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Effect of Heat Stress, Hypoxia and Hypoxia-hyperoxia on Free Radical Production in mice *Mus musculus*

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The purpose of this study was to examine the effects of heat stress, hypoxia and hypoxia-hyperoxia on Free Radicals (FR) production in mice. Three experimental groups were exposed to three environmental conditions namely; heat stress (35-38°C); hypoxia (12-15% O_2) and the third group underwent hypoxia (12-15% O_2) followed by hyperoxia (100% O_2). In comparison with control group, mean FR production increased significantly ($p<0.05$) by 44.89% in the heat stress group, 26.13% in the hypoxia group and by 77% in the hypoxia-hyperoxia group. The partial correlation coefficient ($r = 0.81$), controlling for control group, for the FR induced by heat stress on FR caused by hypoxia was significant ($p<0.05$) with regression coefficient of $R^2 = 0.75$ which was also significant ($p<0.05$). These findings indicated that FR production is related to environmental stress induced by heat stress, reductive stress (hypoxia) and oxidative stress (hyperoxia). Heat stress has common effects with hypoxia on FR production. The effect of hyperoxia is increased if tissue ischemia presents prior exposure to oxidative stress exposure.

Key words: Free radicals, heat stress, hyperoxia, hypoxia

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INTRODUCTION

Oxidative stress has been long recognized to endanger cell which is associated with increments of free radicals. Earliest observations by Arcasoy and smuckler[1] showed acute digoxin intoxication on rat hepatic and cardiac cells. Davis *et al.*[2] was the first to show an electron paramagnetic resonance signal consistent with free radical formation and evidence of lipid peroxidation formation in stressed skeletal muscle cell. Since then Reactive Oxygen Species (ROS) were identified and reported as a result of pathological conditions[3-12].

Mitochondria are easily affected by oxygen toxicity[13-15]. Thus mitochondrion is the main source for free radicals production. Recently our laboratory has demonstrated that Free Radicals (FR) was produced above normal value when whole animals were exposed to oxidative stress-hyperoxia[13]. The increase in FR production was associated with variety of mitochondrial pathology that included mitochondrial swelling, hyperplasia, concentrated cristae and dilution of the inner and outer membranes. However, it is not clear in free radical biology that oxidative stress (hyperoxia) and reductive stress (hypoxia alone) both result in increased ROS production. The potential role of oxidative stress in ischemia and reperfusion (hypoxia-hyperoxia) injury has been more established[16-18] but comparisons with hypoxia alone has not been made. Furthermore, whole body hyperthermia elevates total oxygen consumption and stimulates cellular metabolism[19] which can be a potential cause for cellular ischemia that elevate reducing equivalent, NADH. In support of this tenet, we hypothesized that conscious animal can be subject to severe hypoxic stress during whole body heat stress. Thus exposure of animals to environmental heat stress progressively elevates ROS formation. We have speculated that the common effects of heat stress and hypoxic stress promote cellular oxidative stress, which in turn may be involved in the pathogenesis of hyperthermia and oxygen toxicity. The aim of the present study was to evaluate the effects of heat stress, hypoxia and hypoxia-hyperoxia on free radical production in mice. The secondary purpose was to examine the common relationship between heat stress and hypoxic stress in terms of FR production.

MATERIALS AND METHODS

Experimental design: Forty adult white albino mice *Mus musculus*, with mean weight of 27 g, were divided into four groups randomly, ten each. One group served as control and the second, third and fourth groups served as experimental groups. The first experimental group underwent heat stress (35-38°C) for one month. The second experimental group was exposed to moderate hypoxia (12-15% O_2) for 2 h daily for 4 weeks. The third experimental group underwent hypoxia (12-15% O_2) for 30 min followed by 1.5 h of hyperoxia (100% O_2) daily, for 4 weeks period.

Heat stress calculations: Heating rate at 38°C/min was adjusted electronically, using an open circuit insulated box (Minicoldlab 2203, AC Klassbol, Brumma, Sweden) which contained a servomechanism thermoregulatory device. The thermostat of this system compares the desired set temperature with the actual temperature as follows:

$$\frac{[(\text{Peak temperature attained}) - (\text{Actual temperature}_{(\text{Box})})]}{\text{Total heating time (min)}}$$

When an error signal between the circulating temperature and the set point occurred, the heater is turned on automatically to restore the set point temperature. The set temperature and the actual temperature within the box were recorded every 12 h in order to assure that the box temperature was maintained between 36-38°C.

Statistical analysis: Data represent mean values±SEM. Differences among mean free radical values were analyzed by ANOVA, using SPSS. Significant differences group means were identified using the LSD multiple comparisons F test, with significance that was set at $p<0.05$. Groups for analysis values were defined by treatment of heat stress versus control, hypoxia versus control and hypoxia-hyperoxia versus control.

Blood samples collection and FR measurement: Twenty microliter of blood samples were collected into heparinized capillary from the retro-orbital plexus. Blood samples were mixed with buffered chromogen immediately, in accordance with d-ROMs test procedure (FERAS-II, Italy) and FR was determined as described previously[13].

RESULTS

There were 44.89% increase in FR as a result of heat stress, 26.13% increase in FR induced by hypoxia alone and 77% increase caused by hypoxia-hyperoxia (Table 1).

One way analysis of variance (ANOVA) showed a significant difference ($p<0.05$) among groups means (Table 2). Thus the difference between an individual group mean and the mean of all potential observations

Table 1: Descriptive statistics of free radical production

Groups	N	Mean	Diff. (%)*	SD	95% confidence interval for mean: Lower bound	Upper bound
Control	10	172.70	00.00	6.89	157.11	188.29
Heat stress	8**	248.13	44.89	19.24	202.62	293.63
Hypoxia	10	216.30	26.13	15.97	180.18	252.42
Hypoxia-Hyperoxia	10	303.00	77.00	39.87	212.81	393.19

* Difference (%) as compared to control group

** As two animals died due to hyperthermia, the analysis of heat stress group was limited to 8 animals.

Table 2: One way ANOVA results for free radical production

Source of variation	Model	Sum of squares	Df	Mean square	F	Sig.
Between groups	(Combined)	89911.49	3	29970.493	5.334	0.004
Within groups		191021.08	34	5618.267		
Total		280932.56	37			

Table 3: Multiple comparisons between group means, using LSD

Sources of comparisons: (I) Group membership	(J) Group membership	Mean difference (I-J)
Control	Temperature	-75.425*
	Hypoxia	-43.600
	Hypoxia-hyperoxia	-130.300*
Temperature	Control	75.4250
	Hypoxia	31.825
	Hypoxia-hyperoxia	-54.875*
Hypoxia	Control	43.600
	Temperature	-31.825
	Hypoxia-hyperoxia	-86.700*
Hypoxia-hyperoxia	Control	130.300*
	Temperature	54.875
	Hypoxia	86.700*

* The mean difference is significant at the .05 level

Table 4: Partial correlation coefficients, controlling for control group

	Heat stress	Hypoxia	Hypo.-hypr.
Heat stress	1.0000	0.8149	0.6272
	p=0.0000	p=0.013	p=0.066
Hypoxia	0.8149	1.0000	0.6589
	p=0.013	p=0.0000	p=0.054
Hypo.-hypr.	0.6272	0.6589	1.0000
	p=0.0000	p=0.054	p=0.0000

was significant ($p<0.05$). Multiple comparisons of mean FR, using LSD, showed that the mean FR production in the heat stress group and hypoxia-hyperoxia were significantly different ($p<0.05$), as compared with control group (Table 3). Partial correlation coefficient ($r = 0.8149$), controlling for control group, showed significant ($p<0.013$) correlation between the heat stress FR and hypoxia groups (Table 4). In addition, the regression model of FR production induced by heat stress on FR induced by hypoxia alone was significant ($p<0.05$) and linear (Fig. 1) that can be described by the following regression model:

$$Y = 85.547 + 0.753(X)$$

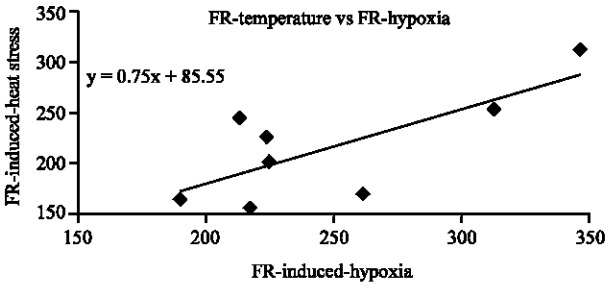

Fig. 1: Regression of FR induced by heat stress on FR induce by hypoxia

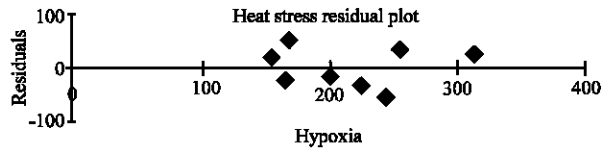

Fig. 2: Residual plot showing that the unexplained variance departed symmetrically from zero

Furthermore, coefficient of determination (R^2) of the regression model was 0.656 which means 66% of the variability in FR during heat stress accounted for by hypoxia alone. The slope (β) of the regression line was significantly ($p<0.05$) different from zero implying predicable heat stress-induced FR production by hypoxia-induced FR production-the predictor variable. The regression model was evaluated using residual sum of squares plot. Clearly, Fig. 2 shows that the residual variance of FR production induced by heat stress was symmetrically depart from zero at all level of the predictor variable-hypoxia. In other words the residual variance of FR stayed about constant at all levels thus the errors associated with the model was random and free of bias.

DISCUSSION

The results of the present study demonstrated that heat stress, hypoxia alone and hypoxia-hyperoxia all had led to an increase in FR production. However, hypoxia caused the smallest increase but the regression of FR changes induced by heat stress was significantly predictable by hypoxia. Recent evidence from our laboratory had shown that increase in FR production was related to mitochondrial pathology in rats' myocyte after hyperoxia exposure[13]. Thus hyperoxia-induced-mitochondrial oxidative stress can be associated with progressive mitochondrial uncoupling[20-23]. Mitochondrial uncoupling elevates the reducing equivalents concentration which in turn leads to progressive accumulation of superoxide ($^1O_2$)[24,25] and hence increased rate of FR levels in the blood. Therefore high

ROS formation is initiated by abnormal electron flow of mitochondrial electron transport which relies on the delicate balance between O_2 supply and demand. Evidently mitochondria structural pathology disturb O_2 balance which becomes above or below critical level in the cell which in turn leads to buildup of reducing equivalents and enhanced ROS formation.

The finding that hypoxia elevated FR strongly suggest that cellular oxidative stress may be elevated by hypoxia. These new findings led us to believe that reductive stress (hypoxia) reflect the influence of elevated reducing equivalents in the cell that drive one electron reduction of O_2 that accelerate the formation of superoxide ($^{1*}O_2$). Vanden[26] had shown that intracellular ROS production in cardiac myocytes was markedly increased at PO_2 of 4 Torr and prior to reperfusion. In addition it had been shown that hypoxia contributed to reduction in diaphragm force generation and in brain ischemia[27] that confirmed the lack of O_2 supply is responsible for the build up of the intracellular reducing equivalents and subsequently enhanced ROS formation.

The second major findings of the present study was elevated FR induced by heat stress. These findings support the fact that whole body heat stress increases ROS formation and hence plasma levels of FR. It has been shown marked increase in ROS production during brief exposure to 42°C[28,29]. Other investigators have shown that hyperthermia leads to oxidants production which was associated with increased levels of heat shock proteins-HSP[12], mitochondrial production of superoxide[30], progressive mitochondria uncoupling[31] immediately followed heat stress. Even though the mechanisms with this regards is not clear but results of the present study confirmed that serum FR was elevated in heat stress and had common changes with hypoxia effects. Furthermore, the regression of FR during heat stress on FR during hypoxia exposure was significant implying that tissue hypoxia had occurred due to heat stress. Notably, cellular hypoxia had been reported in organs of high metabolic capacity and can develop arteriole-venule O_2 diffusional shunting[32] or evolve gradients of tissue oxygenation[33] during periods of low blood flow. These are also tissues that are characteristically injured following thermal challenge[34-38]. On the basis of these results, it can be proposed that cellular hypoxia contributes to hyperthermia-related cellular injury.

The findings that FR production was highest after hypoxia-hyperoxia indicated that oxidative damages were enhanced by reperfusion. The reductive stress associated with oxidant production with subsequent conditions of reoxygenation or reperfusion has been well established[6,7,11]. Furthermore, during reperfusion, not only is the tissue suddenly capable of generating large levels of reactive oxygen, but the metabolites are transiently and suddenly changed, prior to the ability of the tissue to reestablish equilibrium[39].

Based on the findings of the present study, it is not surprising that excessive O_2 (oxidative stress) and decreased O_2 (reductive stress) both resulted in increased FR production. Hypoxia is potential possible mechanism to explain FR-induced hyperthermia.

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