Protocols for Evaluating Antioxidant Defence and Oxidative Stress Parameters in Rat Testis

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Abstract

This article describes the comprehensive methods and steps evaluating different antioxidant defence parameters and oxidative stress parameters of rat testis. Different antioxidant defence parameters include antioxidant enzymes like superoxide dismutase, catalase, glutathione peroxidase, glutathione reductase, glutathione S-transferase along with small antioxidant molecules like ascorbic acids and glutathione contents. Various oxidative stress parameters include hydrogen peroxide, protein carbonyl and lipid peroxide contents. Besides, methods for isolation of different testicular subcellular fractions, germ cell isolation and testis histological preparation were also described.

Protocols

Studying various antioxidant defence as well as oxidative stress parameters of rat testis is critical for evaluating testicular antioxidant defence status under various physiological conditions particularly when there are concerns about male infertility (Sahoo, 2011; 2013a; 2013b; Sahoo et al., 2005; 2007; 2008a; 2008b; 2008c; Sahoo and Roy, 2012). This article describes the comprehensive methods and steps evaluating different antioxidant defence parameters and oxidative stress parameters of rat testis.

1. Chemicals Required

Thiobarbituric acid (TBA), catalase, glutathione reductase (GR), cumene hydroperoxide, sephadex G-25, bovine serum albumin (BSA), 5,5’-dithiobis-2-nitrobenzoic acid (DTNB), trypan blue dye, 1-chloro-2, 4 dinitrobenzene (CDNB) and 4-vinyl pyridine were obtained from Sigma Chemical Company, USA and tert-butyl hydroperoxide was obtained from Merck-Schudant, Germany. Horse radish peroxidase (HRP), reduced nicotinamide adenine dinucleotide (NADH), reduced nicotinamide adenine dinucleotide phosphate (NADPH), ascorbic acid, reduced (GSH) and oxidized (GSSG) glutathione, L-methionine, riboflavin, xylenol orange, guanidine hydrochloride, disodium succinate hexahydrate and sodium dodecyl sulphate (SDS) were purchased from SISCO Research Laboratory, India. Hydroxylamine hydrochloride was obtained from Koch Light Laboratory Ltd., England.

2. Tissue Processing

Homogenization

After removal of rat testis, a 20% (w/v) homogenate of the testis was prepared in cold phosphate buffer (50 mM pH 7.4) containing 0.25 M sucrose with the help of a Potter-Elvehjem type motor driven homogenizer.

Isolation of sub-cellular organelles

The testis homogenate was filtered through four-layered sterilized cheesecloth and the filtrate (referred as crude homogenate, CH) was centrifuged at 600 ´ g for 10 minutes at 4°C to precipitate nuclei and cellular debris. The supernatant was centrifuged at 10,000 ´ g for 20 minutes at 4°C to separate mitochondrial pellet. The mitochondrial pellet was washed three times with ice-cold phosphate buffer (50 mM, pH 7.4) at 10,000 ´ g for 5 minutes at 4°C (Figure 1). The crude homogenate, mitochondrial fraction (MF) and post-mitochondrial fraction (PMF) were processed immediately for various biochemical analyses. All the centrifugation steps were performed in Sigma laboratory centrifuge model 3K30.

3. Assay of succinate dehydrogenase

Purity of mitochondrial fraction obtained by differential centrifugation was assessed by determining the specific activity of succinate dehydrogenase (SDH) in crude homogenate (CH), cell debris and nuclear fraction (NF), mitochondrial fraction (MF) and post-mitochondrial fraction (PMF) (Sahoo et al., 2005). The assay was done by the method of Lambowitz (1979). In brief, the enzyme reaction was started by adding 0.1 ml diluted sample containing around 100 mg protein to 2.9 ml of assay medium (containing 0.05 ml of 1.2 M sodium succinate, 0.05 ml of 2.25 mM DCPIP (2,6-dichlorophenol-indophenol), 2.75 ml of 50 mM phosphate buffer pH 7.4 and 0.05 ml of 60 mM KCN pre-warmed to 37°C). All the reagents were prepared in 50 mM phosphate buffer pH 7.4. The absorbance was read at 600 nm at intervals of 0, 3 and 15 min. A reagent blank (without sample) and a sample blank (without DCPIP) were taken in a similar manner. Another blank reaction was setup where buffered sucrose solution was taken for the assay. The
activity was calculated from the formula
\[
\ln (\text{Abs at 3 min} / \text{Abs at 15 min}) \quad 1000 \\
\frac{\text{In} \times 0.1 \text{ units}}{\text{mg protein}}
\]
The result was expressed as units/mg protein/min.

4. Estimation of protein content
Protein contents of samples were estimated by the method of Lowry et al. (1951). Biuret reagent was prepared freshly by mixing solutions A, B and C in a proportion of 100:2:2. Solution A was 2% (w/v) sodium carbonate (Na₂CO₃) in 0.1 N sodium hydroxide (NaOH) solution; solution B contained 0.5% (w/v) copper sulphate (CuSO₄) in distilled water and solution C contained 1% potassium sodium tartarate (KNaC₂H₃O₆) in distilled water. Commercially available Folin & Ciocalteu’s phenol reagent was used after diluting it with distilled water in a proportion of 1:2 (v/v). Samples were diluted suitably and 0.1 ml of the sample were taken and the volume was made up to 0.5 ml with distilled water. Then 5 ml of biuret reagent was added to the tubes, vortexed and allowed to stand for 10 minutes at room temperature. To this 0.5 ml of Folin-Ciocalteu’s phenol reagent was added, vortexed and then incubated at room temperature for 30 minutes. The color of the reaction was read against a blank at 700 nm.

Bovine serum albumin (BSA) solution (1 mg/ml of distilled water) was used as working standard. It gave a linear curve in the concentration range of 25-125 mg. Protein content was expressed as mg/gram wet weight of tissue.

5. Assay of Oxidative stress parameters
A. Estimation of endogenous and oxidant induced lipid peroxidation
The crude homogenate (20%, w/v homogenate in phosphate buffer pH 7.4, 50 mM) and the mitochondrial fraction of testis were used for the assay of lipid peroxidation and expressed in terms of thiobarbituric acid reactive substance (TBARS). Endogenous lipid peroxidation levels in samples were determined by monitoring the formation of thiobarbituric acid reactive substance in the assay itself. TBARS reagent was prepared by mixing following solutions: (i) 0.2 ml of 8.1% sodium dodecyl sulphate (SDS), (ii) 1.5 ml of 20% acetic acid (pH adjusted to 3.5 with NaOH), (iii) 1.5 ml of 0.8% aqueous solution of thiobarbituric acid (TBA), (iv) 0.1 ml of 0.76% BHT and 0.5 ml of distilled water.

For the assay to 0.2 ml of sample (1-1.5 mg protein) 3.8 ml of the TBA reagent was added. The mixture was vortexed and heated in a water bath at 95°C for 60 minutes using glass balls as condensers. Tubes were cooled down to room temperature and then centrifuged at 1,000 ‘g’ for 10 minutes. The absorbance of the supernatant was measured at 532 nm against an appropriate blank. The concentration of TBARS was calculated from the extinction coefficient of 1.56 × 10⁻⁴M⁻¹cm⁻¹. Result was expressed as nmol TBARS formed per mg protein.

To check the susceptibility of TBARS to various oxidants, the crude homogenate and the mitochondrial fraction were incubated with H₂O₂ (600 nmol/mg protein), tert-butyl hydroperoxide (t-BuOOH, 600 nmol/mg protein) and ferrous sulphate/ascorbic acid (0.25 mM/0.01 mM) at 37°C for 60 minutes in a shaking water bath prior to assay (Radi et al., 1993; Sahoo et al., 2005).

B. Estimation of hydrogen peroxide content
H₂O₂ content was measured in samples according to the method of Pick and Keisari (1981). Briefly, to 1.7 ml of 50 mM phosphate buffer (pH 7.4), 0.1 ml of 5.6 mM phenol red and 50 µl of HRP (20 units/ml) were added. Phenol red and HRP solutions were prepared in 50 mM phosphate buffer pH 7.4. It was vortexed and incubated for 5 minutes at room temperature. To it 0.1 ml sample (0.4-0.5 mg protein) was added followed by 50 µl of 1 N NaOH to stop the reaction. A standard curve of H₂O₂ was plotted in the range of 200-800 µM (20-80 nmol H₂O₂/0.1 ml). The absorbance was read at 610 nm against blank. The H₂O₂ content was expressed as nmol/mg protein. A recovery experiment for H₂O₂ was carried out in the test system and in the samples. Catalase was pre-incubated in ice for 30 minutes after adding 0.17 M ethanol to it. Catalase was added (10 µg/ml) in two successive additions, 1st addition and after an interval of 1 minute, 2nd addition. After 10 minutes incubation absorbance was recorded against a blank containing catalase in buffer. Absorbance was recorded at 610 nm (Sahoo et al., 2008a).

C. Estimation of lipid hydroperoxide
FOX 2 method was used to measure the levels of lipid hydroperoxides (LOOH) in samples (Hermes-Lima et al., 1995; Gay and Gebicki, 2002). FOX 2 Reagent was prepared by mixing following reagents: (i) 0.1 ml of 2.5 mM ammonium ferric sulphate (dissolved in 35.71 mM H₂SO₄), (ii) 0.6 ml of 35.71 mM H₂SO₄, (iii) 0.1 ml of 1 mM xylene Orange and (iv) 0.1 ml distilled water. To 0.9 ml of FOX 2 reagent 100 µl of test sample was added, vortexed and incubated at room
temperature for 1 hour. Then it was centrifuged to remove any flocculated materials and absorbance of supernatant was read at 552 nm against a blank. Cumene hydroperoxide was taken as the standard and a linear curve was obtained in the concentration range of 25-200 pmols/ml. The LOOH content was expressed as nmol lipid hydroperoxide/mg protein (Sahoo et al., 2007).

D. Estimation of protein carbonyl content

The protein carbonyl content in the CH, MF and the PMF was estimated according to the method of Levine et al. (1990). Following reagents were used for protein carbonyl assay: (i) PDEP buffer: 50 mM phosphate buffer (pH 7.4) containing 0.2% digitonin, 2 mM EDTA and 0.46 mM phenyl methane sulphonil fluoride (PMSF), (ii) 10% streptomycin sulphate in 50 mM phosphate buffer, (iii) 2.5 M HCl, (iv) 10 mM 2,4-dinitrophenyl hydrazine (DNPH) dissolved in 2.5 M HCl, (v) 20% trichloroacetic acid (TCA), (vi) absolute ethanol/ethyl acetate solution 1:1 ratio (v/v) and (vii) 6 M guanidine hydrochloride in 20 mM K2HPO4, pH adjusted to 2.3 with concentrated HCl.

To 0.9 ml sample (1.5-2 mg protein), 0.9 ml PDEP buffer was added followed by 0.2 ml of 10% streptomycin sulphate solution. It was incubated for 10-15 minutes, centrifuged at 1000 ´ g for 10 minutes and supernatant was used for assay and protein estimation. To 0.8 ml of the supernatant, 0.8 ml of 10 mM dinitrophenyl hydrazine solution was added. A blank was prepared by treating 0.8 ml of supernatant with 0.8 ml of 2.5 M HCl. The tubes were allowed to stand at room temperature in dark for 1 hr, with vortexing every 15 minutes. Then 1.6 ml of 20% trichloroacetic acid was added by keeping the tubes in ice. Tubes were incubated for 10 minutes and then centrifuged at 1000 ´ g for 10 minutes. Supernatant was discarded. Pellet was washed three times with 1 ml ethanol-ethyl acetate to remove free reagent, allowing the sample to stand for 10 minutes before centrifugation and discarding the supernatant each time. The precipitate was re-dissolved in 2 ml of guanidine solution. Vortexed and the absorbance was read at 366 nm against 2.5 M HCl treated sample as blank. The carbonyl content was calculated from the molar absorption coefficient of 22000 M⁻¹cm⁻¹. The result was expressed as nmol per mg protein (Sahoo et al., 2005).

6. Assay of non-enzymatic small antioxidant molecules

A. Estimation of ascorbic acid content

The ascorbic acid content in samples was measured by the method of Mitsui and Ohta (1961). Phosphomolybdate is stochiometrically reduced by ascorbic acid in the presence of inorganic phosphorous to give a characteristic molybdenum blue colour.

A 10% (w/v) homogenate (CH) of testis was prepared in 5% (w/v) metaphosphoric acid, centrifuged at 1000 ´ g for 30 minutes at room temperature. The supernatant was taken for assay. 2 ml each of ice chilled 2% sodium molybdate and 0.15 N H2SO4 was taken in test tubes having glass stoppers. To this mixture 1ml of 1.5 mM Na2HPO4 and 1 ml of sample were added. The reaction was started by placing tubes in water bath maintained at 60 °C for 40 minutes. The tubes were then rapidly cooled in running water, centrifuged and the absorbance of the supernatant was recorded at 660 nm within 60 minutes against blank. Ascorbic acid was taken as standard a linear curve was obtained in the concentration range of 2.5-50 µg/ ml. Results were expressed as mg per g wet weight of tissue.

B. Estimation of Non-protein -SH and Protein –SH content

The estimation of Non-protein and protein thiol content in samples was done by the method of Sedlak and Lindsay (1968) and Wudarczyk et al. (1996), respectively. In brief, the mitochondrial and post-mitochondrial fractions were precipitated in ice-cold 5 % trichloroacetic acid containing 0.01 N HCl and then subjected to centrifugation at 1000 ´ g for 15 minutes. Pellet was used to determine protein-SH and supernatant was used to estimate non-protein –SH contents. Pellet was dissolved in 8 M guanidine HCl (Sample: Guanidine HCl=1:1). From this 0.1 ml of sample was taken and 1.35 ml of distilled water was added. To this 1.5 ml of 0.4 M Tris buffer pH 8.9 was added followed by 50 µl of 0.01 M DTNB (dissolved in methanol). The mixture was incubated for 15 minutes at room temperature. A reagent blank (without sample) and a sample blank (without DTNB) were prepared in a similar manner.

For non-protein-SH, 0.1 ml of supernatant was taken, to it 1.35 ml distilled water and 1.5 ml of 0.4 M Tris buffer, pH 8.9, was added followed by 50 µl of 0.01 M DTNB (prepared in methanol). The mixture was incubated for 15 minutes at room temperature. A reagent blank (without sample) was taken in a similar manner.

The absorbance of the supernatant was read at 412 nm. Reduced glutathione was taken as the standard, which gave a linear curve in the concentration range of (12.5-100) x 10⁻³ µmoles/0.1 ml (0.125-1 mM). Results were expressed as µmol per gram wet weight of tissue.
(Sahoo et al., 2007).

C. Estimation of total, oxidized and reduced glutathione level

The estimation of total, oxidized and reduced glutathione level was estimated according to the method of Tietze (1969) and Griffith (1980).

The mitochondrial and post-mitochondrial fractions were precipitated in ice cold 5% trichloroacetic acid containing 0.01N HCl and then subjected to centrifugation at 1000 × g for 15 minutes. Supernatant was used to determine total GSH equivalent, GSSG and GSH contents. Following reagents were used for the assay:(i) 50 mM phosphate buffer containing 7 mM EDTA, pH 7.4, (ii) 0.3 mM NADPH prepared in 50 mM phosphate buffer pH 7.4 containing 7 mM EDTA, (iii) 6 mM DTNB, (iv) GR (50 units/ml), (v) 2-vinyl pyridine and (vi) 8 µM GSH.

For the assay of total GSH equivalent, 1.4 ml of 0.3 mM NADPH was taken in a cuvette. To this 0.2 ml of 6 mM DTNB was added. 0.1 ml of the sample (suitably diluted) was added to it followed by 0.28 ml 50 mM phosphate buffer pH 7.4 containing 7 mM EDTA. Finally 20 µl of GR (50 units/ml) was added to the cuvette and the rise in absorbance was noted at 1-minute interval till 6 minutes. A reagent blank (without sample) was taken in a similar manner. For GSSG assay, the GSH present in the solution is readily derivatized by adding 2 µl of neat 2-vinyl pyridine for 100 µl solution, mixing it vigorously for about 1 minute. GSH is fully derivatized after 30 minutes at 25 °C. The assay was performed in a similar manner as that of total GSH equivalent. The absorbance was recorded at 412 nm. The DAbs$^{6}\text{nm}$ of the sample was noted.

T GSH equivalent = 2 GSSG + GSH

Reduced glutathione was taken as the standard, which gave a linear curve in the concentration range of 2-32 µM (0.2-3.2 nmol/0.1 ml). The results were expressed as nmol per gram wet weight of tissue (Sahoo et al., 2007).

7. Assay of antioxidant enzyme activity

The mitochondrial and post-mitochondrial fractions were obtained by sub cellular fractionation as mentioned in section 3.4.2. Mitochondrial fraction was freeze thawed three times and then centrifuged at 10,000 × g for 5 minutes. One ml of freeze-thawed mitochondrial supernatant was passed through a 5 ml sephadex G-25 column. Similarly 1 ml of the post-mitochondrial supernatant was also passed through a 5 ml sephadex G-25 column. The eluted mitochondrial and post-mitochondrial fractions thus obtained were used for the assay of superoxide dismutase, glutathione peroxidase, glutathione reductase and glutathione S-transferase. For catalase assay the PMF was used directly without passing through sephadex G-25 column.

A. Assay of superoxide dismutase (SOD) activity

SOD activity was determined according to the method of Das et al. (2000) using a negative assay system for detection of SOD by the photo reduction of riboflavin. In this method, superoxide radicals generated are allowed to react with hydroxylamine hydrochloride to produce nitrite, which in turn reacts with sulphanilic acid to produce diazonium compound that forms red azo compound by reacting with naphthalamine. The azo compound has absorption maxima at 543 nm. Superoxide dismutase scavenges superoxide radicals produced by photo reduction of riboflavin. The activity of superoxide dismutase is inversely proportional to the formation of red azo compound.

In brief, the 1.4 ml of cocktail was prepared by adding following reagents: (i) 1.11 ml of 50 mM phosphate buffer pH 7.4, (ii) 0.075 ml of 20 mM L-methionine, (iii) 0.04 ml of 1% (v/v) triton X-100, (iv) 0.075 ml of 10 mM HAC, (v) 0.1 ml of 78.125 µM EDTA. To 1.4 ml of this prepared cocktail 0.1 ml of sample (containing 50-100 µg protein) and 0.1 ml of 40 µM riboflavin were added. A blank prepared without riboflavin and a control without sample was run with each set. The tubes were exposed to two 20 W fluorescent lamps fitted parallel to each other in an aluminum foil coated wooden chamber for 10 minutes. After incubation period 1 ml of Griess reagent was added. The Greiss Reagent was prepared by adding equal volumes of 0.1% N-(1-Naphthylethylene diamine) and 1% sulfanilamide in 5% orthophosphoric acid.

The absorbance of tubes was read at 543 nm against the blank. Enzyme activity was expressed as units/ mg protein. One unit of enzyme activity is calculated from the value (Vc/V) - 1 (Where Vc is the absorbance of the control and V is the absorbance of the sample) (Sahoo et al., 2005; 2008a; 2008b).

B. Assay of catalase (CAT) activity

Catalase activity was measured in the PMF by the method of Aebi (1974). To 0.5 ml of sample 5 µl of absolute ethanol was added and incubated for 30 minutes in ice bath. To 0.45 ml of this aliquot 50 µl of 10 % triton x-100 (1% final concentration) was added (Cohen et al., 1970). Catalase forms an inactive complex with H$_2$O$_2$, which is called Complex II. Ethanol reverses the inactivation, which occurs during the time interval from the preparation of tissue homogenate to the subsequent assay of catalase activity. Triton x-100
increases observable catalase levels, as the activation is complete. The enzyme reaction was started by adding 0.1 ml of sample (0.4-0.5 mg protein) to 2.9 ml of 50 mM phosphate buffer, pH 7.0 containing 12 mM H$_2$O$_2$. The absorbance was recorded at 240 nm immediately at 15 seconds interval till 2 minutes. A blank was taken without sample. Absorbance was read at 240 nm. ?A/ min was calculated. Calculation was done by taking the extinction coefficient of H$_2$O$_2$ of 43.6 M$^{-1}$ cm$^{-1}$ (Sahoo et al., 2005). Activity was expressed as nmol NADPH oxidized per min per mg protein considering NADPH extinction coefficient as 6.22 mM$^{-1}$ cm$^{-1}$ (Sahoo et al., 2008a; 2008b).

C. Assay of glutathione peroxidase (GPx) activity

Glutathione peroxidase activity was assayed in sephadex G-25 column passed samples according to the method of Paglia and Valentine (1967). Following reagents were used for the assay: (i) 50 mM phosphate buffer, pH 7.4, (ii) 30 mM GSH, (iii) 4.5 mM KCN, (iv) 30 mM NaN$_3$, (v) 4.5 mM NADPH, (vi) GR (10 units/ml), (vii) 7.5 mM cumene hydroperoxide and (viii) 7.5 mM tert-ButOOH. All the reagents were made in 50 mM phosphate buffer, pH 7.4. A cocktail was prepared with first four reagents in the ratio 23: 1:1:1:1:1:: phosphate buffer: GSH: KCN: NaN$_3$. To 2.6 ml cocktail taken in a cuvette, 0.1 ml each of NADPH, GR and sample (0.1-0.15 mg protein) were added at interval of one minute for stabilization. Cumene or tert-butyl hydroperoxide was added and fall in the absorbance was recorded at 2 minutes interval for 6 minutes at 340 nm. A sample blank was run in parallel. The two isozymes of GPx, selenium-dependent and non-selenium-dependent were estimated by using cumene and tert-butyl hydroperoxides as substrates. Cumene hydroperoxide was used as substrate to measure the total (both selenium-dependent and non-selenium-dependent) GPx activity, whereas tert-butyl hydroperoxide was used as the substrate for selenium-dependent GPx only. Activity was expressed as nmol NADPH oxidized/min/mg protein considering NADPH extinction coefficient as 6.22 mM$^{-1}$ cm$^{-1}$ (Sahoo et al., 2008a; 2008b; 2008c; Sahoo, 2012; Sahoo and Roy, 2012).

D. Assay of glutathione reductase (GR) activity

The sephadex-column passed samples were used for the assay of GR by the method of Massey and Williams (1965) in which the rate of GSSG conversion to GSH was measured by following the decrease in absorbance of NADPH at 340 nm. In brief, to 2.7 ml of 50 mM phosphate buffer pH 7.4, 0.1 ml each of 120 mM GSSG and 4.5 mM NADPH were added. After one minute, reaction was initiated by adding 0.1 ml sample (containing 0.05-0.1 mg protein) to it. The absorbance was recorded at 2 minutes interval till 6 minutes at 340 nm. A parallel sample blank was also run. Enzyme activity was expressed as nmol NADPH oxidized per min per mg protein considering NADPH extinction coefficient as 6.22 mM$^{-1}$ cm$^{-1}$ (Sahoo et al., 2008a; 2008b).

E. Assay of glucose-6-phosphate dehydrogenase (G6PD) activity

The post-mitochondrial fractions were used for the assay of G6PD by the method of Lee (1982) by monitoring increase in absorbance at 340 nm due to reduction of NADP to NADPH. The assay relies upon the G6PD catalyzed oxidation of glucose 6-phosphate to gluconate 6-phosphate using the coenzyme NADP that is reduced to NADPH.

In brief, the reaction was initiated by adding 0.1 ml sample (containing 0.25-0.5 mg protein) to 1.9 ml of assay mixture containing following reagents: (i) 1752 µl of 0.1 M Tris HCl buffer pH 7.6, (ii) 134 µl of 0.1 M magnesium chloride, (iii) 6 µl of glucose-6-phosphate (10 mg/ml) and (iv) 8 µl of NADP (10 mg/ml). The change in absorbance was monitored at 20 seconds interval till 2 minutes at 340 nm. A parallel sample blank was also run. G6PD activity was expressed as nmol NADP reduced per min per mg protein considering extinction coefficient of NADPH as 6.22 mM$^{-1}$ cm$^{-1}$ (Sahoo et al., 2007).

F. Assay of glutathione S-transferase (GST) activity

The column-passed samples were used for the assay of GST by the method of Habig et al. (1974). In brief, to 2.7 ml of 0.1 M phosphate buffer pH 6.5, 0.1 ml each of 30 mM GSH and 0.1 ml sample (containing 12.5-25 µg protein) were added to make the reaction mixture and the reaction was initiated by adding 0.1 ml of 15 mM CDNB (dissolved in ethanol) to it. Absorbance was recorded at 340 nm at one-minute interval till 6 minutes. Enzyme activity was expressed as nmol CDNB conjugate formed/min/mg protein considering extinction coefficient of CDNB as 9.6 mM$^{-1}$ cm$^{-1}$.

8. Germ cell isolation for assaying germ cell antioxidant defence parameters

Testes were immediately dissected out of rats without damaging the capsule and kept in 5 ml of cell isolating media consisting of 50 mM phosphate buffered saline (PBS), pH 7.4 with 5% fetal bovine serum (FBS) and collagenase Type IV (0.5 mg/ml) at room temperature (25°C). Testes were decapsulated and minced into fine pieces and the mixture was flushed with a 10 ml pipette for about 15-20 times, at a moderate speed. The cell suspension was filtered through a fine nylon
cloth having mess size of 100 µm and then filtered through glass wool. The cell suspension was centrifuged at 200 x g for 10 min at room temperature. The supernatant was discarded and the cell pellet was suspended in cell isolating media (Figure 2). Germ cell viability was assessed by trypan blue exclusion test and viability of cells was found to be 94 - 96 %. The germ cells were frozen at -80°C in cell isolating media, pH 7.4 containing 0.1 M EDTA as described earlier by Bauche et al. (1994) for assay of antioxidant enzymes. For enzyme assay frozen germ cells were thawed and sonicated for 3 x 10 sec at 4°C using a micro-ultrasonic cell disrupter. Sonicated samples were centrifuged at 1,000 x g for 10 min at 4°C to obtain supernatant and antioxidant enzyme assays could be done in supernatants (Sahoo et al., 2006).

9. Histology analysis

Reagents & Compositions:

(i) Formol sublimate: 37-41% Formaldehyde solution: 100 ml
(ii) Alcohol (C2H5OH) for dehydration: Absolute, 90%, 70%, 50%, 30%
(iii) Xylene as clearing agent
(iv) Xylene : Paraffin for proper infiltration: 1:1
(v) Pure paraffin of melting temperature: 58° C to 62° C
(vi) Egg albumin
(vii) Iodine alcohol
   Iodine crystal: 5g
   70% alcohol: 100ml
(viii) Harris's Hematoxylin:
   Hematoxylin crystals: 1 gm
   Absolute alcohol: 50 ml
(ix) Ammonium or Potassium aluminium sulfate: 50 gm
   (Ammonium or Potassium Alum)
(x) Distilled water: 1000 ml
(xi) Mercuric oxide (red): 2.5 gm
(xii) Working eosin solution
   Eosin Y 1% stock solution prepared in 90% ethanol
   Glacial acetic acid: few drops
(xiii) 5% Hypo solution
   Distilled water: 100 ml
   Sodium thiouphosphate: 5g
   (xiv) Lugole's solution
   Iodine crystal: 1 g
   Potassium iodide: 2 g
   Distilled water: 100 mls

In brief, testis was fixed in formol fixative. Following 2 hr of fixation, two poles of the testis were cut and following 4 h of fixation, each testis was cut into two halves with a fine blade and left for 24 hr at 4° C for proper fixation. Following fixation, testis was dehydrated in graded doses of alcohol as 30%@50%@70% @ 90% for 30min in each fraction@ 100% for 1hr using two changes. After dehydration cleaning of alcohol from the tissue was done using two changes of xylene for 30min each. Proper paraffin infiltration was done by incubating testis in different mixtures of xylene and paraffin (1:1, 1:2, 1:4 etc) first and then in two changes of pure melted paraffin at 60° C hot oven for 1h each. With the help of metal 'L-Mold' paraffin block was prepared using melted Bees wax-paraffin mixture. Section was cut using a simple microtome at 5m. Sections were spread on albumin-coated slides and dried on a slide warmer (45° C). The slides were deparaffinized, rehydrated in a series of alcohol bath in descending order as 100% @ 90% @ 70% @ 50% @30% and H2O for 10 min in each step. Then, slides were then placed in Lugol's solution for 5 min. and then washed with water followed by placing slides in 5% sodium thiosulphate solution to remove iodine followed by hematoxylin staining for 2 minutes. Then the slides were washed carefully in slow running tap water for 5-10 minutes. Sections were dehydrated in a series of alcohol bath in ascending order as 30% @ 50% @ 70% and 90% for 5 min for each step and after that followed by counter staining with eosin for 15 minutes with dehydration in absolute alcohol using two changes. The slides were dipped in xylene with a second change for 5 minutes. Finally, sections were mounted in DPX, covered with glass coverslip and observed under microscope (Sahoo et al., 2008a).

10. Sperm analyses

Assay of sperm anomalies

Viability of epidydimal spermatozoa was assessed by trypan blue exclusion assay (Alvarez and Story, 1984) with little modification. Anomalies in sperm head structure was assayed by Eosin Y staining as described below (Wyrobek and Bruce, 1975).

A. Preparation of spermatozoa

Spermatozoa were collected from caudae epididymes of mature rats by flushing with 10 ml rat sperm suspension medium containing 140 mM NaCl, 0.3 mM KCl, 0.8 mM Na2HPO4, 0.2 mM KH2PO4 and 1.5 mM D-glucose (pH adjusted to 7.3 by adding 0.1 N NaOH), centrifuged at 100g for 2 minutes, and resuspended in 10 ml of fresh medium.

B. Trypan blue exclusion assay

A fraction of the sperm suspensions (100 µl) was mixed with equal volume of 1% Trypan blue in the same medium. The resulting suspension was loaded into a haemocytometer slide and the number of sperms was counted in WBC counting chamber. At this concentration of Trypan blue (0.5%), the dye was
completely excluded by intact sperms, which appeared bright and colourless; but was taken up by damaged sperms, which showed blue heads. The sperm number was expressed per ml suspension.

C. Assessment of anomalous sperm head

An aliquot of each sperm suspension (original) was mixed with 1% Eosin-Y in a proportion of 10:1 (v/v) and 30 minutes later, smears were made, allowed to dry in air, and sperm heads were examined at 400-fold magnification following the classification of Wyrobek and Bruce (1975). For each suspension, 200 sperms were examined and 5 such suspensions prepared from 5 matured rats were included in each group (1000 sperms per group) (Sahoo et al., 2007).

References

Illustrations
Illustration 1

Steps for separation of mitochondrial and postmitochondrial fractions

1. **Testis homogenate (20%, w/v)**
2. Filter through four-layered cheesecloth
3. **Crude homogenate (CH)**
4. Centrifuge at 600 x g for 10 minutes at 4°C
   - Pellet (cell debris, nuclear fraction NF)
   - **Supernatant**
5. Centrifuge at 10,000 x g for 20 minutes at 4°C
   - Pellet (Mitochondrial fraction, MF)
   - **Supernatant (Post-mitochondrial fraction, PMF)**
6. Wash three times
   - **Mitochondrial fraction (MF)**
Illustration 2

Steps for isolation of germ cells from rat testis

Rat

→

Testis

→

Kept at room temperature in cell isolating media containing 5% FBS and collagenase type IV (0.5mg/ml) in PBS

→

Decapsulated, minced and thoroughly agitated

→

Cell suspension

→

Filtered through fine nylon cloth (100 μm) and glass wool

→

Centrifuged at 200 x g for 10 min at room temperature (25°C)

→

Germ cells (pellet)

→

Suspended in 5% FBS

→

Trypan blue exclusion test (viability 94-96%)
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