Original Article

Vulnerability of Prepubertal Mice Testis to Iron Induced Oxidative Dysfunctions *In Vivo* and Functional Implications

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Abstract

Background: The present study describes the susceptibility of prepubertal testis of mice to prooxidant induced oxidative impairments both under *in vitro* and *in vivo* exposure conditions.

Materials and Methods: Following in vitro exposure to iron (5,10 and 25 μM), oxidative response measured in terms of lipid peroxidation and hydroperoxide levels in testis of pre pubertal mice (4 wk) was more robust compared to that of pubertal mice (6 wk).

Results: Further, in an *in vivo* study, pre pubertal mice administered (i.p) sub lethal doses (12.5, 25 and 50mg/100g bw/d, 5d) of Iron dextran, showed significant induction of oxidative stress response in testis cytosol and mitochondria manifested as lipid peroxidation, generation of reactive oxygen species, hydroperoxide levels and enhanced protein carbonyl levels (a measure of protein oxidation). Diminished levels of GSH and total thiols in both cytosol and mitochondria of testis suggested an altered redox state. Significant perturbations in the activities of antioxidant enzymes such as glutathione transferase, glutathione peroxidase and SOD were discernible suggesting the ongoing oxidative stress in vivo. These oxidative impairments were accompanied by functional implications in testis as reflected in the altered activities of dehydrogenases and reduced activities of both 3 β- and 17 β-hydroxysteriod dehydrogenase.

Conclusion: Collectively, these data provide an account of the susceptibility of prepubertal testis to iron-induced oxidative stress, associated functional consequences and this model is being further exploited for understanding the implications on the physiology of testis and consequent effect on fertility.

Keywords: Prepubertal Mice, Testis, Iron, Oxidative Dysfunctions, Fertility

Introduction

Epidemiological evidences emphasize that oxidative stress can play a vital role in the etiology of male infertility (1, 2). Several recent findings ascribe oxidative stress as a major cause for male reproductive dysfunctions (3-6). To assess the effect of prooxidants on male reproductive system, generally sexually mature rodents are employed as a result of which our understanding on the susceptibility of developing testis (prepubertal /pubertal stage) is rather limited. Such a basic understanding appears highly relevant given the fact that the mammalian testis is highly sensitive to oxidative mediated free radical damage. Further, testicular cells are highly susceptible to oxidative stress as they are intimately associated with the free radical generating phagocytic germ cells (7) and more importantly the germ cell plasma membrane contains a higher amount of polyunsaturated fatty acids which are more prone to oxidation by free radicals (8).

In order to understand whether developing testis exhibits increased vulnerability to oxidative stress, we chose iron and assessed the oxidative induction response in testis of prepubertal and pubertal mice. The selection of iron as the prooxidant was based on the fact that it is widely used to induce oxidative stress both in vitro and in vivo. Our own recent findings have shown the genotoxic consequences associated with oxidative damage in the testis of sexually mature mice subjected to iron intoxication (9).

While iron is an essential element for normal cell function and metabolism, an excess of cellular iron becomes highly toxic by inducing reactive oxygen species (ROS) production (10). It is one of the major catalytic metals responsible for formation of reactive oxygen species (ROS) in vivo (11). Organ damage arising from chronic iron overload is remarkable for the range of tissues affected and often slow and insidious onset of organ dysfunction. Foremost amongst the organs and cell types affected by iron overload are liver, heart, kidney, pancreatic beta cells and testis (12). Iron -induced liver damage and failure are associated with both primary and secondary hemochromatosis, common genetic disorders of iron metabolism (13). Multiple lines of evidence implicate redox active transition metals as mediators of oxidative stress in a variety of diseases

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(14, 15). It is unequivocally demonstrated that oxidative stress leads to an increase in iron uptake (16). Treatment of experimental animals with Iron-dextran (ID) closely resembles hemochromatosis secondary to iron-loading anemia and high oral intake of iron (16). Experimental data have shown accumulation of iron in testis on exposure to TCDD (17), Cadmium (18), Zinc deficiency conditions (19) and exposure to organic hydroperoxides (3).

Based on our earlier findings, we hypothesized that the exposure of pre pubertal mice to prooxidants may cause enhanced testicular oxidative stress and are likely to have significant effect on testis functions viz., steroidogenesis and spermatogenesis. This hypothesis was tested by examining the oxidative induction response in testis of pre pubertal and pubertal mice following in vitro exposure to Iron. Further in vivo studies were conducted in pre pubertal mice (4 wk old) to establish the occurrence of oxidative impairments (measured in terms of lipid peroxidation, generation of reactive oxygen species (ROS) and hydroperoxide levels) following short-term exposure to Iron. Alterations in the redox status, perturbations in the levels of antioxidant enzymes / non-enzymic antioxidants and protein carbonyl content (a measure of protein oxidation) were also determined. The occurrences of oxidative impairments were examined in both cytosol and mitochondria in the pre pubertal testis. Further, the functional implications in testis were investigated by determining the activity of various dehydrogenases and steroidogenic marker enzymes.

Materials and Methods

Chemicals

Iron Dextran (batch # 18H25175; Ferric hydroxide dextran complex, 100mg/ml containing 0.5% phenol as preservative), thiobarbituric acid, 1,1,3,3-tetramethoxypropane and other fine biochemicals were obtained from M/s Sigma Chemical Co., St Louis, USA. All other chemicals were of analytical grade.

Animals and care

Male mice (4-wk and 6-wk old, CFT-Swiss strain) were randomly drawn from the stock colony of 'Institute animal House facility'. Mice were held in polypropylene cages kept on racks built of slotted angles and were housed in a controlled atmosphere with a temperature range of 25±5ºC and mean relative humidity of 50 ± 5%. The animals were maintained on commercial mouse pellets (Ms Gold Mohur, supplied by M/s Lipton India Pvt. Ltd., India) ad libitum and had free access to water during a week of acclimatization and throughout the studies.

Experimental design and conduct

Iron-induced oxidative response in vitro in pre pubertal/ pubertal testis

Fresh testis obtained from both pre pubertal (4 wk old) and pubertal (6 wk old) male mice were used. Cytosol, mitochondria and microsomes were prepared as described below. Ferrous sulfate was employed as the prooxidant to measure the induction of oxidative response in vitro. All incubations were carried out in sterilized tissue culture plates (12 wells of 24 diameters) at 37ºC in a shaker incubator. Suitable aliquots of cytosolic fraction, or mitochondria or microsomes of testis were incubated with varying concentration of Fe (5, 10 and 25μM) for 30min. Following the incubation period, iron-induced TBARS and hydroperoxide levels were determined by methods as described under the in vivo study.

Iron-induced oxidative impairments in vivo in pre pubertal mice

Animal experiments were conducted strictly in accordance with approved guidelines by the "Institute Animal Ethical Committee" regulated by the Committee for the purpose of Control and Supervision of Experiments on Animals (CPCSEA), Ministry of Social justice and Empowerment, Government of India, India. Initially, groups of prepubertal mice were administered (i.p) acute doses of iron dextran (ID) at 12.5, 25 and 50mg/ 100g bw and sacrificed at 24hrs of post administration to assess the induction of oxidative damage in testis and liver. For further studies, prepubertal mice (n=6) were administered ID at doses of 0, 12.5, 25 and 50mg/100g bw /d for five consecutive days. During the experimental period, food intake was monitored daily and individual body weights were recorded prior to sacrifice. Both control and treated animals were sacrificed by decapitation at 24hrs after the administration of the last dose and testes were excised immediately, weights were recorded and processed for various biochemical investigations.

Preparation of cytosol, mitochondria and microsomes

Testis was homogenized in phosphate buffer (0.1M. pH 7.4) using a glass-Teflon grinder and the homogenate was centrifuged at 100xg for 10min to yield a pellet that was discarded and the supernatant was used as the cytosol. Mitochondria and microsomes were prepared as per the methods described earlier (20, 21).

Measurement of oxidative parameters

Lipid peroxidation was assessed in tissue cytosol and mitochondrial fractions by measuring the thiobarbituric acid reactive substances (TBARS) and quantified as malondialdehyde levels by using 1,1,3,3-tetramethoxypropane (TMP) as the standard (22). Briefly, the reaction mixture contained 0.2 ml of tissue homogenate or mitochondria (1mg protein), 1.5ml of acetic acid (pH 3.5, 20%), 1.5 ml of 0.8 % thiobarbituric acid (TBA, 0.8%w/v) and 0.2ml SDS (8%w/v). The mixture was boiled for 45min and TBARS adducts were extracted into 3ml of 1-butanol. The TBARS formed was measured by a UV-Visible spectrophotometer at 532ηm.

ROS generation in tissue cytosol and mitochondria was assayed using dihydrodichloro- fluorescein diacetate, a non-polar compound that, after conversion to a polar derivative by intracellular esterases, can rapidly react with ROS to form the highly fluorescent compound dichlorofluorescein (23, 24). Briefly, the homogenate was diluted 1:20 times with ice-cold Locke's buffer to obtain a concentration of 5mg tissue/ml. The reaction mixture (1ml) containing Locke's buffer (pH 7.4), 50µl homogenate or 0.2mg mitochondria and 10µl of DCFH-DA (5µM) was incubated for 15min at room temperature to allow the DCFH-DA to be incorporated into any membrane-bound vesicles and the diacetate group cleaved by esterases. After 30min of further incubation, the conversion of DCFH-DA to the fluorescent product DCF was measured using a spectrofluorimeter with excitation at 484ηm and emission at 530ηm. ROS formation was quantified from a DCF- standard curve and data are expressed as pmol DCF formed /min /mg protein.

Hydroperoxide levels in tissue cytosol and mitochondria were measured as described earlier (25). Briefly, tissue cytosol or mitochondrial fractions were added to FOX1 reagent (ferrous oxidation with xylenol orange), mixed, vortexed and incubated at room temperature for 30min. The absorbance was read at 560nm and unit was expressed as µmoles of H_2O_2 per mg cytosolic or mitochondrial protein.

Determination of Reduced glutathione (GSH), ascorbic acid and α-tocopherol

Measurement of GSH was done according to the fluorimetric method of Hissin and Hilf (26). Briefly, tissue was homogenized (5%w/v) in 25% HPO_3 and centrifuged at 10,000 g for 15 min. An aliquot of the supernatant was added to the reaction mixture containing phosphate buffer, (0.1M, pH 8.0, 5 mM EDTA) and 100µl of O-phthalaldehyde (50µg/ml) and incubated at room temperature for 15min. The fluorescence was measured at an excitation wavelength 350 ηm and emission 420 ηm.

Tissue vitamin C concentrations were analyzed using the 2,4-dinitrophenyl-hydrazine method of Omaye et al., (27). 1 mL tissue homogenate (25%, in 0.01 mol/L phosphate buffer, pH 7.4) was mixed with an equal amount of 0.62-mol/L trichloroacetic acid (TCA) and then centrifuged for 10min at 3500Xg. The supernatant (0.25mL) and 0.1mL DTC solution (0.4 g thiourea, 0.05g $CuSO_4 5H_2O$, and 3 g 2, 4-dinitrophenyl hydrazine in 4.5mol/L H_2SO_4) were mixed and incubated in a 37ºC water bath for 3hrs. After incubation, 0.75mL ice-cold 8.17mol/L H_2SO_4 was added and allowed to stand at room temperature for 30min and the absorbance measured at 520ηm. Vitamin E was estimated by the method of Zaspel and Csallany (28) with minor modifications. 100mg of testis was homogenized in 1.15% KCl and extracted with n-hexane and centrifuged. The n-hexane layer was separated and transferred into a separate tube and dried under Nitrogen atmosphere at 30-40ºC and stored at -18ºC until used. The dried sample was resuspended in a suitable volume of methanol and injected into reverse phase HPLC system C18 equipped with UV detector and measured at 280ηm. Tissue Vitamin E content was quantified using alpha-tocopherol standard and expressed as µg Vitamin E/mg tissue.

Determination of Protein carbonyls and iron levels

Protein carbonyl were determined in supernatants obtained after centrifugation of tissue homogenates at 10,000 rpm for 15min by measuring the hydrazone derivatives between 360 and 390ηm (e=21.0mM-1cm-1) according to the method described earlier (29).

The iron level in the tissue was measured by the method of Vacha et al., (30). To 0.2mL of plasma/ 10% testicular homogenate are added 1mL of water, 2mL of 10% TCA and mixed thoroughly and allowed to stand for 5min. Following centrifugation (2000rpm for 15min), 2mL of the clear supernatant is transferred to a clean tube and 2mL of bathophenanthroline solution was added, mixed and allowed to stand for at least 5min . The optical density of the developed color was measured at 540nm.

Activities of antioxidant enzymes

Testis samples were homogenized in 50mM phosphate buffer (pH 7.4) and sonicated over ice. The activities of enzymes viz., Catalase, SOD, GPX, GST and GR were determined in 1000xg supernatants of testis homogenates. Catalase (EC 1.11.1.6) activity was assayed by the method of Aebi (31) and the activity was expressed as µmol H_2O_2 consumed /min/mg protein. SOD (EC 1.15.1.1) activity was measured spectrophotometrically (32) by monitoring the inhibition of ferricytochrome-c reduction using xanthine-xanthine oxidase as the source of O_2. One unit of SOD is defined as the amount of protein required to inhibit 50% of the rate of cytochrome-c reduction. The activity of Glutathione peroxidase(EC 1.11.1.9) activity was determined using the t-butyl hydroperoxide as the substrate according to the method described (33) and the activity was expressed as ηmoles of NADPH oxidized /min/mg protein (e340 =6.22mM/cm) . Glutathione transferase (EC 2.5.1.18) was assayed at 340ηm by measuring the rate of enzyme catalyzed conjugation of reduced glutathione with 1-chloro-2-4-dinitro benzene (CDNB) according to the method of Guthenberg (34). Enzyme activity was expressed as ηmoles of s-2,4-dinitrophenyl glutathione formed/min/mg protein.

Measurement of 3β-HSD and 17β-HSD activity

3β-HSD activity in testis was determined by measuring the rate of conversion of pregnanolone into the progesterone according to the method of Qujeq (35). Briefly, the enzyme was assayed in a reaction mixture containing tris-HCl buffer (0.15M, pH 7.25), 400µM NAD, 200µM pregnanolone, Nitro blue tetrazolium (953µM)

in a total volume of 3.20ml. The reaction was started by adding the enzyme (250µg protein) and incubated at 37ºC for 60min. A control incubation of the mice \testis homogenate and NAD, without addition of pregnanolone was carried out. At the end of the incubation period, the reaction was stopped by the addition of 2.0ml of phthalate buffer (pH 4.25), centrifuged at 5000Xg for 30min, and the supernatant was read at 570ηm in a spectrophotometer. Enzyme activity was calculated from the standard curve of NADH and expressed as ηmol NADH formed/h/mg protein.

17β-HSD activity in testis was measured as per the method of Jarbak (36). One testis of each animal was homogenized in 20 % spectroscopic grade glycerol containing 5mM potassium phosphate and 1mM EDTA at a tissue concentration of 100mg/mL homogenizing mixture and centrifuged at 10,000g for 30min at 4ºC. The supernatant (1mL) was mixed with1mL of 44µM sodium pyrophosphate buffer (pH 10.2), 40uL of ethanol containing 0.3µM of testosterone and 960L of 25mg% BSA, making the incubation mixture a total volume of 3mL. Enzyme activity was measured after the addition of 1.1µM NAD to the tissue supernatant mixture in a spectrophotometer cuvette at 340ηm against blank (without NAD). One unit of enzyme activity is equivalent to a change in absorbance of 0.01/min at 340ηm.

Measurement of dehydrogenases

Testis was homogenized in ice-cold tris buffer (10mM, pH 7.0, containing 0.1% cetyltrimethyl-ammonium bromide). The crude homogenates were centrifuged (10,000 rpm for 30min at 4ºC) and the supernatants were used for enzyme assays. Lactate dehydrogenase (isoenzyme-X) was assayed according to the method of Goldberg and Hawtrey (37) using α-ketovalerate as the substrate. The activities of Glucose-6-phosphate dehydrogenase (G6PDH) and Sorbitol dehydrogenase (SDH) were determined following the methods described in Bergmeyer (38) and the enzyme activity was expressed as µmol of product formed/min/mg protein. Protein content in testis was determined according to the method of Lowry et al., (39) using bovine serum albumin as the standard.

Data analysis

Data on the in vitro induction of lipid peroxidation measured as MDA and hydroperoxide levels were obtained in triplicates and are presented as percent increase over the controls. The data obtained from in vivo study is presented as group mean±SD for each experimental group (n=6). The data was analyzed employing one way Analysis of variance (ANOVA) (SPSS Statistical package) followed by Duncan's New Multiple Range test (DMRT) and a p-value less than 0.05 was set as the minimum level of significance.

Results

Induction of lipid peroxidation in vitro

The pattern of LPO induction induced by iron in cytosol, mitochondria and microsomes of both pre pubertal and pubertal testis is presented in Fig 1A. While, testis of both age groups showed concentration related elevation in MDA levels, the response of pre pubertal testis was more robust. The cytosolic fraction of prepubertal testis showed consistently higher levels of induction (75, 100 and 210% increase over basal levels) compared to that of pubertal testis (15, 70 and 110%). Likewise, the mitochondrial fractions of prepubertal testis also responded in a similar fashion. The lowest concentration of Fe (5µM) yielded a marked increase in mitochondria (pre pubertal, 140%; pubertal, 24%), while at the highest concentration the induction was dramatic (pre pubertal, 395%; pubertal, 158%). Interestingly, the induction pattern in microsomes of testis was also higher among pre pubertal mice (50, 74 and 158%) compared to that of pubertal (23, 65 and 110%).

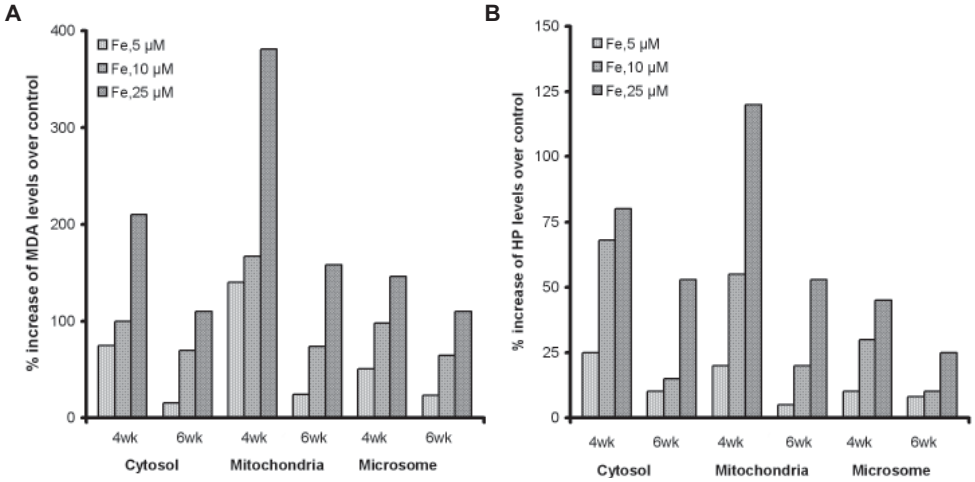

Fig 1: Percent increase in lipid peroxidation measured as malondialdehyde levels (A) and hydroperoxide levels (B) in testis cytosol, mitochondria and microsomes of prepubertal (4 wk) and pubertal (6 wk) mice exposed to various concentrations of FeS04 in vitro.

The pattern of HP levels formed in cytosol, mitochondria and microsomes of both prepubertal and pubertal testis is presented in Fig 1B. While, testis of both age groups showed concentration related elevation in HP levels, the response of prepubertal testis was more robust. The cytosolic fraction of pre pubertal testis consistently showed higher levels of induction (25, 68 and 80% increase over basal levels) compared to that of pubertal testis (10, 15 and 53%). The mitochondria of prepubertal testis also responded in similar fashion. The lowest concentration of Fe (5µM) yielded a marginal increase in mitochondria (pre pubertal, 20%; pubertal, 5%), while at the highest concentration the induction was higher (pre pubertal, 120%; pubertal, 53%). Interestingly, the induction pattern in microsomal fractions of testis was also relatively higher among pre pubertal testis.

In vivo study in pre pubertal mice

Administration of ID pubertal mice at sub lethal doses did not induce any clinical signs of toxicity or mortality. Further, there was no significant effect on body weights or testicular weights of treated mice (Table 1).

Table 1: Effect of administration Iron dextran (ID) (i.p, 5 days) on the body weight, liver and testis weights of prepubertal mice

I D[1] (mg/100gbw)	Body Wt (g)	Organ Wt (g)	
	Final	Testis	Liver
0	28.83±0.63[a]	0.139±0.01[a]	1.15±0.10[a]
12.5	27.98±0.60[a]	0.141±0.02[a]	1.49±0.02[a]
25	28.80±0.47[a]	0.138±0.02[a]	1.78±0.05[ab]
50	28.75±0.42[a]	0.142±0.01[a]	1.70±0.05[bc]

[1] Mice were administered (i p) at these dosages on the consecutive days

Values are mean±SE (n=6), data analysed by Duncan's multiple range test. Means of the same column followed by different letters differ significantly.

ID at the lowest dose failed to induce any significant oxidative damage in testis. However, at higher doses, the testicular MDA levels were enhanced by 39% and 100% over the endogenous levels in the cytosol, while the mitochondrial MDA levels were elevated (60%) only at the high dose (Fig 2 A). The ROS levels in cytosol were elevated marginally, while significant elevations were evident in mitochondria (44 and 101%) (Fig 2B). However, ID treatment induced only a marginal increase in the hydroperoxide levels (Fig 2C).

Significant depletion of GSH content in testis was evident only at highest dose (cytosol, 14-15%: mitochondria, 17-19%). Further, the total thiol content in testis were diminished (12- 25 %) at higher doses in both cytosol and mitochondria (Table 2). While the ascorbic acid content was significantly (15-20%) reduced in the testis, the reduction in vitamin E levels were more robust (18-31.6%) at higher doses. Iron levels in testis were also markedly elevated at higher doses. Measurement of protein carbonyl content showed elevations in both cytosol (75 and 150 %) and mitochondria (43 and 74%) suggesting significant oxidative damage to proteins.

In testis, the activity of GST was significantly reduced in both the compartments (cytosol, 39 -29%; mitochondria, 12-15 %), while the activity of catalase was unchanged (Table 3). In contrast, the activities of both SOD and GPX were elevated. The activity of SOD was increased marginally (cytosol, 12-16%; mitochondria, 15-26%). The elevation in the activity of GPX were more robust in the cytosol (18 and 34%); although, the activity was not altered in the mitochondria.

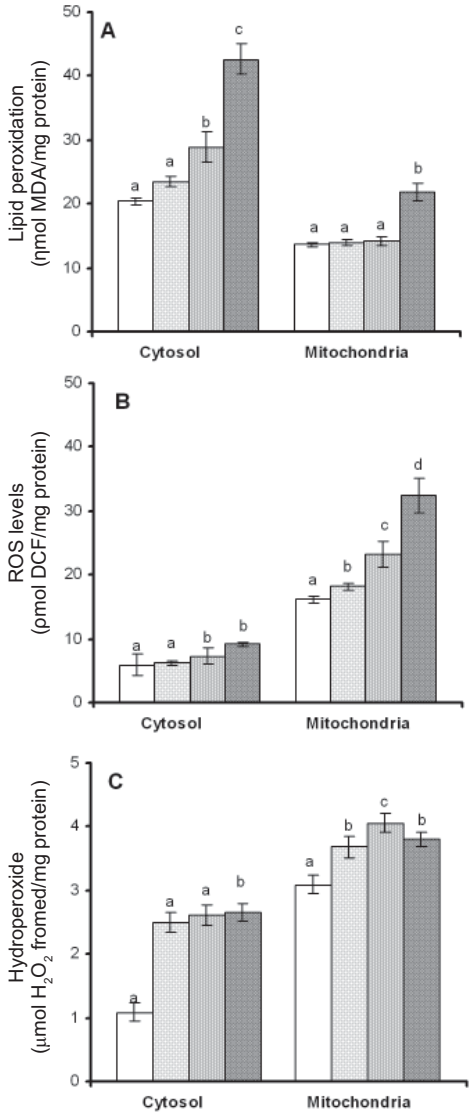

Fig 2: Status of Lipid peroxidation, measured as malondialdehyde levels in cytosol/mitochondria (A), generation of ROS levels (B) and hydroperoxide levels (C) in testis of control and iron dextran administered (12.5, 25 and 50 mg/100 g bw/d for 5 days) prepubertal mice.

Table 2: Effect of iron dextran treatment on reduced glutathione, non-enzymic antioxidants, iron levels and protein carbonyl content in testis of prepubertal male mice

Parameter	Iron Dextran (mg bw/day)			
	0	12.5	25	50
GSH (cyto)[1]	13.21±0.21[a]	11.61± 0.29[a]	11.43±0.29[a]	10.29±0.14[b]
GSH (mito)[1]	0.61±0.09[a]	0.54±0.02[a]	0.49±0.03[a]	0.53±0.04[b]
Total thiols (cyto)[2]	59.30±1.65[a]	57.55±2.39[b]	51.93±1.72[bc]	43.98±2.50[cd]
Total thiols (mito)[2]	50.90±1.41[a]	48.00±0.73[ab]	44.64±1.82[bc]	41.88±1.13[c]
Ascorbic acid[3]	7.91±0.42[a]	7.18±0.10[a]	6.88±0.39[ab]	6.44±0.11[c]
Vitamin E[4]	0.117±0.12[a]	0.112±0.70[a]	0.095±0.70[b]	0.080±0.50[a]
Total Iron[5]	139.00± .24[a]	153.00± .20[a]	227.7±11.16[b]	412.0±15.0[c]
P.carbonyls (cyto)[6]	9.25±0.75[a]	10.88±0.81[a]	16.25±1.11[b]	23.25±1.25[c]
P.carbonyls (mito)[6]	36.58±1.73[a]	38.55± 0.71[a]	51.50±1.71[b]	63.90±1.89[c]

Values are mean ± SE (n=6), data analysed by Duncan's multiple range test.
Means of the same row followed by different letters differ significantly.
1-µg/mg protein, 2-µmol DTNB oxidised /mg protein, 3-µg/mg tissue, 4-µg/mg tissue, 5-µg/mg tissue, 6-η mol/mg protein

Table 3: Effect of iron dextran treatment on the activity of antioxidant enzymes in testis cytosol and mitochondria of prepubertal male mice

ID mg/100g bw	Enzyme Activity			
	CAT[1]	**GST[2]**	**SOD[3]**	**GPx[4]**
Cytosol 0	3.23±0.20[a]	0.583±0.05[a]	52.87±1.57[a]	0.026±0.006[a]
12.5	3.06±0.81[a]	0.489±0.04[b]	54.17±0.49[b]	0.024±0.003[a]
25	3.00±0.09[a]	0.456±0.04[b]	59.46±1.19[b]	0.030±0.003[b]
50	2.73±0.075[a]	0.353±0.04[b]	61.24±1.47[b]	0.035±0.003[c]
Mitochondria 0	2.93±0.065[a]	0.349±0.015[a]	164.35±2.25[a]	0.0146±0.002[a]
12.5	2.80±0.24[a]	0.334±0.005[ab]	162.76±3.88[a]	0.0140±0.001[a]
25	2.58±0.28[a]	0.310±0.005[b]	185.65±4.13[ab]	0.0148±0.002[a]
50	2.73±0.18[a]	0.294±0.02[b]	193.97±3.56[b]	0.0161±0.001[a]

Values are mean±SE (n=6), data analyzed by Duncan's multiple range test.
Means of the same column followed by different letters differ significantly.
1- ηmol H_2O_2 /min/mg protein
2- µ mol of conjugate formed/min/mg protein
3- Units/mg protein
4- ηmoles of NADPH oxidized/min/mg protein

Table 4: Effect of iron dextran treatment on the activity of dehydrogenases in testis of prepubertal male mice

Parameter	Iron Dextran (mg bw/day)			
	0	**12.5**	**25**	**50**
SDH[1]	0.164±0.01[a]	0.158±0.01[a]	0.140±0.03[a]	0.126±0.02[b]
LDH[2]	0.094±0.02[a]	0.098±0.01[a]	0.076±0.02[b]	0.072±0.02[b]
G6PDH[3]	0.027±0.02[a]	0.021±0.01[b]	0.022±0.01[b]	0.020±0.01[b]
SDH[4]	128±2.04[a]	120±2.00[a]	106±1.20[b]	102±1.40[b]

Values are mean ± SE (n=6), data analysed by Duncan's multiple range test.
Means of the same row followed by different letters differ significantly.
1- Sorbitol dehydrogenase -µ mol NADH oxidized/min/mg protein
2- Lactate dehydrogenase -µ mol NADH oxidized/min/mg protein
3- Glucose 6 phosphate dehydrogenase-µ mol NADH reduced/min/mg protein
4- Succinate dehydrogenase (mitochondria) µ mol formazan formed /mg protein

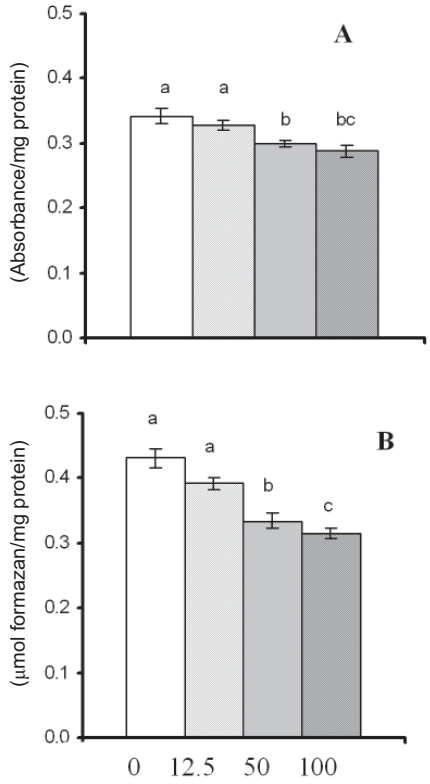

Fig 3: Activities of 3 β- hydroxysteriod dehydrogenase (A) and 17 β- hydroxysteriod dehydrogenase (B) enzymes in testis of prepubertal mice administered Iron dextran (12.5, 25 and 50 mg/100 g bw/d for 5 days)

As shown in Fig 3, iron treatment reduced the activities of both 3β- and 17β-HSD to a significant extent. The percent decrease in the activity of the enzyme varied between 17 and 19 % compared to the basal levels. At the higher dose, the activities of Sorbitol and lactate dehydrogenase were significantly elevated (Table 4). The activity of Succinate dehydrogenase in the mitochondria was not altered significantly. However, there were marginal to moderate changes in the activities of cytosolic dehydrogenases. On the other hand, the activity of G6PDH was reduced at both doses (23%).

Discussion

Our basic knowledge of the vulnerability of prepubertal /pubertal testis to oxidative stress is limited because mainly adult animals (sexually mature) have been assessed for reproductive toxicants. Nevertheless, it is widely accepted that prepubertal/pubertal rodents are more susceptible to xenobioticss due to lower levels of detoxification enzyme systems (40). In recent times, the potential relationship between oxidative impairments in the testis and testicular dysfunctions are being understood (41, 6, 9). The present study was designed to test the hypothesis that the testis of prepubertal rodents may be more vulnerable to oxidant damage.

To test this notion, initially, we have addressed the question whether their exists a differential susceptibility of prepubertal and pubertal testis to iron induced oxidation under in vitro conditions. Our results showed that the prepubertal testis was indeed more susceptible as the degree of iron-induced oxidative alterations and was more robust as determined in various subfractions such as cytosol, mitochondria and microsomes. The underlying reason for this differential response is not clear and is apparent that further studies are required in this direction. Nevertheless, it could be related to the lower levels of antioxidant capacity of the prepubertal testis and lower testosterone levels. Since the prepubertal testis showed higher vulnerability, we further examined the occurrence of oxidative dysfunctions in prepubertal mice in vivo and its functional implications.

Our findings of increased lipid peroxidation in both cytosol and mitochondria of testis, compromised concentrations of reduced GSH, non-enzymic antioxidants (ascorbic acid and α-tocopherol) and perturbations in enzymic defenses, and increased protein carbonyls clearly indicate that enhanced oxidative stress is present under iron exposure. The extent of lipid peroxidation was a similar degree in both cytosol and mitochondria in testis suggesting a uniform induction of oxidative damage. Interestingly, a similar uniform response was also evident in the hepatic tissue, although the degree of peroxidation was more robust compared to that of testis (data not shown). Mitochondria are an important source of ROS production and also the primary targets of ROS-induced toxicity. Our observation in testis mitochondria viz., enhanced ROS/MDA levels, reduced GSH levels and altered activities of antioxidant enzymes are suggestive of the impaired mitochondrial ROS metabolism. These findings are consistent with elevated lipid peroxides, malondialdehyde, and ROS production in the testis mitochondria of adult rodents exposed to various prooxidant xenobiotics (3, 5, 6, 9).

Thiols are generally considered as the primary defense molecules that can directly quench the free radicals or chelate metal ions almost, instantaneously (42). Depletion of reduced GSH and total thiol levels in testis of iron treated mice further suggests that a state of oxidative stress exists in vivo. GSH is important in the regulation of cellular redox state and a decline in the cellular levels is considered to be good indicator of oxidative stress. GSH is very vital as an antioxidant defense because besides serving as a substrate in the GSH-PX reaction; it also acts as free radical scavenger and helps to regulate the thiol disulfide concentration of a number of glycolytic enzymes such as glyceraldehydes-3-phosphate dehydrogenase and Ca^{2+}-ATPases, thus indirectly maintaining the intracellular Ca^{2+} homeostasis. More importantly, GSH regenerates other scavengers such as ascorbic acid and α-tocopherol.

Significant depletion of key non-enzymic antioxidants viz., ascorbic acid and α-tocopherol among iron treated mice indicated that antioxidant machinery is

overwhelmed in the prepubertal testis. Ascorbic acid is potent water-soluble antioxidant in biological fluids, scavenging physiologically relevant reactive oxygen and nitrogen species, there by preventing oxidative damage to vital biomolecules. The decrease in the levels of ascorbic acid could also be related to decrease in the availability of reduced GSH. In contrast, α- tocopherol, the major lipid-soluble antioxidant and an integral component of plasma lipoprotein scavenges lipid peroxyl and alkoxyl radicals in biological membranes by preventing the formation of LPO products. Recycling of tocopheroxyl radical to tocopherol is achieved by reaction with ascorbic acid and GSH. In the present study, dramatic depletion of α-tocopherol in the prepubertal testis is suggestive of the extent of oxidative stress and also a deficient recycling process owing to the deficient GSH and ascorbic acid levels. These findings corroborate with earlier findings in testis of adult rats administered high acute doses of iron dextran (43) in which the levels of lipid soluble antioxidants such as α-tocopherol, ubiquinol-9 and ubiquinol-10 were inversely correlated with the extent of oxidation.

Following prooxidant exposure, alterations in antioxidant defense enzyme activities are known to be tissue-specific. In the testis of ID treated mice, elevated lipid peroxidation was accompanied by decrease in the activities of GST and GPX (cytosol and mitochondria), while the activity of catalase was unchanged. However, a marginal increase in the activity SOD was evident in both cytosol and mitochondria. SOD acts as a first line of defense against deleterious effects of oxyradicals in cells by catalyzing the dismutation of endogenous cytotoxic superoxide radicals to H_2O_2 which is further removed by GSH-PX. Up-regulation of SOD activity in both cytosol and mitochondria in the testis may represent an adaptive response under diabetic condition. More importantly, GSH-PX plays a much greater role in detoxification of hydrogen peroxide than catalase. Increase in the activity of GSH-PX in cytosol of ID mice testis reflects a response to an increase in the rate of hydrogen peroxide formation and the reduction in reduced GSH levels. The decrease in the levels of GSH causes a proportional increase in H_2O_2 by GSH-PX. Further, a decreased activity of GST in prepubertal testis may be partly due to the lack of its substrate (GSH). The enzymic antioxidant machinery in ID treated mice showed a similar response in liver tissue as well. However GST activity in liver was markedly elevated both in cytosol and mitochondria clearly indicating the predominant role played by the enzyme under Iron overload situation.

Protein carbonyls are employed as useful biomarkers of ROS mediated protein oxidation (29) and elevated levels of oxidized proteins in animal tissues under various oxidative stress situations are documented (44). ROS can lead to oxidation of amino acid residue side chains, formation of protein-protein cross linkage and oxidation of protein backbone resulting in protein fragmentation. Further, protein carbonyls are introduced into the protein by reaction with aldehydes such as MDA and 4-HNE, which are the end products of lipid peroxidation. In the present study, the prepubertal testis of ID treated mice showed higher levels of protein carbonyls in both cytosol and mitochondria suggesting high rate of protein oxidation in both compartments. This is consistent with our earlier findings in testis of adult mice administered ID (9).

Although adult testis is endowed with sufficient antioxidative defenses (7), earlier workers have shown altered steroidogenesis as a consequence of increased oxidative stress with xenobiotics (9), alcohol (45) and experimentally-induced diabetes (46). In the testis, various xenobiotics are known to be metabolised by cytochrome P 450 enzymes, radical metabolites of ROS are known to be produced and altered steroidogenesis by itself can generate free radicals. Hence, even minor alterations in ROS levels and their detoxification can substantially affect the spermatogenetic process since germ cells are susceptible to peroxidative damage (47, 3). In the current model of oxidative stress in prepubertal mice, following iron administration, we did observe significant decrease in the activity of both 3 β-HSD and 17 β-HSD clearly indicating altered steroidogenesis. These were also associated with alteration in the activity of dehydrogenases in the testis suggesting functional implications. How exactly iron reduces the activities of 3 β-HSD and 17 β-HSD enzymes is not clear, since we have not measured RNA levels of genes known to regulate the activity of these enzymes. It is quite likely that high levels of ROS generated by iron may damage the proteins themselves. This thinking is supported indirectly by the fact that the protein carbonyl content was significantly enhanced in the testis. Further, iron may directly interact with the enzymes and inhibit their activities.

Determinations of testicular enzyme activities generally associated with intracellular metabolic function have been widely used for identifying the initial biochemical lesions and the progression of testicular injury (48). In the present study, activities of enzymes such as, Sorbitol dehydrogenase, Succinic dehydrogenase, LDH and G6PDH were measured as functional indicators of testis. Decreased activity in the testis of ID mice suggests an altered cellular physiology. The enhanced lipid peroxidation in testis mitochondria of ID treated mice testis is likely to result in disintegration of the mitochondrial membrane. Under iron exposure conditions, it is quite likely that the end products of lipid peroxidation may inactivate the enzymes of electron transport chain.

While our results clearly show the vulnerability of prepubertal testis to oxidative stress, the present model has a limitation since one cannot demonstrate the functional implications in terms of sperm production and development. However, our investigations in pubertal mice (6 wk old) have shown that iron dextran administered at dosages of 25 and 50 mg/100 g bw on alternate days for two weeks induces marked oxidative stress and

various testicular dysfunctions. Interestingly, a marked reduction in mating efficiency and fertilizing ability of such males has been observed (Thyagaraju and Muralidhara, Unpublished). Our further efforts are directed towards a better understanding of the correlations between repeated oxidative stress in pubertal testis and the functional implications which arise subsequently during adulthood.

Conclusion

We have provided significant evidence for the first time that the prepubertal mice testis are highly vulnerable to iron-induced oxidative damage and associated with various functional impairments. The vulnerability of prepubertal testis to oxidative impairments emphasizes the importance of obtaining basic data on the consequences of chronic oxidative stress in the testicular milieu during prepubertal /pubertal period, since the fertility of such males is likely to be affected. Our future studies employing this model are aimed to characterize the effect of chronic prooxidant exposures in pubertal animals with special emphasis on sperm physiology/ function as well as nature/ specificity of DNA lesions which are likely to occur in vivo. Only such investigations would yield reliable information on the role of oxidative impact during the early stages of testicular development and its contribution towards male subfertility / infertility and further help us in formulation of suitable therapeutic approaches to protect the male gonad under such situations.

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