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A critical period in lifespan of male rats coincides with increased oxidative stress

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ABSTRACT

The oxidative stress theory of aging has provided the best possible explanation for the processes which accompany aging and has received much support, however, in the last few years there have been questions regarding the validity of this theory. We have conducted experiments to determine an array of oxidative stress parameters in blood of male rats at various intervals (1, 4, 8, 12, 18 and 24 months) during their entire lifespan. Established protocols were used to measure plasma antioxidant capacity, erythrocyte plasma membrane redox system (PMRS), lipid and protein oxidation in erythrocytes and plasma, and erythrocyte glutathione (GSH). Our results on the total plasma antioxidant potential, PMRS in erythrocytes, protein and lipid peroxidation, and intracellular reduced GSH provide evidence that oxidative stress is minimal till approximately one-third of the total lifespan (8 months) and there is a spurt in oxidative stress between 8 and 12 months. The identification of a period (corresponding to 8–12 months) in the lifespan of rats coinciding with an spurt in oxidative stress is an interesting finding. No such report is available in humans or in any other model systems during aging.

1. Introduction

Despite considerable research effort, aging continues to be the most intriguing biological phenomenon. Although till date no genes have been identified which cause aging, certain genes have been shown to control lifespan through modulation of signaling pathways (Bartke, 2008; Finch & Ruvkun, 2001). Denham Harman's free radical theory of aging in 1956 provided a convincing argument for the accumulation of oxidative damage being responsible for the progressive and functional deterioration with age (Harman, 1956). This theory which later became the 'Oxidative Stress theory of aging' was the best possible explanation for the processes which accompany aging and received continued support (Barja, Cadenas, Rojas, Lopez-Torres, & Perez-Campo, 1994; Beckman & Ames, 1998; Droge & Schipper, 2007) however not all available data mainly concerning lifespan determinations and effect of antioxidant supplementation could validate this theory (Buffenstein, Edrey, Yang, & Mele, 2008; Perez et al., 2009). The oxidative stress theory now does not seem invincible and remains embattled. It is thus imperative that further evidences are generated to verify the usefulness of this theory in the light of current knowledge.

Reactive oxygen species (ROS) production is inseparable from aerobic metabolism; however living systems have evolved to limit the deleterious effect of ROS through several enzymatic and non enzymatic antioxidant systems. There is considerable evidence for the oxidative damage to macromolecules under normal physiological conditions (Gil et al., 2006; Inal, Kanbak, & Sunal, 2001; Sohal & Weindruch, 1996) suggesting that antioxidant repair mechanisms cannot completely avoid ROS mediated oxidative insult (Gil del Valle, 2011; Lenaj, 2001). The shift of redox balance toward oxidative state during aging has been linked to the development of state of chronic inflammation (Hensley, Robinson, Gabbita, Salsmasn, & Floy, 2000; Stadtman, 2004) and predisposes the development of various clinical conditions (Kuor-o, 2001).

Age related decline in plasma antioxidant capacity has been reported in humans (Rizvi & Maurya, 2007), this alteration has been shown to correlate with markers of lipid and protein oxidative stress both in plasma and erythrocytes (Pandey & Rizvi, 2010). In earlier reports we have shown the upregulation of erythrocyte PMRS, which is involved in transferring of reducing equivalents from inside the cell to extracellular acceptors, as a function of human age (Rizvi, Jha, & Maurya, 2006), the increased activity of PMRS has been related to regeneration of ascorbate in the plasma. It has been hypothesized that the increased activity of erythrocyte PMRS is a protective mechanism for mitigating the increased oxidative stress (Rizvi, Pandey, Jha, & Maurya, 2009), a link between erythrocyte PMRS activity and lifespan has also been hypothesized (Rizvi, Kumar, Chakravarti, & Singh, 2011).

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The study on aging related biochemical parameters in humans is dependent on many variables such as genetic factors, temperature, activity, and nutrition. Thus the conclusions drawn from human based studies may not be very relevant when validation of oxidative stress theory of aging is at stake. The present study was undertaken to determine markers of oxidative stress of plasma and erythrocytes in male rats, kept in controlled laboratory conditions, at different ages ranging from 1 month to 24 months. We report the age dependent changes in plasma total antioxidant capacity, PMRS, protein carbonyl, advanced oxidation protein products (AOPP), reduced GSH, and lipid peroxidation product malondialdehyde (MDA).

2. Materials and methods

2.1. Chemicals

(2,4,6-Tri(2-pyridyl)-s-triazine (TPTZ). 4,7-Diphenyl-1,10-phenanthroline disulfonic acid disodium salt (DPI), reduced GSH, 2,4-dinitrophenylhydrazine (DNPH), and dithiobis nitro benzoic acid (DTNB) was procured from Sigma Aldrich, India. All other chemicals were of analytical grade available from Merck, India and HIMEDIA Labs, India.

2.2. Animal model and study protocol

The experiment was carried out with 48 male Wistar rats of 1, 4, 8, 12, 18 and 24 months old, containing eight animals in each age group ($n = 8$). They were housed in a temperature controlled room (25 ± 5 °C) with 12-h light–dark cycles. All rats were fed with a normal laboratory diet of nutrient rich pellets containing total energy as fat, protein and carbohydrates, and had free access to drinking water.

2.3. Collection of blood, isolation of red blood cells and plasma

During experimental period, rats were sacrificed under light anesthesia. Blood samples were collected by cardiac puncture into 10 unit/ml heparin rinsed anticoagulant syringes, and then red blood cells were pelleted by centrifugation at $800 \times g$ for 10 min at 4 °C. After the removal of plasma (immediately frozen at −80 °C until use for biochemical assays), buffy coat, and the upper 15% of packed red blood cells (PRBCs), the RBCs were washed twice with cold phosphate buffered saline (PBS) (0.9% NaCl and 10 mmol L^{-1} Na_2HPO_4; pH 7.4) and then used for experiment. All protocols for experiments were approved by the Animal Care and Ethics Committee of University of Allahabad.

2.4. Measurement of biochemical parameters in blood

Lipid profile, SGOT, SGPT, creatinine, and urea were measured using reagent kits from Erba Diagnostics, Mannheim, Germany. Blood glucose values were determined using an Accu-Chek Active Glucometer (Roche Diagnostics, Mannheim, Germany).

2.5. Erythrocyte membrane isolation

Erythrocyte 'ghosts' from leukocyte-free RBCs were prepared by following the method of Dodge, Mitchell, and Hanahan (1963), with slight modifications. Briefly, washed and packed erythrocytes were lysed by adding 10 volume of 5 mM phosphate buffer pH 7.4 (at 4 °C). After leaving on ice for 30 min, the erythrocyte membranes were packed by centrifugation at $20{,}000 \times g$ for 10 min at 4 °C and the hemoglobin-containing supernatant was removed. The erythrocyte membranes were then washed three times by suspending in fresh buffer followed by centrifugation under the same conditions. Finally, the membranes were suspended in hypotonic 5 mM buffer followed by centrifugation under the same conditions and then resuspended in 5 mM phosphate buffer pH 7.4. Protein was estimated in red cell membrane preparation following the method of Lowry, Rosebrough, Farr, and Randall (1951), using bovine serum albumin as a standard.

2.6. Measurement of total antioxidant activity by FRAP

The total antioxidant potential of the plasma was determined using a modification of the ferric reducing ability of plasma (FRAP) assay reported by Benzie and Strain (1996). FRAP reagent was prepared from 300 mmol L^{-1} acetate buffer, pH 3.6, 20 mmol L^{-1} ferric chloride and 10 mmol L^{-1} 2,4,6-tripyridyl-s-triazine made up in 40 mmol L^{-1} hydrochloric acid. All three solutions were mixed together in the ratio 10:1:1 (v/v/v) respectively, 3 ml of FRAP reagent was mixed with 100 μl of plasma and the contents were mixed thoroughly. The absorbance was read at 593 nm at 30 s intervals for 4 min. Aqueous solution of known Fe (II) concentration in the range of 100–1000 μmol L^{-1} was used for calibration. Using the regression equation the FRAP values (μmol Fe (II)/L) of the plasma was calculated.

2.7. Measurement of erythrocyte PMRS activity

The activity of the erythrocyte PMRS was measured by the reduction of ferricyanide as described earlier (Rizvi et al., 2006). Briefly, packed RBC (0.2 ml) were suspended in PBS containing 5 mM glucose and 1 mM freshly prepared potassium ferricyanide to a final volume of 2.0 ml. The suspensions were incubated for 30 min at 37 °C and then centrifuged at $800 \times g$ at 4 °C. The supernatant collected was assayed for ferrocyanide content using 4,7-diphenyl-1,10-phenanthroline disulfonic acid disodium salt, absorption was recorded at 535 nm ($\epsilon = 20{,}500\ M^{-1}\ cm^{-1}$). The results are expressed in μmol ferrocyanide/ml PRBC/30 min.

2.8. Determination of membrane and plasma protein carbonyls

Erythrocyte membrane protein carbonyls were measured according to procedure of Levine et al. (1990). Erythrocyte membrane samples (0.2 mL) in PBS/0.4 mL plasma were taken in 2 tubes as test and control samples. A total of 4.0 mL of 10 mmol L^{-1} 2,4-DNPH, prepared in 2 mmol L^{-1} HCl, was added to the test sample, and 4.0 mL of 2 mmol L^{-1} HCl alone was added to the control sample. The contents were mixed thoroughly and incubated for 1 h in the dark at 37 °C. The tubes were shaken intermittently every 10 min to facilitate reactions with proteins. After shaking, 20% trichloroacetic acid (TCA) (w/v) was added to both tubes, and the mixture was left on ice for 10 min. The tubes were then centrifuged at $850 \times g$ for 20 min to obtain protein pellets. The supernatant was carefully aspirated and discarded. The protein pellets were washed 3 times with ethanol–ethyl acetate (1:1, v/v) solution to remove unreacted DNPH and lipid remnants. Finally, protein pellets were dissolved in 6 mmol L^{-1} guanidine hydrochloride and incubated for 10 min at 37 °C. The insoluble materials were removed by centrifugation. Carbonyl content was determined by taking the spectra of the supernatant at 370 nm. Each sample was read against the blank. The carbonyl content was calculated using an absorption coefficient of 22000 mol L^{-1} cm^{-1}, and data were expressed in nmol mg^{-1} protein.

2.9. Assay of AOPP

Determination of AOPP levels was performed by modification of the method of Witko-Sarsat et al. (1996). 2 ml of plasma was diluted with 1:5 in PBS, 0.1 ml of 1.16 M potassium iodide was then added to each tube, followed by 0.2 ml acetic acid after 2 min. The