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Superoxide dismutase functional regulation in neonatal hypoxia: Effect of glucose, oxygen and epinephrine

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Hypoxia is one of the major causes of damage to the fetal and neonatal brain and cardiac functions. In earlier studies, we have reported the brain damage caused by hypoxia and resuscitation with oxygen and epinephrine and have found that glucose treatment to hypoxic rats and hypoxic rats treated with oxygen shows a reversal of brain damage. The neonatal rats are shown to be deficient in free radical scavenging system, which offers a high risk of oxidative stress. In the present study, we induced hypoxia in neonatal Wistar rats and resuscitated with glucose, oxygen and epinephrine. Heart tissue and cerebral cortex were used to study the kinetics of superoxide dismutase activity in experimental groups of rats to assess the free radical status. Results showed that glucose supplementation in hypoxia (Hx + G) and hypoxic + oxygen (Hx + O) had an efficient free radical scavenging capability, compared to all other experimental groups. The observation was ascertained by studying the activity of catalase, another antioxidant enzyme in the body. Our results suggested that in neonatal rats during hypoxic condition, damage to heart and brain was more prominent in all groups, except when supplemented with glucose. These findings may have clinical significance in the proper management of heart and brain function.

Keywords: Superoxide dismutase, Neonatal hypoxia, Free radical, Catalase, Glucose, Oxygen, Epinephrine

Hypoxia is one of the most common reasons for neonatal morbidity and mortality, causing reduced oxygen supply to the vital organs[1] and injury to the developing brain[2-5]. Newborn babies are frequently exposed to hypoxia and ischemia during the perinatal period as a result of stroke or problems with delivery or respiratory management after delivery[6]. Free radicals or reactive oxygen species (ROS) are formed under hypoxic conditions. Cells have an enzymatic antioxidant pathway against ROS which are generated during oxidative metabolism. Superoxide dismutase (SOD) catalyzes the formation of hydrogen peroxide from superoxide radicals, which is removed by a reaction catalyzed by catalase (CAT) and glutathione peroxidase (GPx)[7].

Toxicity by oxygen radicals has been suggested as a major cause of heart disease, cellular damage and brain damage. As oxygen is a major determinant of cardiac gene expression and a critical participant in the formation of ROS, its role is essential in understanding the pathogenesis of cardiac dysfunction[8]. Xanthine oxidase (XO)-derived superoxide induces endothelial dysfunction, thus impairing pulmonary arterial relaxation and contributes to vascular remodeling in hypoxia-exposed neonatal rats[9].

The brain and nervous system is especially prone to oxidant damage for a number of reasons[10]. The membrane lipids are especially rich in polyunsaturated fatty acid side chains, which are prime targets for free radicals attack. The brain has only moderate amounts of CAT (EC 1.11.1.6), SOD (EC 1.15.1.1) and GPx and also is relatively lacking in vitamin E. Some areas of the brain are rich in iron ions which are released from injured cells or bleeding in the reperfused area and may enhance lipid peroxidation. One particular role of oxygen free radicals in brain injury appears to involve reperfusion after cerebral ischemia[11].

SOD is essential for biological defense against superoxide anion. The degree of SOD activity is an important parameter to assess the free radical scavenging capability. Approaches to prevent/treat hypoxic damage in neonates[12] are important for neonatal intensive care. The immature brain is particularly susceptible to free radical injury, because of its poorly developed scavenging systems and high

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availability of iron for the catalytic formation of free radicals.

During neonatal hypoxia, traditional resuscitation practices include the routine administration of 100% oxygen, epinephrine and glucose[13-16]. In the present study, resuscitation with glucose, epinephrine and oxygen as the sequence of administration has been employed to study the free radical scavenging capability in heart tissue and cerebral cortex to understand the significance of scavenging of ROS from two very important organs during hypoxic condition.

Materials and Methods

Chemicals

SOD from bovine erythrocytes was purchased from Sigma Chemical Co., USA. All other reagents were of analytical grade purchased locally.

Animals

Wistar adult rats (body wt 180 to 200 g) and Wistar neonatal rats of 4-days old (body wt 6.06 g ± 0.45) procured from Amrita Institute of Medical Sciences, Cochin were used for the experiment. All groups of neonatal rats were maintained with their mothers under optimal conditions of 12 h light and 12 h dark periods and fed standard food and water *ad libitum*.

Induction of acute hypoxia in neonatal rats

Induction of hypoxia and supplementation of glucose, oxygen and epinephrine was according to the procedure described previously[17]. Wistar neonatal rats used for the experiments were grouped into seven as follows: Group I or control rats were given atmospheric air (20.9% oxygen) for 30 min; Group II: hypoxia was induced by placing the rats in a hypoxic chamber provided with 2.6% oxygen for 30 min; Group III: hypoxic neonatal rats were injected 10% dextrose (500 mg/Kg body wt) intra-peritoneally (i.p.); Group IV; hypoxic rats were supplied with 100% oxygen for 30 min (Hx + O); Group V; hypoxic rats were injected 10% dextrose (500 mg/kg body wt) intra-peritoneally and treated with 100% oxygen for 30 min (Hx + G + O); In Group VI hypoxic rats, 10% dextrose (500 mg/ Kg body wt) and epinephrine (0.1 µg/Kg body wt) were injected i.p. and then treated with 100% oxygen for 30 min (Hx + G + E + O). The experimental animals were maintained in the room temperature for 1 week.

Tissue preparation

Control and experimental neonatal rats were sacrificed on the 10th day after the induction of hypoxia by decapitation. The brain regions and body parts were dissected out quickly over ice according to the procedure described previously[18] and the tissues were stored at $^{-}70^{0}$C for the experiments. All animal care and procedures were in accordance with Institutional and National Institute of Health guidelines.

Kinetics of SOD and CAT

The ability of the flavonoid to inhibit the reduction of nitroblue tetrazolium (NBT) by superoxide generated by the reduction of photoreduced riboflavin and oxygen was assayed. SOD assay was done in heart and cerebral cortex homogenate as described previously[19]. The SOD concentration (U/mg) that established IC_{50} (50% inhibition of the reaction) was determined using a standard SOD (2000-10000 U/mg protein). Then, the dilution rate of heart and cerebral cortex homogenates that established IC_{50} was determined and the unit (U/mg) of the extract was calculated by the SOD concentration that established IC_{50} determined using standard SOD. One unit of SOD was defined as the amount of protein that inhibited the rate of NBT reduction by 50%. Kinetic parameters V_{max} and K_m were calculated from the data of SOD assay measured at substrate concentrations of 0.03, 0.06, 0.12, 0.15 and 0.2 m*M*.

CAT assay was done in heart and cerebral cortex homogenate as described previously[20]. The reaction mixture contained 40 mM H_2O_2 in a 50 mM phosphate buffer, pH 7.0, and 0.1 ml pure enzyme in a total volume of 3 ml. CAT activity was estimated by measuring the decrease in absorbance of H_2O_2 at 240 nm. V_{max} and K_m were calculated from the data of CAT assay measured at substrate concentrations of 0.03, 0.06, 0.12, 0.15 and 0.2 m*M*.

Results

SOD activity in heart and cerebral cortex of neonatal rats

The SOD activity showed a significant increase in V_{max} (p<0.001) and a significant decrease in K_m (p<0.01) in adult rats compared to neonates, indicating the poorly developed enzyme system in the neonates. In hypoxic group (Hx), a significant decrease in V_{max} (p<0.01) and a significant increase in K_m (p<0.01) was observed, showing decreased activity of SOD with less affinity under hypoxic condition. In glucose supplemented hypoxic group (Hx + G), an increase in V_{max} (p<0.001) and a decrease in K_m (p<0.001) were observed compared to hypoxic group, showing the enhanced free radical

Table 1—SOD activity in the heart and cerebral cortex of control and experimental rats

[Values represent mean ± S.E.M of 4-6 separate experiments]

Conditions	Heart		Cerebral cortex	
	V_{max} (U/mg protein)	K_m (μM)	V_{max} (U/mg protein)	K_m (μM)
Control adult	38.25 ± 0.12***†††	1.15 ± 0.08**††	38.56 ± 0.10***	1.04 ± 0.15**
Control neonate	20.94 ± 0.19	1.40 ± 0.05	29.0 ± 0.10	1.53 ± 0.05
Hx	19.67 ± 0.05**	1.85 ± 0.05**	20.05 ± 0.05***	1.50 ± 0.30
Hx + O	26.13 ± 0.10***†††	1.75 ± 0.10*†	35.15 ± 0.05***†††	1.35 ± 0.15**††
Hx + G	23.52 ± 0.10***†††	1.0 ± 0.10**†††	28.25 ± 0.15**††	1.52 ± 0.02
Hx + G + O	21.09 ± 0.05†††	1.40 ± 0.05†††	28.80 ± 0.10†††	1.25 ± 0.02***†††
Hx + G + E + O	19.43 ± 0.07***¶¶	0.80 ± 0.05***†††	19.30 ± 0.10***††¶¶	1.50 ± 0.05¶

*p<0.05, ** p<0.01, ***p<0.001, when compared with control

† p<0.05, †† p<0.01 ††† p<0.001, when compared with hypoxic group

¶¶ p<0.01, when compared with Hx + G + O

Hx, Hypoxic rats; Hx + O, Hypoxic rats oxygen treated; Hx + G, Hypoxic rats glucose treated; Hx + G + O, Hypoxic rats glucose and oxygen treated; Hx + G + E + O, Hypoxic rats glucose, epinephrine and oxygen treated.

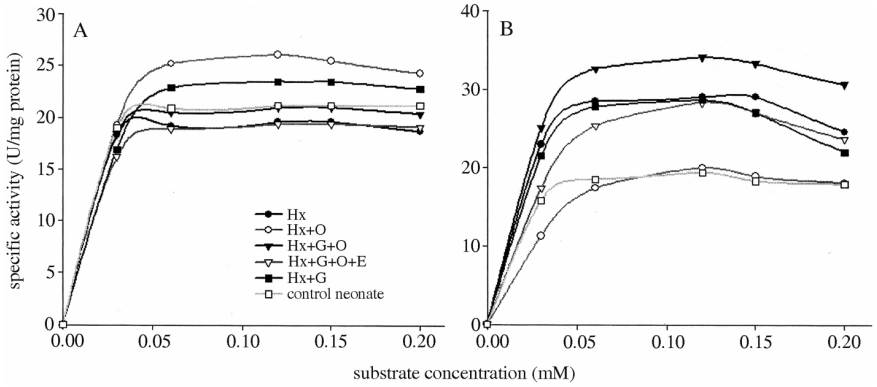

Fig. 1—SOD activity in the heart (A) and cerebral cortex (B) of control and experimental rats [Kinetic parameters V_{max} and K_m were calculated from the data of SOD assay measured at substrate concentrations of 0.03, 0.06, 0.12, 0.15 and 0.2 m*M*]

scavenging capability due to glucose supplementation. In Hx + O group, both V_{max} (p<0.001) and K_m (p<0.05) showed a significant increase compared to control. In Hx +G + O group, V_{max} and K_m showed a reversal to near control value with significant increase in V_{max} (p<0.001) and a significant decrease in K_m (p<0.001) compared to hypoxic group. In Hx + G + E + O group, V_{max} (p<0.001) showed a significant decrease to near hypoxic value with a decreased K_m (p<0.001), when compared to control neonate (Table 1, Fig. 1A).

In cerebral cortex, SOD activity increased (p<0.001) with an increased affinity (p<0.01) in adult rats compared to neonates. In hypoxic group (Hx), SOD activity showed a significant decrease in V_{max} (p<0.001), whereas in oxygen supplemented hypoxic rats (Hx + O) a significant increase in V_{max} (p<0.001) was observed compared to control. The SOD activity in glucose supplemented group (Hx + G) showed reversal to the near control level with a significant increase in V_{max} (p<0.01) compared to the hypoxic group. In Hx +G + O group, V_{max} showed a significant increase (p<0.001) compared to hypoxic group, showing a reversal to near control level. Hx + G + E + O group showed a significant decrease in V_{max} (p<0.001) to near hypoxic level (Table 1, Fig. 1B).

CAT activity in heart and cerebral cortex of neonatal rats

In heart of adult Wistar rats, CAT showed a higher activity (p<0.001) with a higher affinity (p<0.05) compared to neonatal control rats. In hypoxic group (Hx), a significant decrease in V_{max} (p<0.01) with a

Table 2— CAT activity in the heart and cerebral cortex of control and experimental rats
[Values represent mean ± S.E.M of 4-6 separate experiments]

Conditions	Heart		Cerebral cortex	
	V_{max} (U/mg protein)	K_m (μM)	V_{max} (U/mg protein)	K_m (μM)
Control adult	44.0 ± 0.20***†††	3.30 ± 0.01*††	48.85 ± 0.15***	3.50 ± 0.10***
Control neonate	27.00 ± 0.02	2.65 ± 0.15	33.10 ± 0.09	2.20 ± 0.10
Hx	15.50 ± 0.05**	1.80 ± 0.01*	19.60 ± 0.04***	1.55 ± 0.05**
Hx + O	12.50 ± 0.05**	2.50 ± 0.10	18.35 ± 0.03***	2.35 ± 0.05††
Hx + G	26.50 ± 0.10††	2.45 ± 0.25	30.50 ± 0.05**†††	2.40 ± 0.01††
Hx + G + O	21.50 ± 0.05†	2.45 ± 0.01	29.90 ± 0.10**†††	2.50 ± 0.01††
Hx + G + E + O	17.15 ± 0.10***¶¶	2.55 ± 0.01††¶¶	18.20 ± 0.10***††¶¶	1.80 ± 0.01¶¶

*p<0.05, ** p<0.01, ***p<0.001, when compared with control
† p<0.05, †† p<0.01 ††† p<0.001, when compared with hypoxic group
¶¶ p<0.01 when compared with Hx + G + O
Hx, Hypoxic rats; Hx + O, Hypoxic rats oxygen treated; Hx + G, Hypoxic rats glucose treated; Hx + G + O, Hypoxic rats glucose and oxygen treated; Hx + G + E + O, Hypoxic rats glucose, epinephrine and oxygen treated.

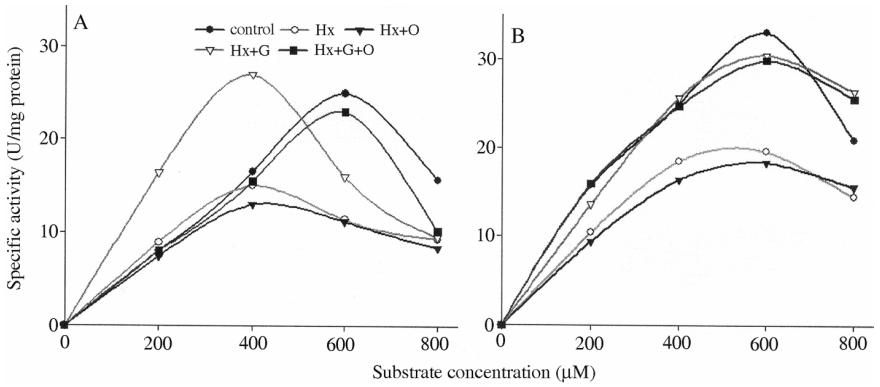

Fig. 2— CAT activity in the heart (A) and cerebral cortex (B) of control and experimental rats [V_{max} and K_m were calculated from the data of CAT assay measured at substrate concentrations of 0.03, 0.06, 0.12, 0.15 and 0.2 m*M*]

decreased affinity (p<0.05) was observed, which accounted for the free radial injury during hypoxic shock. In glucose supplemented hypoxic group (Hx + G), V_{max} showed a significant increase (p<0.01) compared to hypoxic group, showing a reversal of the activity to control level. In Hx + O group, both V_{max} (p<0.01) exhibited a significant decrease compared to control. In Hx +G + O group, V_{max} and K_m showed a reversal to near control value with significant increase in V_{max} (p<0.05) compared to hypoxic group. In Hx + G + E + O group, V_{max} (p<0.001) showed a significant decrease to near hypoxic value (Table 2, Fig. 2A).

In cerebral cortex, the CAT activity showed a significant increase in V_{max} (p<0.001) and a significant decrease in K_m (p<0.001) in adult rats compared to neonates. In hypoxic (Hx) and oxygen supplemented hypoxic (Hx + O) group. CAT activity showed a significant decrease in V_{max} (p<0.001) compared to control. The V_{max} of glucose supplemented Hx + G and Hx +G + O groups showed reversal to the near control level with a significant increase in V_{max} (p<0.001) compared to the hypoxic group, showing a reversal to near control level. Hx + G + E + O group showed a significant decrease in V_{max} (p<0.001) to near hypoxic level (Table 2, Fig. 2B).

Discussion

Oxygen and/or glucose deprivation alters electrical transmission in the brain and generates free radicals, which may mediate neuronal death[21]. Free radical production has been proposed to be involved in the

pathogenesis of ischemia-reperfusion neuronal damage[10,22-25]. Damage to lipids, proteins, and nucleic acids has been observed concomitantly with their production, ultimately resulting in cell function impairment and death[26]. SOD plays an important role in protection against oxygen toxicity in mammalian systems[27]. Major pathway in cardiac tissue for detoxification of reactive oxygen metabolites is via the concerned action of SOD and selenium-dependent GPx[28].

Administration of SOD can reverse the pronounced deleterious effect on large artery NO-mediated vasorelaxation in rats due to oxidative stress, suggesting that a systemic state of inadequate antioxidant reserve exists in heart failure[29]. SOD mimetics M40403 and M40401 have protective effects against hypoxicischemic brain injury and suggest the involvement of superoxide anion in neuronal cell injury during hypoxia-ischemic injury[30]. SOD activity is lower in neonates than in adults[31, 32]. Our study confirmed the enhanced free radical scavenging ability in the heart tissue of adult rats, compared to neonates with greater affinity.

Studies have demonstrated that free radicals are formed under hypoxic conditions in newborn piglet brain[33]. The SOD activity decreases in the cerebral cortex of rats subjected to 24 h of reperfusion, following 2 h of cerebral ischemia[34]. The rat endothelial cells can produce SOD inhibittable ROS which are augmented by hypoxia/reoxygenation[35]. We have observed the free radical scavenging capability in the heart and cerebral cortex of neonatal rats exposed to hypoxia and found that both SOD and CAT activities are significantly less than that of normal neonates, indicating that limitation in oxygen supply is associated with reduction in antioxidant enzymes like SOD and CAT.

The resuscitation with 100% oxygen is a common practice to encounter severe neonatal hypoxia during delivery. Hyperoxia with 100% oxygen after hypoxia-ischemia can cause more damage in the cerebral cortex than room air in newborn rats[36]. 100% oxygen generates abnormally high levels of ROS which cause dysfunction of defensive antioxidant system of cells by altering enzyme activity[37] and act as a factor for neurodegeneration[38]. Hypoxemic piglets resuscitated with 100% oxygen have also shown increased cerebral injury, cortical damage and early neurological disorders[36,39-41]. In our study, the increased SOD and CAT activities with decreased affinity observed in Hx + O group indicates the decreased function of SOD, which might add to more damage due to the free radical formation. This highlights the role of free radicals in causing damaging effects during 100% oxygen administration for neonatal hypoxia.

We observed that glucose supplementation to hypoxic and hypoxic treated with oxygen has an efficient free radical scavenging capability compared to all other experimental groups. Hypoxic neonates treated with glucose have shown higher SOD and CAT activities, suggesting an increased antioxidant capability in presence of glucose. The combination of glucose, epinephrine and oxygen as resuscitation in hypoxic condition has shown a decreased SOD and CAT activity, indicating that free radical toxicity is high in heart and cerebral cortex, due to the administration of epinephrine.

Reduction in blood glucose levels and substantially increased cerebral glucose utilization has been observed as a result of hypoxic stress in experimental rats[42,43]. In response to a stressor, glucose metabolism becomes subject to control by the two major neurohormonal stress systems, i.e. the sympathoadrenal system and hypothalamus-pituitary-adrenal axis. Both catecholamines and cortisol via increasing hepatic glucose output and decreasing muscular glucose uptake[44,45] enhance blood glucose concentration with the actions of catecholamines, mainly of epinephrine being faster than those of cortisol.

Unlike the adult, where glucose supplementation prior to or during hypoxia-ischemia accentuates tissue injury, glucose treatment of perinatal animals subjected to a similar insult substantially reduces the extent of tissue injury[44]. Hypoxia induced expressional and functional changes in NMDAR1 receptors of neuronal cells in neonatal rats are corrected by supplementation of glucose alone or glucose, followed by oxygen during the resuscitation to prevent the glutamate related neuronal damage[17]. Post- hypoxic glucose supplement also reduces an elevated brain lactate level which is responsible for cerebral infarction occurring during the hypoxia[43].

Glucose supplementation helps to reverse the SOD and CAT activities in hypoxic neonates to near normal levels in both heart and cerebral cortex homogenates, suggesting that glucose supplementation alone or in combination with oxygen as a better resuscitation method. In hypoxic condition, glucose

supplemented groups have shown better recovery from damage to heart and brain, which has clinical significance in the proper management of heart and brain function in the later stages of life. The neonatal brain is especially at risk of free radical mediated injury, because neuronal membranes are rich in polyunsaturated fatty acids and the human newborn has a relative deficiency of brain SOD and GPx. The understanding of neonatal factors involved in the pathogenesis of "oxygen free radical diseases" will lead to the development of new therapies for prevention and treatment of these neonatal diseases.

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