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Effects of Hyperoxia on Free Radical Production

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ABSTRACT. It is clear that environmental changes associated with industrial development influence life systems. Reactive gases, such as carbon monoxide, carbon dioxide, and nitric oxide contribute to elevating oxygen radical species. Previous work on pigeon in our laboratory has shown that hyperoxia exposure, two hours daily for 6 weeks period, caused cell injury and mitochondria pathological changes such as mitochondria fusion, hyperplasia, swelling, cristae disorientation, metrical density, and ring shape. These pathological abnormalities were attributed to oxidative damage to lipid membrane and hence increased lipid peroxidation. It is not clear whether the combined effect of aging and hyperoxia relate differentially to free radical (FR) production. Here we report FR changes in two groups of rats (young vs. old) exposed to hyperoxia (100% O_2), for 4 - 6 hours daily for 4.5 week. Baseline FR for the young and the old groups were 445.5 U Carr and 409.0 Carr U, respectively, which were not significantly ($P > 0.05$) different. Hyperoxia elevated blood FR for the young to 611.0 U Carr and for the old to 608.25 U Carr. These changes in FR were significant ($P < 0.05$) for the two groups. Thus, hyperoxia exposure mediate mitochondria damage independent of age. Based on the results of the present study, it can be concluded that hyperoxia exposure lead to the elevation of reactive oxygen species (ROS) which mediate inhibition of mitochondrial respiration in rats.

Key Words: *Free radicals, hyperoxia, mitochondria, oxidative stress.*

Introduction

Industrial development and modern life style has resulted in accumulation of reactive gases and metals vapors in the atmosphere. The potential of oxidative stress of these gases and vapors may be enhanced by the presence of high level of oxygen (hyperoxia). Hyperbaric oxygen (HBO) has been used in a variety of medical treatments. HBO has also been accepted for long term therapy for intoxication, soft tissue infections, and traumatic ischemia. Information regarding the side effects of using HBO as therapy is lacking in order to better deliver healthy care and improve quality of human life. Hyperoxia exposure is related to cellular damage which is mainly induced by free radical production and the subsequent peroxidation of biomolcules. The chemistry of oxygen and its derivatives has been extensively reviewed (Wood, 1988; Halliwell & Gutteridge, 2003). The diatomic oxygen, O_2 is reactive itself due to the fact that its two unpaired electrons are located in

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different molecular orbital and possess a parallel spins (Gilbert and Colton, 1999). Thus oxygen can accept two electrons, in absence of antiparallel spins relative to the unpaired electrons in O_2. As a result, O_2 accepts electrons, one at a time, to form other biological radicals such as hydroxyl radical (*OH) and the superoxide radical ($*O_2$) which together with the hydrogen peroxide (H_2O_2) and the singlet oxygen ($^{1*}O2$), are known as reactive oxygen species (ROS). Therefore in an animal body one-and two- electron reduction of O_2 generates $*O^-_2$ and hydrogen peroxide (H_2O_2) respectively. Moreover, in the presence of free transition metals; $^*O^-_2$ and H_2O_2 together generate the extremely reactive hydroxyl radical ($^*OH$) which in turn is responsible for initiating the oxidative destruction of biomolcules; lipid, and protein.

Furthermore, mitochondria are easily affected by oxygen toxicity (Ogburn *et al.*, 1988; Yan *et al,* 1994; Haffor *et al,* 2002, Haffor, 2004). It has been shown that the growth of houseflies in an atmosphere of 10% O_2 markedly reduced their mean and maximum life span and inereased the rate of accumulation of protein carbonyls in whole body extracts (Sohal *et al.,* 1993) and in isolated mitoehondria (Sohal & Dubey, 1994). Similarly, elevated atmospheric O_2 decreased the mean and maximum life spans of nematodes (Honda *et al.,* 1993).

Most studies are in agreement that mitochondria are the main source for free radicals produetion (Bioveris & Chance, 1973; Cutler, 1986; Dizdarglu, 1992a; Ku & Sohal, 1993; Sohal & Dubey, 1994; Barja, 1999). In addition, mitochondria DNA (mtDNA) damage occurs much faster than does nuclear DNA because mitochondrial DNA is not protected by proteins, rather it is attaehed to the inner mitochondrial membrane (Dizdaroglu, 1992b; Bekman & Ames, 1997). The damaged mtDNA aeeumulates in the cells progressively because damaged mitochondria replicate faster than undamaged mitochondria. Thus replicated damaged mitochondria retain damage with time passage, aging.

Furthermore, free radicals promote formation of cross link between biomolcules (Dyer *et al.,* 1991; Barlett & Stadman, 1997; Iqbal *et al.,* 1999; Braun, 2000; Haffor, *et al.,* 2002) whieh in turn reduce movement of molecules for normal chemieal reactions. It is not clear how the combined effects of hyperoxia and aging influencc free radical hyperoxia on free radieal production in two groups of rats; old and young.

Materials and Methods

Rats and Hyperoxia Treatment

Sixteen male rats, 8 old with mean age 2.5 years and 8 young mean age of 7 weeks. Both groups underwent hyperoxia exposure for 4-5 hrs daily for 4.5 weeks period.

Hyperoxia exposure

Experimental groups underwent hyperoxia cxposure for 2 hours daily for six weeks period. Experimental animals were placed in a elosed 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 liter per minute (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 3 LPM to ensure that the concentration of oxygen in the box remains equal to 100% O_2.

Blood Samples Collection

Blood samples were colleeted from the retro-orbital plexus using heparinized capillary tube into heparinized chilled glass. The vials were centrifuged immediately at 3,000 rgm for 10 minutes. Plasma was then separated and then analyzed for free radical concentration.

Colorimetric Test for Radical Determination

Free radical was measured in plasma, using the d-ROMs-2 test kits (FRAS-II, 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 (FRAS-II, 2002). 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:

$$H_2O_2 + Fe^{+\cdot} = {}^{*}OH + OH^{-} + Fc^{+-}$$

Thus, the hydrogen peroxides of biological sample (whole blood) generate free radicals (alcoxy and peroxyl radicals) after exposure to a transitional metal (F^{-+} / $Fe^{-++)}$. When a correctly buffered chromogen substance (N, N-diethyl-phenylendiamine) lead to the reduction of hydrogen peroxides which in turn 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 corresponds to 0.08 mg H_2O_2 / 100 ml (FRAS II, Italy, 2002).

Results

Baseline mean free radicals were 439.5±42.7 and 409±17.33 CARR U for the young and old groups, respectively. These values correspond to 35.00 and 32.72mg H_2O_2/100mL which were greater than average human values of about 25mg/100ml (FRAS-2, 2002). The reason that our values were some what higher was due to the fact that blood samples were taken on normal feeding condition. Thus the elevated basclinc FR could be attributed to the influence of feeding or hypercaloric factor.

The results of student *t*-test showed that mean free radicals production levels increased significantly ($P<0.05$) in both groups after exposure to hyperoxia (Table 1). Before and after exposure to hyperoxia, the differences in free radicals production between the young and the old groups were not significant ($P > 0.05$). The finding of no significant difference among aging groups was not surprising. It is very important to realize that our measurements were based on the rate of free radicals production rather than removal rate. Evidently exogenous hyperoxia result in an over production in reactive oxygen species (ROS) which should be related to free radicals production rate which is independent of clearance rate. The later, removal rate is affeeted by endogenous rather than exogenous mechanisms. It is true that aging could havc altered oxidative imbalance differently in different aging groups but via endogenous mechanisms that are subject to change with time, aging. Thus aging can be a cause for increased free radicals levels via slowing down in elimination rate rather than production rate, having all conditions met.

Table (1). Free radicals concentration before and After Exposure to Hyperoxia.

Animal	Young Group FR (Carr U)*		Old Group FR (Carr U)*	
	Before Exposure	After Exposure	Before Exposure	After Exposure
1	492	652	430	460
2	430	552	372	564
3	468	810	422	638
4	392	510	412	592
5	380	418	409	638
6	414	456	413	610
7	450	764	401	756
8	490	726	415	608
Average	439.50	611.00	409.25	608.25
SD	42.70	147.80	17.33	82.64

*Values are average of triplicate measurements.

Discussion

The major findings of the present study showed that hyperoxia resulted in an increased rate of free radicals production. There is a general agreement that mitochondria are the major oxygen radicals producing sites. Mitochondria release hydrogen peroxide to the cytosol, leading to imbalance between its generation and elimination by cellular antioxidants. It has been shown that cellular oxidative damage was related to cross links between lipid accumulation (Beuchat & Chong, 1998), protein (Barlett & Stadman, 1997; Iqhal *et al.,* 1999; Braun, 2000) and DNA (Beckman and Ames, 1997).

Free radicals age-related studies have focused on antioxidant rather than exogenous oxidants factors. Early comparative studies suggest that antioxidants were longevity determinants (Cutler, 1986). It has been reported (Ogburn *et al.,* 1988; Yan *et al.,* 1994) that, when tissue antioxidants were directly studied as a function of mean life span (MLSP) it was proved that FR production is lower in long-lived than in short-lived. The more long-lived is undergone, the smaller are tissue levels of endogenous antioxidants. Thus the rate of FR production in tissues must he low in long-lived animas. This would result in lower oxidative damage and slow rate of aging. This is not the case in pigeon. Comparisons of different tissues, liver, kidney, heart, would provide a question. How could birds live longer with high aerobic metabolic rate and then strong ROS generations. In addition, Pigeon MLSP is nine folds higher (35yrs) than rats (4yrs), whereas basal metabolic rate (BMR) and body size are similar (Holmes and Austand, 1995). It was found that pigeons had less mitochondrial ROS generation than rats in all the organs studied that include brain, liver, lungs, heart and kidney (Ku & Sohal, 1993; Sohal *et al.,* 1993). It was also found that pigeons had less ROS in respiratory chain. Thus it is the rate of ROS production rather than removal rate which correlates negatively with MLSP. Moreover, it was found that difference in H_2O_2 production with pyruvate / malate between the two species (pigeon vs. rats) disappeared after addition of rotenone, which fully reduces complex I with those substrates (Barja, 1999). Based on ultrastructure, mitochondria pathological changes induced by hyperoxia in pigeon (Haffor *et al.,* 2002), we believe that the continued rise in free radical production along with failure of defense mechanisms to effectively neutralize H_2O_2 toxic intermediates and to prevent significant free radical injury have become an important issue. The results are cross-links among the protein strands, and they then

resemble strip which becomes tangled together. The cross-linkage is extremely stable and long lasting. Alternatively, the amino acid/glucose portion may join with each other on the same or different protein molecules-maillard reaction. Forming glucose cross-linkage, are more probable because maillard reaction glucose reacts without the use of enzymes.

Oxidative damage to nucleic acids includes adducts of base and sugar groups (Dizdarglu, 1992a; Dizdarglu, 1992b, Epe, 1996), single-and double-links to other molecules (Halliwell, 1992). In addition exogenous hyperoxia places an oxidative stress during oxidation in the respiratory chain located in the mitochondrial crestae as evident by increasing FR production in the present study.

In conclusion, exogenous hyperbaric oxygen resulted in an increased rate of free radicals production. Further research is needed to identify the molecular basis of hyperoxia induced oxidative stress among species with different age groups.

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تأثير الضغط الأوكسجيني المحدث انتاج الجذور الحرة

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المستخلص. بات واضحاً أن التغيرات البيئية المصاحبة للتقدم الصناعي ذات أثر ملموس على حياة المجتمع . فالغازات النشطة مثل أول وثاني أكسيد الكربون، وأكسيد النيتروجين تساهم في رفع مستوى الجذور الحرة في الجسم. في دراسة سابقة معملية ظهر أن زيادة التعرض للأكسجين تسبب أضرارًا للخلايا وتغيرات مختلفة مثل انحراف الأعراف وتشوه الماتريكس في المتيوكوندريا. هذه الشذوذات تنسب إلى أكسدة الدهون في أغشية الميتوكوندريا. حتى الآن فإن التأثير المشترك للعمر والأكسجين على انتاج الجذور الحرة وشذوذات الميتوكوندريا ليس معروفاً. في الدراسة الحالية نرصد تأثير التعرض للأكسجين الزائد على إنتاج الجذور الحرة في مجموعتين من الجرذان (فتية وهرمة). أوضحت النتائج أن التعرض للأكسجين تسبب فى زيادة إنتاج الجذور الحرة في المجموعتين بالمقارنة مع ماقبل التعرض، فلقد كان متوسط الجذور الحرة للمجموعة الفتية 439.50 والهرمة 409.25 CarrU. وبعد تعريض الحيوانات للأكسجين ازداد معدل الجذور الحرة زيادة معنوية ($p<0.05$) إلى 611.00 فى المجموعة الفتية و608.25 فى المجموعة الهرمة . وعلى هذا فإن التعرض للأكسجين يؤدي إلى زيادة إنتاج الجذور الحرة سواء فى صغارأو كبارالعمر. ولذا يمكن أن نستنتج أن التعرض للأكسجين الزائد يؤدى إلى كبح التنفس الداخلي للميتوكوندريا في الجرذان.