respectively (Hitoshi et al. 1997).

MTT reduction assay

Testis mitochondria (0.01 mg protein) were added to Mannitol–Sucrose–HEPES buffer (pH 7.4) containing sodium succinate (20 mM) and MTT (5 mg/ml). Following incubation at 37°C for 60 min, the formazan crystals were dissolved in aqueous 10% SDS–45% DMF buffer (pH 7.4). The absorbance was measured at 570 nm and expressed as OD/mg protein (Berridge and Tans 1993).

Statistical analysis

Data are expressed as mean ± SE ($n = 6$) and were analyzed employing one-way analysis of variance (ANOVA) followed by Tukey's test for comparison of means to determine the significance of differences among the groups. P values ≤ 0.05 were considered as statistically significant.

Results

Effect of D-Asp on body and organ weights

At the administered doses, D-Asp did not induce any clinical symptoms or mortality. Further, there was no effect on the body weight gain among the treated animals. However, the weights of liver and testis were marginally (11–13%) decreased.

In vitro susceptibility of testis mitochondria to D-Asp

The ability of D-Asp to induce lipid peroxidation was determined in freshly prepared mitochondria isolated from prepubertal rat testis and was compared with induction response obtained in the cytosol (Fig. 1a, b). Mitochondria exhibited a significant increase in lipid peroxidation at

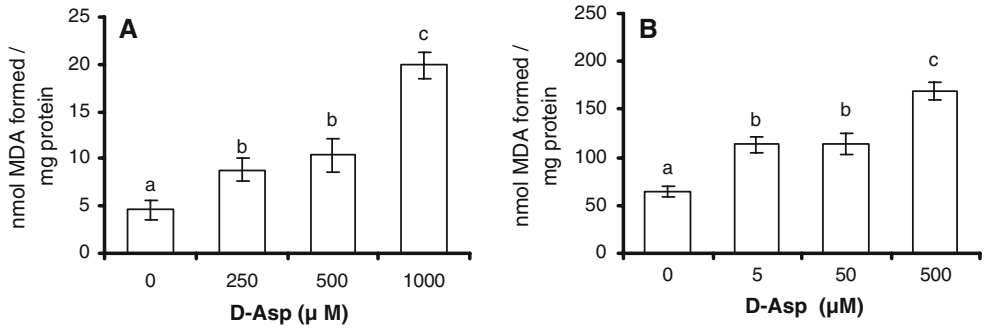

Fig. 1 Concentration-related increase in lipid peroxidation measured as malondialdehyde (MDA) levels in cytosol (**a**) and mitochondria (**b**) of prepubertal rat testis following exposure to D-aspartic acid (D-Asp) in vitro; each value represents the mean ± SE ($n = 3$). Statistical significance among groups was determined by one way analysis of variance (ANOVA) followed by Tukey's test for comparison of means. Bars assigned with different letters are statistically significant. $^{b}P < 0.01$, $^{c}P < 0.001$

lower concentrations of 5 and 50 μM (twofold), while the highest concentration (500 μM) induced an optimum response (threefold). However, in cytosol, D-Asp-induced lipid peroxidation was evident only at higher concentrations (beyond 250 μM).

Induction of oxidative stress in vivo

A marked enhancement in the levels of oxidative stress markers was discernible in the testis of D-Asp administered rats (Fig. 2a–c). D-Asp caused a robust elevation in ROS generation in mitochondria (74, 85% over control) as compared to the cytosol (30, 46% over control). Further, the MDA levels were uniformly enhanced in both cytosol (30, 55%) and mitochondria (33, 50%). Likewise, the hydroperoxide levels were also significantly increased in cytosol (20, 41%) and mitochondria (28, 40%).

Perturbations in GSH and activities of antioxidant enzymes

In testis of D-Asp treated rats, the GSH levels were markedly enhanced in both cytosol (45, 50%) and mitochondria (14, 30%) (Fig. 3a), while the total thiol levels were unaltered. A dose-related increase (58, 75%) in catalase activity was accompanied with marked elevations in GPx levels in cytosol (32, 40%) and mitochondria (25, 37%). Concomitantly, the activity of GST was elevated in cytosol (47, 40%) and mitochondria (10, 29%), while the activity of SOD was unaltered (Table 1).

Alterations in activities of functional enzymes

D-Asp caused significant increase in the activities of cytosolic LDH (27, 30%) and 3β-HSD (12, 20%), while the activity of MDH was marginally reduced (15%) (Table 1). Further, D-Asp also caused significant increase (21, 31%) in the activity of D-AspO, while the nitric oxide synthase activity (NOS) measured as NO levels were enhanced only at the lowest dose (30%) (Fig. 3b).

Alterations in mitochondrial enzyme activities and membrane integrity

D-Asp treatment caused significant perturbations in the activities of various mitochondrial enzymes (Table 2). The

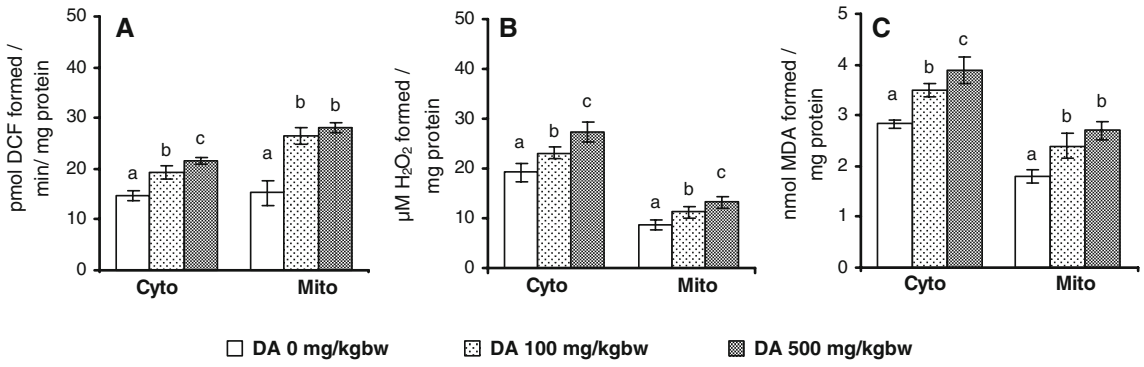

Fig. 2 Effect of D-aspartic acid (D-Asp) administration on the generation of reactive oxygen species (ROS) levels (**a**), hydroperoxide levels (**b**) and malondialdehyde (MDA) levels (**c**) in cytosol (cyto) and mitochondria (mito) in testis of prepubertal rats; each value represents the mean ± SE ($n = 6$). Statistical significance among groups was determined by one way analysis of variance (ANOVA) followed by Tukey's test for comparison of means. Bars assigned with different letters are statistically significant. $^{b}P < 0.05$, $^{c}P < 0.01$

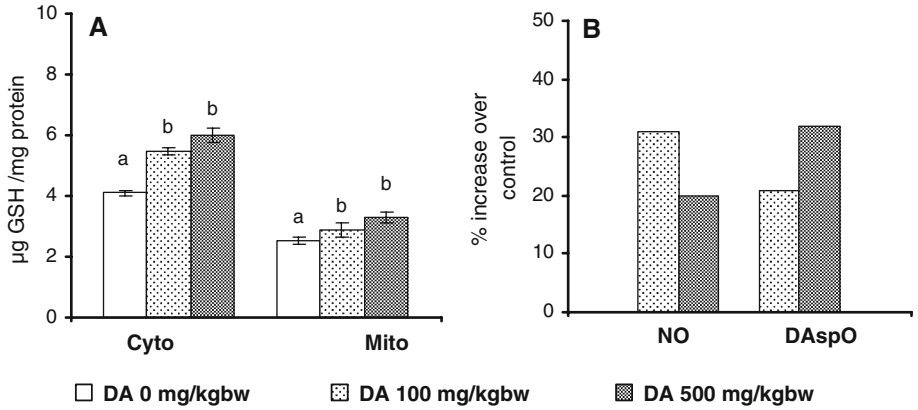

Fig. 3 Enhancement of glutathione (GSH) levels in cytosol (cyto) and mitochondria (mito) (**a**) and percent increase in nitric oxide (NO) release and activity of D-aspartate oxidase (D-AspO) (**b**) in testis of prepubertal rats administered with D-aspartic acid (D-Asp); each value represents the mean ± SE ($n = 6$). Statistical significance among groups was determined by one way analysis of variance (ANOVA) followed by Tukey's test for comparison of means. Bars assigned with different letters are statistically significant. $^{b}P < 0.05$ and $^{c}P < 0.01$

Table 1 Activities of antioxidant enzymes and functional enzymes in testis cytosol (cyto) and mitochondria (mito) of prepubertal rats administered D-aspartic acid for 7 days

	D-Aspartic acid (mg/kg bw/d)		
	CTR	100	500
Catalase[A]	0.012 ± 0.02[a]	0.019 ± 0.02[b]	0.021 ± 0.03[b]
Glutathione peroxidase (cyto)[B]	1.06 ± 0.02[a]	1.40 ± 0.03[b]	1.49 ± 0.02[b]
Glutathione peroxidase(mito)[B]	0.08 ± 0.01[a]	0.10 ± 0.02[b]	0.11 ± 0.01[b]
Glutathione-S-transferase (cyto)[C]	1.23 ± 0.08[a]	1.81 ± 0.11[b]	1.78 ± 0.05[b]
Glutathione-S-transferase (mito)[C]	0.72 ± 0.02[a]	0.73 ± 0.04[a]	0.93 ± 0.13[b]
Lactate dehydrogenase (cyto)[D]	0.044 ± 0.0[a]	0.056 ± 0.0[b]	0.057 ± 0.01[c]
Malate dehydrogenase (cyto)[D]	0.32 ± 0.06[a]	0.27 ± 0.005[b]	0.26 ± 0.008[b]
3β-hydroxy steroid dehydrogenase[E]	0.29 ± 0.01[a]	0.33 ± 0.05[a]	0.35 ± 0.02[b]

Each value represents the mean ± SE ($n = 6$). Statistical significance among groups was determined by one way analysis of variance followed by Tukey's test. Values within row with no common superscripts are statistically significant. $^{b}P < 0.05$

^{c} $P < 0.01$ compared to control

[A] µmol H_2O_2 decomposed/min/mg protein; [B] µmol NADPH oxidized/min/mg protein

[C] µmol GS-DNB conjugate formed/min/mg protein; [D] µmol NADH oxidized/min/mg protein; [E] µmol NADH formed/min/mg protein

activity of aldehyde dehydrogenase was significantly decreased (34, 20%) indicating increased accumulation of toxic aldehydes. While the activities of SDH and MDH remained unaffected, the activity of citrate synthase was consistently elevated (15, 43%). On the other hand, a differential response was evident in the activity levels of ETC enzymes. While D-Asp had no significant effect on NADH-cyt C reductase, the activity of succinate-cyt C reductase was significantly elevated (38, 51%) along with a marginal (16%) increase in the activity of Mg^{2+} ATPase at the highest dose. These alterations in enzyme activities were further reflected in the enhanced rate (43, 36%) of MTT reduction by mitochondria among D-Asp treated rats (Fig. 4a). Interestingly, mitochondria from D-Asp treated rats showed increased susceptibility to membrane damage and leakage as evidenced by opening of membrane transition pore and loss in membrane potential (Fig. 4b, c). We also found significant increase in intracellular Ca^{2+} levels at the lower dose (Fig. 4d).

Discussion

Data on the possible physiological consequences of excessive intake of D-Asp either in experimental animals or humans is limited. Several vegetables, marine foods and beverages contain exceptionally high quantities of D-Asp (Friedman 1999) and their consumption is likely to result in an increased accumulation in various tissues including testis. Hence, the potential damaging effects of exogenous

Table 2 Alterations in the activities of mitochondrial enzymes in testis of prepubertal rats administered D-aspartic acid for 7 days

	D-Aspartic acid (mg/kg bw/d)		
	CTR	100	500
Aldehyde dehydrogenase[A]	5.46 ± 0.7[a]	3.63 ± 0.2[b]	4.34 ± 0.9[b]
Citrate synthase[B]	19.5 ± 1.2[a]	22.5 ± 0.6[a]	28.0 ± 2.0[b]
Succinate dehydrogenase[C]	7.9 ± 0.2[a]	7.6 ± 0.11[a]	7.4 ± 0.03[a]
Malate dehydrogenase[D]	0.55 ± 0.04[a]	0.50 ± 0.03[a]	0.49 ± 0.06[a]
NADH-cyt C reductase[E]	0.074 ± 0.01[a]	0.079 ± 0.05[a]	0.078 ± 0.02[a]
Succinate-cyt C reductase[D]	0.098 ± 0.01[a]	0.135 ± 0.05[c]	0.148 ± 0.02[c]
Mg^{2+} ATPase (Complex V)[E]	46.7 ± 1.8[a]	45.7 ± 0.8[a]	54.0 ± 0.3[b]

Each value represents the mean ± SE ($n = 6$). Statistical significance among groups was determined by one way analysis of variance followed by Tukey's test

Values within row with no common superscripts are statistically significant. [b] $P < 0.05$

[c] $P < 0.01$ compared to control

[A] ηmol NADH oxidized/min/mg protein; [B] ηmol DTNB oxidized/min/mg protein

[C] mmol/mg protein; [D] μmol Cyt C reduced/min/mg protein; [E] μg P_i formed/min/mg protein

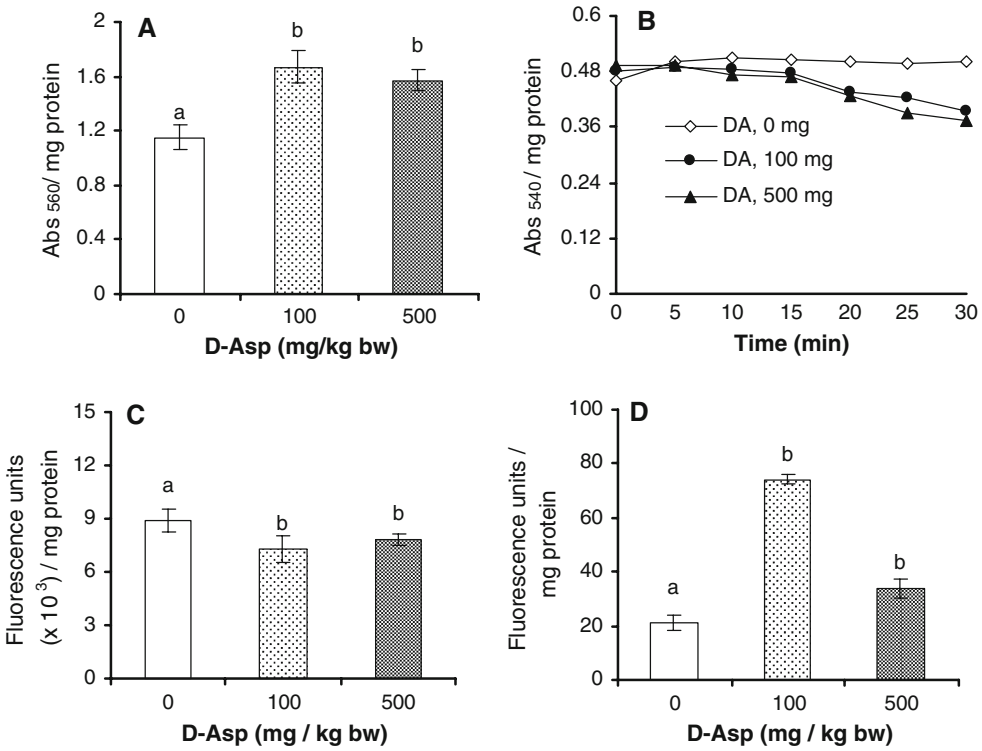

Fig. 4 Alterations in mitochondrial function measured as increase in MTT reduction (**a**), mitochondrial membrane pore opening (**b**), membrane potential (**c**) and cytosolic Ca^{2+} levels (**d**) in testis of prepubertal rats administered with D-aspartic acid (D-Asp). Each value represents the mean ± SE ($n = 6$). Statistical significance among groups was determined by one way analysis of variance (ANOVA) followed by Tukey's test for comparison of means. Bars assigned with different letters are statistically significant. [b]$P < 0.05$, [c]$P < 0.01$

D-amino acids following systemic administration has received wide attention (Hashimoto and Chiba 2004). Recent studies have shown remarkable augmented D-Asp levels in the pineal and pituitary glands following its intravenous administration suggesting a possible accumulation of D-Asp derived from intestinal bacteria or food in specific tissues (Morikawa et al. 2007). Previously, exogenously administered D-Asp was shown to accumulate in testis of rats with concomitant increase in testosterone and luteinizing hormone levels in blood (D'Aniello 1996). However, implications of excess D-Asp intake on testis physiology have not been comprehensively investigated.

The dosage selected for this study was based on a preliminary study in which D-Asp administered up to dosage of 50 mg/kg bw/d for 7 days had no significant impact on the oxidative stress markers in rat testis. Employing similar doses, earlier workers have reported physiological changes in testis and release of hormones in adult male rats (D'Aniello et al. 1996). Further, from a nutritional point of view, the lower dose (100 mg/kg bw) is an approximate

estimate of human exposure based upon ingestion of a mixed food diet (dairy products, fish, fruits and vegetables). However, the higher dosage (500 mg/kg bw) represents an exaggerated dose in order to assess the potential implications of D-Asp on testicular physiology.

Our principal findings suggest that exogenous D-Asp induces a marked elevation in oxidative stress markers in testis of prepubertal rats. Induction of oxidative stress following D-Asp exposure could be primarily related to its metabolism by inducible D-aspartate oxidase (D-AspO). Under normal physiological conditions, D-Asp is metabolized by D-AspO into oxaloacetate, ammonia and hydrogen peroxide. Hydrogen peroxide is immediately hydrolyzed either by catalase and or glutathione peroxidase thereby minimizing the peroxidative injuries. Hence, the enhanced activities of catalase and glutathione peroxidase in testis observed in the present study clearly implicate a possible upregulation in D-Asp metabolizing enzymes. This thinking is consistent with our earlier findings of enhanced oxidative response in testis of rats exposed to D-Asp in vitro under conditions of compromised catalase and glutathione peroxidase activities (Chandrashekar and Muralidhara 2008). Further, alterations in the activity of D-AspO explain the potential tissue damaging effects of D-Asp, which is consistent with previous reports of impaired metabolism of D-Asp by D-AspO leading to its accumulation in tissues and or organs (D'Aniello et al. 1993a, b).

D-Asp treatment caused significant oxidative dysfunctions in testis mitochondria as revealed by elevated ROS generation, hydroperoxide levels and MDA levels. These findings are consistent with earlier reports of oxidative stress mediated effects of other D-amino acids on mitochondrial functions (Gonzalez-Hernandez et al. 2003; Corte's-Rojo et al. 2007; Orozco-Ibarra et al. 2007). In the present study, testis mitochondria exhibited robust oxidative induction response even at low D-Asp concentrations (5–50 μM) under in vitro exposures. Testis is bestowed with a variety of antioxidant enzymes capable of metabolizing toxic aldehydes (MDA and HNE) generated during oxidative stress conditions. Glutathione-S-transferase and aldehyde dehydrogenase are particularly involved in the catalysis of the conjugation of HNE to glutathione and NAD^+ dependant oxidation of HNE to 4-hydroxy-2-nonenoic acid (Traverso et al. 2002). In the present study, irrespective of the dosage, testis mitochondria of D-Asp exposed rats exhibited reduced ALDH activity and enhanced glutathione-S-transferase activity, a finding which suggests a preferential regulation of aldehyde metabolizing enzymes.

Tissue sulfhydryls such as, protein thiol groups, glutathione etc., are major cellular antioxidants, whose altered levels serve as good indicators of the ongoing oxidative stress (Bauche et al. 1994). Glutathione, a redox active compound besides acting as a potent antioxidant also functions as a signal in the upregulation and expression of detoxification gene/s (Kirlin et al. 1999). In the present study, D-Asp treatment significantly enhanced glutathione levels with concomitant increase in the activities of glutathione-dependent enzymes which favor the detoxification of free radicals generated upon D-Asp metabolism. Additionally, higher ROS levels in a tissue can subsequently generate toxic reactive intermediates such as glutathione sulfenic acid and glutathiyl radicals which can form mixed disulfides with cellular proteins and alter their function (Ying et al. 2007).

In the present study, we also observed a significant increase in the NO levels in testis cytosol following D-Asp exposure. NO, a free radical metabolite of L-arginine is highly lipophilic, readily diffusible molecule which is demonstrated to possess a dual regulatory role under physiological and pathological conditions (Choi et al. 2002). Although the precise mechanism by which D-Asp causes enhanced NO levels is not clear from the present data, it may be directly related to the D-Asp-mediated upregulation of NO Synthase activity. The results of a recent report on induction of nitric oxide synthase by D-Asp in boar testis support such a possibility (Lamanna et al. 2007a). Further, over-production of NO can be potentially deleterious by activating caspase mediated apoptotic pathways (Choi et al. 2002).

Although earlier evidences suggest the involvement of D-Asp in upregulating testosterone biosynthetic pathways (D'Aniello et al. 1996; Lamanna et al. 2007b), in the present study we observed only a marginal increase in the activity of 3β-HSD. This could be attributed to the age of rats employed for the study and down regulation of steroidogenesis by high levels of NO (Kostic et al. 1998). D-Asp induced physiological changes in testis were also evidenced by significant elevation in the activity of LDH (an inner mitochondrial membrane bound enzyme) which is often considered as a marker of mitochondrial membrane integrity. Thus, enhanced activity of cytosolic LDH probably suggests extensive membrane damage and also a possible interference in normal physiology of sertoli cells (Pant et al. 1997). Further, this effect is also suggestive of a possible shunting of the metabolites of glycolytic pathway towards the lactate formation and a consequent depletion in cytosolic NADH levels. This finding is consistent with reduced activity of MDH, which catalyzes the entry of cytosolic NADH into mitochondria.

D-Asp treatment had no significant effect on the activities of TCA cycle enzymes in testis excepting for the elevated activity of citrate synthase. D-Asp elicited differential effects on the ETC enzyme activities particularly succinate-cytochrome C reductase and Mg^{2+} ATPase which were markedly enhanced in contrast to the unaltered levels

of NADH-cytochrome C reductase. This was further reflected by the increased rate of MTT reduction in testis mitochondria of D-Asp treated rats. However, the reasons for the differential effects on ETC enzymes are not clear.

In the present study, mitochondrial dysfunctions were studied by further examining two interrelated events such as transmembrane potential and membrane transition pore (MPT) opening in the presence of Ca^{2+} (Kroemer et al. 1997). Marked reduction in the membrane potential and associated increased MPT opening is suggestive of the potential of D-Asp to cause perturbations in mitochondrial membrane integrity. This finding in testis mitochondria corroborates well with previous observations of D-amino acid-induced hepatic mitochondrial dysfunctions in vitro (Gonzalez-Hernandez et al. 2003). Induction of MPT is shown to occur due to the dissipation of the difference in voltage between the mitochondrial membrane possibly due to free radical-mediated increase in intracellular concentration of calcium (Armstrong et al. 2004; Gunter et al. 2004). Further, the ETC may also generate more free radicals due to loss of specific components through the pore opening (Luetjens et al. 2000). Thus, it is likely that D-Asp induced specific mitochondrial membrane alterations in testis may probably result from altered calcium homeostasis.

In conclusion, the present investigation demonstrates for the first time the potential adverse effects of D-Asp administration and the participation of oxidative stress mediated mechanism/s in the observed biochemical effects caused by D-Asp in testis cytosol and mitochondria of prepubertal rats. However, further investigations are necessary to obtain basic insights into the functional implications of long term exposure to D-Asp on prepubertal testis.

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