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Comparison of Glutathione Peroxidase Activity and Free Radicals Production in the Lungs and the Brain of Rats During Graded Hyperoxia

Ismaeel Bin-Jaliah

The purpose of this study was to examine the behavior of glutathione peroxidase (GPx) activities and Free Radicals (FR) production in the brain and the lung during graded hyperoxia exposure. Twenty-four adult male rats, matched with age and body weigh, were randomly assigned to four groups. The first group served as control and the second, third and fourth were exposed to hyperoxia for 24, 48 and 72 h, respectively. Following the exposure period for each group animals were sacrificed and both lungs and brain tissues were homogenized for GPx and FR determinations. GPx activity was determined by Randox protocol (Randox, UK) and FR was determined using dROM method (H and D, Italy). Results showed that mean (±SD) GPx activity in the lungs increased from the baseline control of 12898.33±6034.77 to 20083.62±2734 (UL^{-1}) during hyperoxia exposure for 24; then dropped to 5467.77±1159.53 and 8271.80±1347.67 ($U\ L^{-1}$) during hyperoxia exposure for 48 and 72 h, respectively. Whereas mean (±SD) GPx activity in the brain increased from the baseline control of 5467.80±2852.65 to 13841.72±1245.67 and 14594.82±6711.44 ($U\ L^{-1}$), during hyperoxia exposure for 24 and 48 h, respectively; then dropped to 4346.17±343.34 ($U\ L^{-1}$), during 72 h exposure. The sustained increased in GPx up-to 48 hr in the brain provided evidence of delayed protection against ROS. The average (±SD) FR production in the lung increased from the baseline control of 176.67±33.79 to 274.33±33.37, 260.00±62.54 and 320.00±114.91 (UL^{-1}) during hyperoxia exposure for 24, 48 and 72 h, respectively. The average (±SD) FR production in the brain increased from the baseline control of 73.33±20.18 to 132.17±21.77 during hyperoxia exposure for 24 h and then dropped to 94.33±14.56 and 65.33±21.12, during 48 and 72 h, respectively. Tukey-Kramer multiple comparisons between the lung and the brain showed that the lungs had higher rate of FR formation at all levels of hyperoxia exposures, which suggest more mechanisms that had contributed to FR formation in pnueumocyte, as compared with neurocyte. Based on the results of the present study antioxidant supplements are recommended for traumatic brain injury and hypoxemia lung injury patients subjected to oxygen therapy.

Key words: Brain, GPx, lung, hyperoxia, ROS

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INTRODUCTION

Oxygen toxicity had attracted the attention in physiology since the early work by Bean (1945) and Comroe *et al*. (1945). Hyperoxia inducts reactive oxygen species (Heberlein *et al*., 2000; Hitka *et al*., 2003; Haffor and Al-Johany, 2005) which alter cellular components and thus impair various ionic conductance's that regulate cell excitability and exchangeability (Colton and Colton, 1986; Upham *et al*., 1997; Bickford *et al*., 1999). Studies of hyperoxia confirmed that ROS are reported to target certain neurotransmitter and neuromodulator systems and thus to alter chemical synaptic function (Zhang *et al*., 1995; Bitterman and Bitterman, 1998; Dean and Mulkey, 2000; Mulkey *et al*., 2003). Following exposure to hyperoxia, the lungs show morphologic changes that are similar to pulmonary inflammation, atelectasis and oedema formation, leading to irreversible loss of respiratory function. Furthermore, lung inflammation is associated with infiltration of circulating neutrophils (Crapo, 1986; Jankov *et al*., 2003; Jafari *et al*., 2004) with potential source for ROS formation.

Glutathione Peroxidase (GPx), is a free radical scavenger, initially reported that neutralized the highly toxic hydroxyl radical (Paglia and Valentine, 1967), an observation which has been confirmed by several authors (Allen and Balin, 2003; Pollack *et al*., 2005; Al-Johany and Haffor, 2007). Previous studies demonstrated that GPx is critical antioxidant in the peroxidase system of cytochrome-C, quinones and ascrobate that prevented mitochondrial swelling which are accompanied by the production of hydrogen peroxide and malonaldhyde and intermediate of free radicals initiated lipid peroxidation (Hunter *et al*., 1964; Lesnefsky *et al*., 2001; Haffor *et al*., 2002; Haffor, 2004; Haffor and Al-Johang, 2005; Haffor and Alhazza, 2007).

The question that arises is how fast it takes for ROS intermediates to react with membrane lipids and start a series of pathologic antioxidants response in the brain, as compared with the lung tissues. The clinical relevance of this issue relates to the controversial question of whether hyperoxic exposure intervals contribute to irreversible deleterious in the CNS control of pulmonary vascular effects or an earlier direct pneumocyte phagocyte infiltration mechanism. The purpose of this study was to examine the changes in glutathione peroxidase activity (GPx) and free radicals (FR) production in the brain and the lung tissues during graded hyperoxia exposure for 24, 48 and 72 h. In particular, we emphasize the use of hyperoxia under normal baric O_2 as in intensive care model for studying the impact on the cellular mechanisms by which acute oxidative stress (ROS) can potentially alters neuronal and pneumocyte physiological activities. Some of this work has been published previously in abstract form (Bin-Jaliah, 2007).

MATERIALS AND METHODS

Experimental design and hyperoxia exposure: The experiments were conducted during the spring of 2007 in the College of Science, King Saud University, as part of collaborative initiative with the aim to establish a High Altitude and Stress Physiology Laboratory (HASPL). Twenty-four adult wister albino male rats, *Rattus norvigicus*, matched with age and body weigh were randomly assigned to four groups, six animals each. The first group served as control and the second, third and fourth were exposed to hyperoxia for 24, 48 and 72 h, respectively. Animals of the experimental groups were placed in a closed box that has an inlet flow which was connected to 100% O_2 tank, medical grade, on which a regulator was connected to maintain flow at 5 L min^{-1} (LPM). The out flow of the regulator passed through a humidifier in order to saturate the inspired air with H_2O. The outlet ventilation rate of the box was adjusted at 5 LPM to ensure that the concentration of oxygen in the box remains equal to 100% O_2 and maintain normal flow and normal barometric pressure at 767 mmHg. The temperature inside the box was adjusted at room temperature (22-24°C). Animals of the control and experimental groups were sacrificed and the lungs and brain were isolated and homogenized immediately in 0.9 saline solutions (4:1 ratio).

Glutathione peroxidase: Samples from all groups were used for the determination of GPx activities using Randox protocol (Randox, England). This method is based on the detoxification of hydrogen peroxide by the oxidation of reduced glutathione according to the following reaction:

$$2GSH + H_2O_2 \xrightarrow{GPx} GSSG + 2\,H_2O.$$

Free radical determination: Free radicals production was measured, using the d-ROMs-2 test kits (Health and Diagnostic, Italy) according to the manufacturer's instructions. The test measures the levels of hydroperoxides (R-OOH) which are generated by peroxidation of biological compounds; lipid, amino acids, nucleic acids. This test is based on the principle of the ability of hydrogen peroxides to generate free radicals after reacting with some transitional metals (Fe_2^+/Fe_3^+), according to Fenton's Reaction as follows:

$$H_2O_2 + Fe^{++} = *OH + OH^- + Fe^{++}$$

Thus, the hydrogen peroxides of biological sample (whole blood) generate free radicals (alcoxy and peroxyl radicals) after exposure to a transitional metal (Fe^{++}/Fe^{+++}). When a correctly buffered chromogen substance (N, N-diethyl-phenylenediamine) lead to the reduction of hydrogen peroxides which in turns colored as radical cation. Color intensity was read using spectrophotometer with peak absorbance of 505 nm. In the d-ROMs test, results were expressed in CARR units (CARR U). One CARR U relates to 0.08 mg H_2O_2/100 mL.

Statistical analysis: Mean group differences for the dependent variables; glutathione peroxidase (GPx) and free radicals (FR) were evaluated using one-way analysis of variance (ANOVA) to reveal the main effect of each group on the dependent variables. Tukey-Kramer multiple comparisons were used to compare differences between each means pairs.

RESULTS

The mean final body weights (±SD) of the four groups; control, hyperoxia-24 h, hyperoxia-48 h and hyperoxia-72 h; at the end of the experiment were 185.67±6.41, 188.66±11.39, 192.33±10.09 and 186.83±12.35 g, respectively. Results of paired t-test indicated that values were not significantly ($p>0.05$) different from body weights prior to the experiment.

Exposure to hyperoxia resulted in increasing mean (±SD) GPx activity from the baseline control of 12898.33±6034.77 to 20083.62±2734 (U L^{-1}), during hyperoxia exposure for 24; then dropped to 5467.77±1159.53 and 8271.80±1347.67 (U L^{-1}), during hyperoxia exposure for 48 and 72 h, respectively. The average (±SD) FR production in the lung increased from the baseline control of 176.67±33.79 to 274.33±33.37, 260.00±62.54 and 320.00±114.91 (CARR) during hyperoxia exposure for 24, 48 and 72 h, respectively (Table 1).

GPx activity in the brain increased, from the mean baseline control of 5467.80±2852.65 to 13841.72±1245.67 and 14594.82±6711.44 (U L^{-1}), during hyperoxia exposure for 24 and 48 h, respectively; then dropped to 4346.17+343.34 (U L^{-1}), during 72 h exposure. The mean (±SD) FR production in the brain increased from the baseline control of 73.33±20.18 to 132.17±21.77 during hyperoxia exposure for 24 h and then dropped to 94.33±14.56 and 65.33±21.12, during 48 and 72 h, respectively (Table 2).

Results of one-way (ANOVA) analysis of variances (Table 3) showed significant ($p<0.05$) differences among groups' means in both lungs and brain for glutathione peroxidase (GPx) activities and free radicals (FR) productions. Post-hoc Tukey-Kramer multiple comparisons procedures (Table 4) were conducted to simultaneously examine comparisons between all possible pairs of group means. When hyperoxia exposure administered for 24 h, it elevated GPx activity significantly ($p<0.05$), then dropped significantly ($p<0.05$) at 48 h in the lungs and remained lower following 72 h exposure but the difference was not statistically significantly ($p>0.05$) lower than control group. In the brain, when hyperoxia was administered for 24 h, it elevated GPx significantly ($p<0.05$) and remained higher significantly ($p<0.05$) following hyperoxia exposure for 48 h, then dropped to control value following 72 h of hyperoxia exposure. Despite the observed progressive rise in FR production in the lungs, with increasing hyperoxia exposure but the difference was significantly ($p<0.05$) higher following hyperoxia exposure for 72 h only. In the brain, when hyperoxia administered for 24 h FR production increased significantly ($p<0.05$), then dropped following exposure for 48 and 72 h, but the difference was not statistically significant ($p>0.05$), as compared with control group. Figure 1a and b displays and summarizes the behavioral mean changes for GPX activity and FR production in the lungs and the brain during control and hyperoxia exposure for 24, 48 and 72 h.

Table 1: Glutathione peroxidase activity (U L^{-1}) and free radicals production (Carr) in the lungs

	Glutathione peroxidase activity				Free radicals production			
Animal	Control	HP-24	HP-48	HP-72	Control	HP-24	HP-48	HP-72
1	5888.40	19768.20	5047.20	8411.21	204.00	252.00	260.00	252.00
2	8412.00	22712.40	5888.40	8411.13	136.00	240.00	196.00	172.00
3	21030.11	20399.11	7570.81	6729.46	152.00	316.00	212.00	308.00
4	9253.20	21871.00	5047.20	9253.71	152.00	284.00	336.00	492.00
5	18506.40	14931.30	4206.41	6729.72	200.00	245.00	220.00	412.00
6	14300.04	20819.71	5047.13	10094.41	216.00	309.00	336.00	284.00
Average	12898.36	20083.62	5467.86	8271.61	176.67	274.33	260.00	320.00
SD	6034.80	2734.68	1159.43	1347.72	33.79	33.37	62.53	114.91

Table 2: Glutathione peroxidase activity (U L^{-1}) and free radicals production (Carr) in the brain

	Glutathione peroxidase activity				Free radicals production			
Animal	Control	24-HP	HP-48	HP-72	Control	HP-24	HP-48	HP-72
1	7570.80	14931.29	17665.32	4306.91	84.00	108.00	76.00	48.00
2	1682.40	14931.33	14300.41	4105.79	68.00	104.00	88.00	64.00
3	2523.61	14300.39	17665.20	4206.21	104.00	156.00	100.00	48.00
4	5047.20	13987.81	21030.25	5047.13	64.00	144.00	108.00	104.00
5	8412.00	11650.62	15141.63	4206.07	76.00	132.00	112.00	72.00
6	7570.80	13248.90	1766.52	4206.19	44.00	149.00	82.00	56.00
Average	5467.80	13841.72	14594.89	4346.38	73.33	132.17	94.33	65.33
SD	2852.65	1245.67	6711.50	349.14	20.19	21.77	14.56	21.12

Table 3: One way analysis of variance (ANOVA) results

Source of variation	SS	df	MS	F	p-value	F-crit
ANOVA results for FR in the Lungs						
Between groups	64377.83	3	21459.28	4.431599	0.015228*	3.098391
Within groups	96846.67	20	4842.333			
ANOVA results for FR in the Brain						
Between groups	16058.13	3	5352.708	13.91247	3.96E-05*	3.098391
Within groups	7694.833	20	384.7417			
Total	23752.96	23				
ANOVA results for GPx in the lungs						
Between groups	7.34E+08	3	2.45E+08	20.79367	2.32E-06*	3.098391
Within groups	2.35E+08	20	11764425			
ANOVA results for GPx in the Brain						
Between groups	5.26E+08	3	1.75E+08	12.77838	6.89E-05*	3.098391
Within groups	2.74E+08	20	13712640			
Total	8E+08	23				

*: $p<0.05$

Table 4: Tukey-Kramer Multiple comparisons for GPx and FR in the lungs and brain

GPx in the Lungs					
Group 1		Group 1 to Group 2 Comparison		Group 2 to Group 3 Comparison	
Mean	12898.33	Absolute difference	7185.283	Absolute Difference	14615.850
		Standard error of diff	1400.263	Standard Error of Diff	1400.263
Group 2		Critical range	5012.943	Critical Range	5012.943
Mean	20083.62	Means are different		Means are different	
Group 3		Group 1 to Group 3 Comparison		Group 2 to Group 4 Comparison	
Mean	5467.767	Absolute difference	7430.567	Absolute Difference	11811.820
		Standard error of diff	1400.263	Standard error of diff	1400.263
Group 4		Critical range	5012.943	Critical range	5012.943
Mean	8271.8	Means are different		Means are different	
MSW	11764425	Group 1 to Group 4 Comparison		Group 3 to Group 4 Comparison	
Q Statistic	3.58	Absolute difference	4626.533	Absolute difference	2804.033
		Standard error of diff	1400.263	Standard error of diff	1400.263
		Critical range	5012.943	Critical range	5012.943
		Means are not different		Means are not different	
GPx in Brain					
Group 1		Group 1 to Group 2 Comparison		Group 2 to Group 3 Comparison.	
Mean	5467.8	Absolute difference	8373.92	Absolute difference	753.100
		Standard error of diff	1511.767	Standard error of diff	1511.767
Group 2		Critical range	5412.126	Critical range	5412.126
Mean	13841.72	Means are different		Means are not different	
Group 3		Group 1 to Group 3 Comparison		Group 2 to Group 4 Comparison	
Mean	14594.82	Absolute difference	9127.02	Absolute difference	9495.553
		Standard error of diff	1511.767	Standard error of diff	1511.767
Group 4		Critical range	5412.126	Critical range	5412.126
Mean	4346.167	Means are different		Means are different	
MSW	13712640	Group 1 to Group 4 Comparison		Group 3 to Group 4 Comparison	
Q Statistic	3.58	Absolute difference	1121.633	Absolute difference	10248.650
		Standard error of diff	1511.767	Standard error of diff	1511.767
		Critical range	5412.126	Critical range	5412.126
		Means are not different		Means are different	
FR in the Lungs					
Group 1		Group 1 to Group 2 Comparison		Group 2 to Group 3 Comparison	
Mean	176.6667	Absolute difference	97.66667	Absolute difference	14.33333
		Standard error of diff	28.40872	Standard error of diff	28.40872

Table 4: Continued

Group 2		Critical range	101.7032	Critical range	101.70320
Mean	274.3333	Means are not different		Means are not different	
Group 3		Group 1 to Group 3 Comparison		Group 2 to Group 4 Comparison	
Mean	260	Absolute difference	83.33333	Absolute difference	45.66667
		Standard error of diff	28.40872	Standard error of diff	28.40872
Group 4		Critical range	101.7032	Critical range	101.70320
Mean	320	Means are not different		Means are not different	
MSW	4842.333	Group 1 to Group 4 Comparison		Group 3 to Group 4 Comparison	
Q Statistic	3.58	Absolute difference	143.3333	Absolute difference	60
		Standard error of difference	28.40872	Standard error of difference	28.40872
		Critical range	101.7032	Critical range	101.70320
		Means are different		Means are not different	
FR in the Brain					
Group 1		Group 1 to Group 2 Comparison		Group 2 to Group 3 Comparison	
Mean	73.33333	Absolute difference	58.83333	Absolute difference	37.833330
		Standard error of diff	8.007722	Standard error of diff	8.007722
Group 2		Critical range	28.66764	Critical range	28.667640
Mean	132.1667	Means are different		Means are different	
Group 3		Group 1 to Group 3 Comparison		Group 2 to Group 4 Comparison.	
Mean	94.33333	Absolute difference	21	Absolute difference	66.833330
		Standard error of diff	8.007722	Standard error of diff	8.007722
Group 4		Critical range	28.66764	Critical range	28.667640
Mean	65.33333	Means are not different		Means are different	
MSW	384.7417	Group 1 to Group 4 Comparison		Group 3 to Group 4 Comparison	
Q Statistic	3.58	Absolute difference	8	Absolute difference	29
		Standard error of diff	8.007722	Standard error of difference	8.007722
		Critical range	28.66764	Critical range	28.667640
		Means are not different		Means are different	

*Group 1 = Control, Group 2 = Hyperoxia exposure for 24 h, Group 3 = Hyperoxia exposure for 48 h and Group 4 = hyperoxia exposure for 72 h

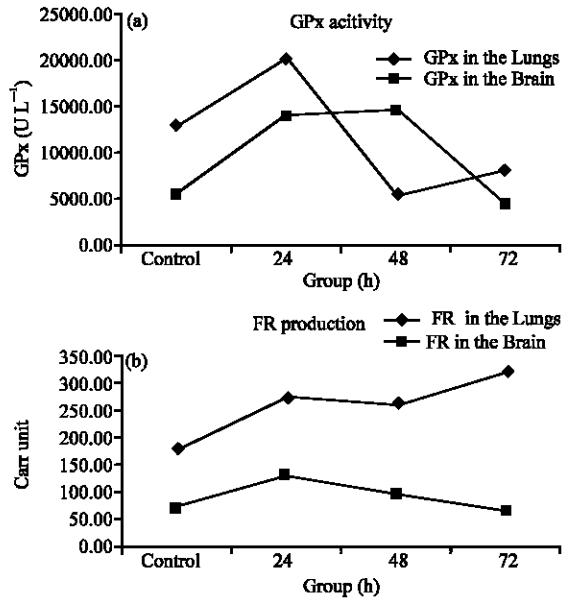

Fig. 1: The Behavior of mean GPx in the lungs and brain (a) and FR production in the lungs and brain (b) during hyperoxia exposure

DISCUSSION

Hyperoxia is believed to generate Reactive Oxygen Species (ROS) and inhibit antioxidants defense. O_2 toxicity is believed to occur when the body's antioxidant defenses are overwhelmed by increased production of ROS. Included in ROS list are superoxide, hydrogen peroxide, hydroxyl radicals and peroxynitrite at high levels of $PtiO_2$ (Demchenko *et al.*, 2001, 2003; Elayan *et al.*, 2000; Torbati *et al.*, 1992). Herein, the present study showed that glutathione peroxidase (GPx) in the lungs was overwhelmed following 24 h of hyperoxia exposure. In the brain, GPx was overwhelmed after 48 h of hyperoxia exposure.

It is clear that dysfunctional mitochondria result in releasing its contents such as oxidative enzymes and hydrogen peroxides to the cytoplasm, in attempt to prevent swelling. When the rate of release of mitochondrial contents exceeds elimination rate of antioxidants defense system, ROS accumulate and FR productions rise. GPx catalyzes specifically detoxification of hydrogen peroxide (Paglia and Valentine, 1967). The severity of hyperoxic-induced cellular injury is time and dose dependent (Hayatdavoudi *et al.*, 1981; Barry and Crapo, 1985; Crapo *et al.*, 1994).

The effects of hyperoxia on the central nervous system (CNS) and risk of bronchopulmonary dysplasia in infants or adult respiratory distress syndrome in adults begins with exposure period over 8 hours (Arieli, 1998; Chavko *et al.*, 1998; Demchenko *et al.*, 2001). In healthy adult risk begins after 48 h (Comroe *et al.*, 1945). Acute exposure to hyperoxia causes tissue and cellular damages in the brain (Huang *et al.*, 2000; Gerstner *et al.*, 2006). In addition, ethanol intake is associated with adaptive

changes in the antioxidant defense enzymes such as increased levels of neural superoxide dismutase and catalase (Montoliu *et al*., 1994). Furthermore, chronic ethanol intake decreases the levels of the GSH (Guerri and Grisolia, 1980; Montoliu *et al*., 1994) and tocopherol content of the cerebellum (Rouach *et al*., 1991). Many clinicians have successfully reduced oxygen toxicity by the antioxidant redoxiredoxin administration (Kim *et al*., 2003) and selenium supplementation (Ebert *et al*., 2006). Others used nitro oxide to prevent cell apoptosis in the lungs (Iben *et al*., 2000; Howlett *et al*., 1999). In the present study, GPx sustained its rise in attempt to defend the integrity of the pneumocyte till 24 h of hyperoxia exposure and up-to 48 h in the brain.

Although increased generation of ROS is evident in lung epithelia cells *in vitro* within 30-60 min of hyperoxia (Manautou and Carlson, 1991; Sanders *et al*., 1993; Parinandi *et al*., 2003), clinical use of normobaric hyperoxia for several hours is frequently considered harmless or even recommended to reduce the risk of post-surgical wound infections (Neubauer *et al*., 1994; Greif *et al*., 2000; Belda *et al*., 2005) and head injury (Brown *et al*., 1988). In humans, the first respiratory symptoms have been reported after 6 h of oxygen exposure (Comroe *et al*., 1945) and ultrastructural alterations such as epithelial cell swelling are evident within 14 h but with hyperoxia of 70% O_2 (Kapanci *et al*., 1972). In rats, animals die within 60-72 h of exposure to 100% O_2, whereas an FIO_2 of 0.85 is sublethal but may cause platelet accumulation within 3 days and increase lung weight within 5 days of exposure (Crapo *et al*., 1980; Barry and Crapo, 1985; Tibbles and Edelsberg, 1996). Herein, results of the present study clearly showed reduction in GPx and higher rate of FR generation beyond 24 h of hyperoxia exposure (100% O_2, medical grade) but did not result in animals' death up-to 72 h.

The severity of hyperoxic lung injury is time- and dose dependent (Crapo *et al*., 1980; Barry and Crapo, 1985). As compared with 95% O_2, an FIO_2 of 0.7 results in less ROS formation but does not protect from lung injury when applied over longer periods because rats exposed to 60% O_2 for 7 days showed reduced lung compliance and perivascular oedema formation (Cragg *et al*., 1986; Caldwell *et al*., 1966; Nishio *et al*., 1998) and 14 day exposure to 60% O_2 causes low-grade epithelial injury and interstitial fibrosis in baboons (Crapo *et al*., 1994). Besides lung epithelial ROS generation, capillary endothelial cells were identified as the source of hyperoxia-induced ROS production (Kuebler *et al*., 2000). Herein, results of the present study showed a steady rise in ROS generation, along with increasing exposure period, yet beyond the onset of lung tissue injury at 24 h, which reflected additional phagocytes defense mechanism of the alveolar macrophages which in turn contributed additively to ROS generation.

CONCLUSIONS

The results of the present study along with the data showing that GPx and FR levels increased in the lungs and the brain tissues but these changes were delayed and reversed in the brain. Steady elevated of ROS formation, with increasing exposure period, in the lungs reflected added pulmonary vascular effects directly related to pneumocyte phagocyte infiltration mechanism, perhaps ROS-related chemotaxic mechanism.

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