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COMPARATIVE TOXICOLOGY OF OZONE AND t-BUTYL HYDROPEROXIDE ON ISOLATED RAT LUNG

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Comparative studies were undertaken on the effects of ozone and t-butyl hydroperoxide (t-BuOOH) on alkane production, glutathione and lactate dehydrogenase (LDH) release from isolated rat lungs. Specifically, ethane and pentane production, as well as glutathione and LDH release were simultaneously determined at different time intervals from the isolated lung preparation in the absence and presence of toxic concentrations of ozone or t-BuOOH. When compared to control conditions, both toxic agents produced a marked decrease in the dry weight to wet weight ratios, reflecting the development of acute pulmonary edema. These toxic agents also caused a marked increase in the efflux of glutathione into the effluent. A much higher glutathione efflux into the effluent was observed during t-BuOOH perfusion in comparison to ozone exposure. In parallel with the enhancement of glutathione release a significant increase in ethane production was also observed during t-BuOOH perfusion. However, the production of either alkane by ozone inhalation was not significantly different from that in the control conditions. Unlike glutathione release, there was no marked increase of LDH release into the effluent following exposure to t-BuOOH or ozone. This suggests that the observed increase in glutathione release caused by t-BuOOH or ozone treatment was not associated with non-specific destruction of cell membrane integrity.

KEY WORDS: Ethane, pentane, t-butyl hydroperoxide, rat lung, glutathione, lactate dehydrogenase.

INTRODUCTION

Although evidence exists for ozone-induced lipid peroxidation of subcellular fractions *in vitro*[1] and in conscious rats fed a vitamin E deficient diet,[2] it is not known whether in parallel with the development of the acute toxic effect of ozone, such as pulmonary edema, there is an increased release of oxidative products.

In order to acquire further insight into this aspect, a newly developed method[3] to measure alkane production, a direct index of lipid-peroxidation, during administration of ozone to isolated lungs was utilized. Specifically, whether the levels of alkanes, glutathione and LDH released by isolated rat lungs are increased in parallel following exposure to an edemagenic concentration of ozone was investigated. The responses to ozone were compared with those of t-BuOOH, a model peroxidative agent.

METHODS

(a) Technique of Lung Perfusion

Male Wistar rats between 300–600 g were used (n = 13). One or two days prior to their use they were brought into the laboratory, given unrestricted availability to food and water. The isolated rat lung was prepared and perfused by a modification of the procedure of Fisher *et al.*[4]

Briefly, in preparation for transfer of the lungs from the chest cavity, the rat was deeply anesthetized. A thoracotomy was performed and the pulmonary artery was cleanly dissected from other mediastinal structures and cannulated for delivery of HEPES (1.096 g/L) buffered salt solution (Na^+ 139, K^+ 5.9, Ca^{++} 1.25 and Mg^{++} 1.2 mM and glucose 10 mM) by a peristaltic pump from a reservoir.

Following the washout of blood, the lid with the attached lung, was inserted into the top of the perfusion chamber as previously described.[3] Because the seal between the lid and the collection chamber was constructed of ground glass, an air-tight connection was established which facilitated alkane accumulation in the head space of the chamber as shown in Figure 1. In addition to two openings for ventilation of the lungs, the lid had three other openings. One was used for collection of headspace air-samples for measurement of alkane content; another was used to deliver perfusate to the lungs. The third one was connected to a water manostat to insure that peak respiratory pressures in the chamber were less than 5–7 cm H_2O while allowing no leakage of air from the collection chamber. These studies have clearly exhibited that the use of the manostat prevents the rapid development of edema and deterioration of the preparation.

The peristaltic pump maintained a constant flow rate of 10 ml/min during the entire experimental period. Upon perfusion of the isolated lungs the perfusate freely drained

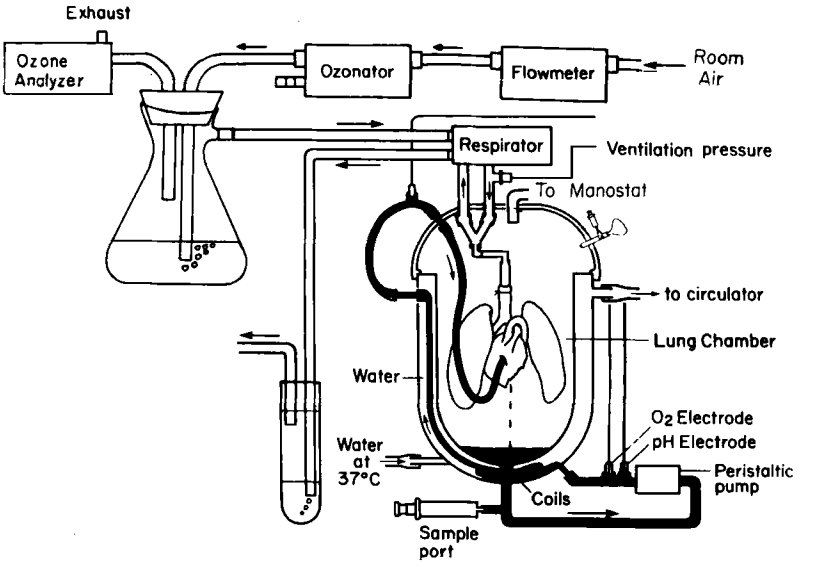

FIGURE 1 A schematic diagram of the alkane collection chamber with lid as used for perfusion of the isolated lungs and recording of tracheal pressure (P_{TR}). See text for details.

into the side arm at the bottom of the chamber allowing sampling for chemical analyses and recirculation through an oxygen electrode before reentering the lungs. Therefore, a continuous record was maintained of oxygen tension of the perfusate. Also, moment to moment performance of the isolated lungs was monitored by continuously measuring ventilation pressure (P_{TR}), with the aid of an electro-mechanical recorder.

(b) Ozone Generation

For exposure of the isolated lungs to filtered room air or ozonated air, the inlet of the respirator was connected to a conical flask containing distilled water to humidify the inspired control or ozonated air (Figure 1) and operated at a rate of 48 strokes/min with a 2 ml tidal volume. To ozonate the inspired gas air was drawn by a membrane pump located in the ozone analyzer at a constant flow rate of 3 L/min through an ultraviolet light source (ozonator) prior to entry into the humidifying flask. When desired the ultraviolet light was turned on and adjusted so as to produce 22–25 ppm of ozone. Because exposure to 1.2 ppm ozone for 24 hours was required to produce pulmonary edema in conscious rats,[5] the ozone concentration was increased 20-fold in order to induce similar effect within one hour. During the equilibration period and experimental period of 60 min, the ozone level was monitored continuously by sampling the humidified gas mixture from the conical flask by an ozone analyzer (Dasibi Environmental Corp, Glendale, California).

(c) Analysis of Air and Effluent Samples

Ethane and pentane that accumulated in the headspace of the alkane collection chamber was measured by withdrawing 5 ml air samples at appropriate intervals. The samples were injected immediately into a sample loop of a gas chromatograph (Carlo Erba Fractovap Model 2151C or Varian 3700) for measurement of alkane peaks. These were compared with known ethane and pentane peaks and quantitated by a graphic approximation method.[6]

Similarly, for measurement of lactate dehydrogenase activity and glutathione content in perfusate, 1 ml effluent sample was withdrawn and an aliquot from each sample was used to determine LDH and glutathione by enzymatic analyses following the methods of Ishikawa and Sies,[7] and Tietze,[8] respectively.

(d) Experimental Protocol

Following transfer of the perfused lungs into the alkane collection chamber and sealing of the chamber, air samples from the headspace were collected at 30, 60 and 90 minutes. Effluent samples were collected for the measurement of LDH at 15, 30, 45, 60, 75 and 90 min, while glutathione samples were collected using three protocols each of which utilized 95 min of perfusion. Approximately 4–5 perfused lungs were used to generate sufficient data for alkane formation, glutathione and LDH release in each protocol. The 95 min of perfusion was divided into 3 periods: an equilibration period (0—30 min) with ventilation in room air and perfusion with HEPES buffer solution; an experimental period (30–60 min) when lungs were ventilated with control gas (room air) and perfused with HEPES solution (protocol 1); or ventilated with ozonated room air and perfused with HEPES solution (protocol 2); or ventilated with

room air and perfused with HEPES solution containing 400 μM t-BuOOH which was infused during this time-period by means of a syringe pump (protocol 3); a post-exposure observation period (60–90 min) when the ventilator was stopped to prevent possible escape of alkanes from the perfused lungs and thereby, to facilitate their accumulation in the collection chamber. At the end of 90 min the respirator was turned on for 5 min in order to determine the effect of ventilation on the expiration of the alkanes that accumulated in the chamber between 60–90 min. Five minutes of ventilation did not seem to effect much loss of alkanes from the chamber. At the end of the experiment the t-BuOOH- or ozone-exposed or control lungs were detached from the system and gently blotted. The wet weight was then determined. Following this procedure the lungs were dried overnight and weighed once again to determine pulmonary tissue water content in order to ascertain whether lungs had shown evidence of edema or not.

(e) Statistical Analysis

Alkane production and release of LDH and glutathione from each group were expressed per gram dry weight of lung for the specified intervals as appropriate. The alkane data for each time-interval were separately compared among 3 groups using an analysis of variance (ANOVA) followed by the application of the Newman–Keuls test utilizing a computer program written for Apple IIe by B. Modrak (Glaxo Inc., Triangle Park, NC). P lower than 0.05 level of significance was considered statistically significant. The LDH and glutathione results were subjected to the t-test.

RESULTS AND DISCUSSION

Figure 2A depicts the oxygen concentration of the perfusate during the 95-min experimental period from a representative control study. There is a rapid equilibration of oxygen with the perfusate after transfer of lungs to the chamber and ventilation with inspired air. Thus, from 0–60 min the perfusate oxygen concentration was stable at 3.6–3.4 μL/ml. When ventilation was discontinued (60–90 min) oxygen concentration in the perfusate decreased to a value of approximately 2.4 μL/ml. Although steady-state oxygen concentration in the perfusate between 0–60 min and drop in oxygen during 60–90 min varied from experiment to experiment, oxygen concentration of the perfusate was not affected by the ozone or t-BuOOH treatment of the lungs.

Tracheal pressure for control, ozone-treated and t-BuOOH perfused lungs is shown in Figure 2B. During control conditions peak inspiratory ventilation pressure was constant. Thus, in control preparation there was not much accumulation of fluid during the experimental period. In contrast, both ozone and t-BuOOH treatment increased tracheal pressure. Tracheal pressure was greater following the inhalation of ozone than during infusion of t-BuOOH. This marked increase in tracheal pressure as caused by the inhaled ozone may be due to its direct effect on airway smooth muscle.

Figure 2C illustrates changes in the dry weight to wet weight ratio of the control lungs at the end of the perfusion period, and challenged with an edemagenic concentration of ozone or 400 μM t-BuOOH. As anticipated from their respective effects on tracheal pressure, both ozone and t-BuOOH treatment caused edema as reflected

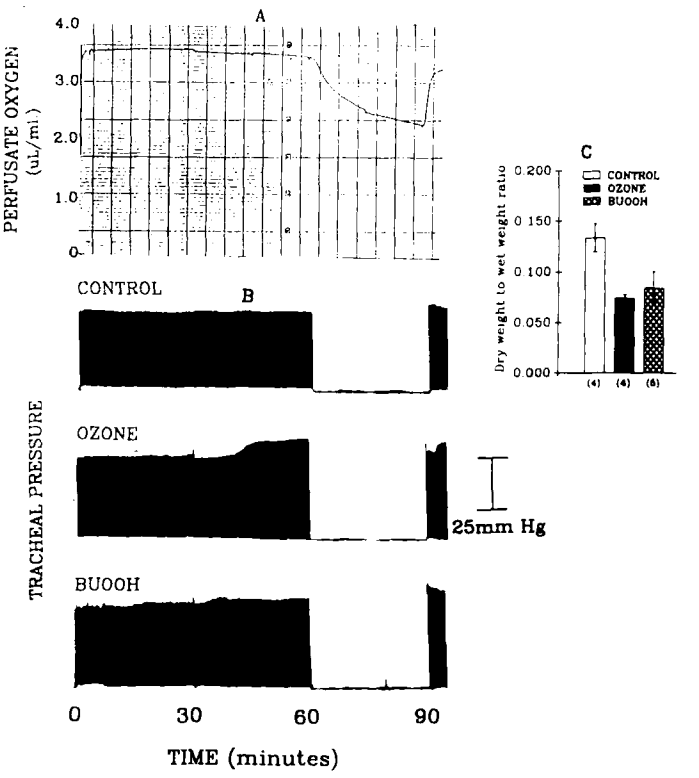

FIGURE 2 A: Oxygen concentration of the perfusate as observed during 0–95 min from a typical control experiment. B: Tracheal pressure as recorded during the experimental period from a typical control, ozone- and t-BuOOH-treated isolated lung. C: Dry weight to wet weight ratio of isolated perfused rat lungs at the end of the control, ozone- and t-BuOOH-treated experiments. The bar and parenthesis represent the mean ± S.E. and the number of experiments in each group.

by a significant decrease of the dry weight to wet weight ratio. Fisher[4] reported that in uncomplicated lung perfusion experiments the dry weight to wet weight ratio was observed to be approximately 0.17. The current study observed a slightly lower (0.134 ± 0.014) ratio for the control experiments, perhaps due to the perfusion of the lungs with an albumin-free medium. However, in contrast to the control value for the ratio, the average ratio for the ozone- and t-BuOOH-treated lungs decreased to 0.075 ± 0.022 ($p < 0.01$) and 0.084 ± 0.004 ($p < 0.1$), respectively. The average wet weight of the lungs increased 2.5-fold from that of the control as a result of edema caused by ozone or t-BuOOH treatment.

Figures 3A, 3B and 3C illustrate gas chromatograms for a variety of alkanes detected from control, ozone- and t-BuOOH-treated experiments. By comparing retention time of the known alkane standards (Messer Griesheim, Duisburg, West Germany), ethane and pentane have been identified as the first and fifth peak after the air peak. As noted in figure 3A, at 0 min a low background level of ethane was detected in the headspace of the alkane collection chamber. Analogous to our previous study,[3] there was little increase in ethane production between 0–90 min. Similarly, there was little pentane production in the control lung between 0–90 min.

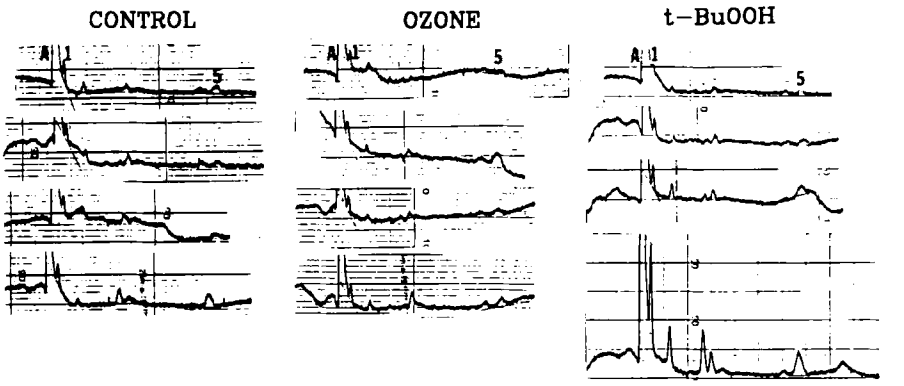

FIGURE 3 Control (A): Gas chromatographic records of air samples collected from a typical isolated lung experiment under control conditions; air samples were collected at 0 (top panel), 30 (upper middle), 60 (bottom middle) and 90 min (bottom) from the headspace of the collection chamber. Ozone (B): Gas chromatographs of air samples collected from a typical ozone exposed isolated lung, at similar time intervals as indicated for the control. T-BuOOH (C): Gas chromatographs of air samples from a typical t-BuOOH-treated isolated lung. Samples have been collected as described above.

As noted in Figure 3B, because of ozone treatment there was not much change in ethane or pentane production. In contrast, t-BuOOH treatment significantly increased ethane production (Figure 3C) between 60–90 min in comparison to the control (Figure 3A) and ozone-treated (Figure 3B) lungs. Thus, when alkanes were produced in excess amounts because of lipid peroxidation, they appeared to release from the surface of the lungs or from the circulating perfusate into the air-tight chamber, and, therefore, accumulated there. These accumulated alkanes were retained in the chamber even after ventilation of the lungs for 5 minutes (data not shown).

Average values of ethane and pentane production from the three groups of experiments are shown in Table I. Of the 13 isolated lungs used in this study, 5 preparations showed no detectable increase in ethane production between 0 and 30 min. The remaining 8 preparations produced 20 ± 9 pmol ethane per gm dry weight. Because of perfusion of t-BuOOH between 30–60 min, ethane production increased to

TABLE I

Average values of ethane and pentane production from three groups of experiments. Ethane and pentane were measured at the end of the period between 0–30, 30–60, 60–90 min.

	Ethane (pmol/gm dry wt.)			Pentane (pmol/gm dry wt.)		
	Time (minutes)			Time (minutes)		
Groups	30	60	90	30	60	90
Control	20 ± 9	–	63 ± 59	198 ± 37	154 ± 42	222 ± 47
Ozone	20 ± 9	37 ± 15	78 ± 31	198 ± 37	202 ± 54	434 ± 112
t-BuOOH	20 ± 9	387 ± 210	1365 ± 471	198 ± 37	237 ± 75	726 ± 354

The results (mean ± S.E.) have been analyzed for statistical difference by ANOVA followed by the Newman-Keuls test. The ethane value for the t-BuOOH group at 90 min is significantly ($p < 0.05$) different from the control and ozone group at different time intervals. The pentane value for the t-BuOOH also showed similar ($p < 0.05$) significant differences from the other two groups.

387 ± 210 and 1365 ± 471 pmol/gm dry weight at 60 and 90 min, respectively. The mean ethane concentration in the chamber remained high (912 ± 312 pmols/gm dry wt.) even after the ventilation was resumed between 90–95 min. In the presence of t-BuOOH the release of pentane also increased markedly between 60 and 90 min.

The average values from four ozone-treated experiments as reported in Table I confirmed the records presented in Figure 3B that there was not much increase in alkane production due to ozone. On the other hand, at 60 min ozone moderately ($p < 0.1$) increased release of glutathione into the effluent relative to the control lungs at a similar time interval (Figure 4). Since ozone does not show much of an effect on alkane production and moderate increase in glutathione release, this implies that by reacting with proteins and/or other elements of the cell surface ozone might have produced oxidative changes which have led to an increased permeability of cell membranes causing edema. Because there was perhaps only limited oxidation of polyunsaturated fatty acids during this acute exposure to ozone and, therefore, limited production of lipid peroxides, this required only limited participation of the glutathione peroxidase/reductase defence system, causing only a moderate increase in glutathione release into the perfusate in comparison to the marked effect of t-BuOOH on this parameter.

The previous study of Nishiki *et al.*[9] appears to support this contention. They demonstrated that when perfused lungs were subjected to hyperbaric oxygenation, a treatment similar to ozone exposure, glutathione was released into the effluent in very low amounts; this effect was enhanced only by using lungs from tocopherol-deficient rats. In view of this observation it is probable that ozone will show much more prominent glutathione release and, consequently, alkane release in lungs from tocopherol-deficient rats.

Figure 5 shows the release of lactate dehydrogenase from the isolated lungs under control conditions, and after treatment with t-BuOOH or ozone. Although there is no statistical difference in LDH release among the 3 groups at 60 or 90 min, the release of LDH was least from the t-BuOOH lungs. This indicates that the observed increase

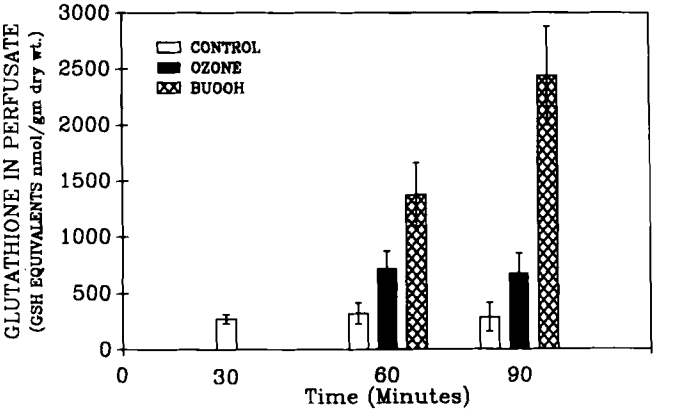

FIGURE 4 Glutathione release into the effluent from four control, four ozone-exposed and five t-BuOOH-perfused lungs. Each point represents a mean ± S.E. of at least 4 individual experiments. The values from the t-BuOOH-treated group were significantly ($p < 0.02$) higher than controls at 30′ vs 60′, 30′ vs 90′, 60′ vs 60′ and 90′ vs 90′. The values from the ozone-exposed group were significantly ($p < 0.05$) higher than the control only at 30′ vs 60′ and 30′ vs 90′.

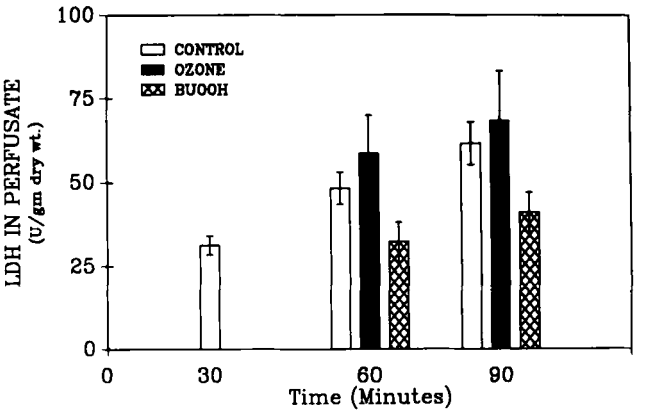

FIGURE 5 LDH release into the effluent from four control, four ozone-exposed and five t-BuOOH-perfused lungs. Each point represents a mean of at least 4 individual experiments.

in glutathione release (Figure 4) from the t-BuOOH-treated lungs was caused by the reaction of glutathione peroxidase and not by oxidative destruction of cell membrane integrity.

Finally, it is essential to mention that the recent study of Seeger *et al.*[10] was also unable to detect any enhancement of alkane release in the exhaled air from the isolated rabbit lungs following exposure to 5 ppm ozone for 30 min and 7 ppm for 60 min. Since two lungs so treated did not show any change in tracheal pressure or lung weight, it is possible that in their study ozone concentrations may not have been sufficient to induce appropriate oxidative stress. However, they also reported negative results, by the application of other peroxidative agents such as high doses of H_2O_2, $FeCl_2$/ascorbate or paraquat, which could not be explained on the basis of the dosage used. Thus, it is possible that in our study most of the volatile hydrocarbons, especially ethane, produced during the oxidative stress, were released from the surface of the lungs or from the perfusate into the air-tight chamber and, therefore, by sampling the chamber headspace air we could measure them. Also, stoppage of ventilation between 60–90 min following 30 min of oxidative stress, facilitated alkane accumulation in the chamber.

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References

1. Roehm, J., Hadley, J.G. and Menzel, D.B. The influence of vitamin E on the lung fatty acids of rats exposed to ozone. *Arch. Environ. Health,* **23,** 234–242, (1982).
2. Dumelin, E.E., Dillard, C.J. and Tappel, A.L. Effect of vitamin E and ozone on pentane and ethane expired by rats. *Arch. Environ. Health,* **33,** 129–134, (1978).
3. Dutta, S., Müller, A., Ishikawa, T., Zimmer M. and Sies, H. Alkane production by isolated rat heart and lung. *Toxicol. Lett.,* **44,** 55–64, (1988).

4. Fisher, A.B. The isolated perfused lung. In Toxicology of Inhaled Materials, edited by H.P. Witschi and J.D. Brain. Vol. 75, pp. 149–179. Berlin, Heidelberg, New York, Tokyo: Springer-Verlag, (1985).
5. Kobayashi, T. Effect of ozone exposure on prostacyclin synthesis in lung. *Prostaglandins*, **26,** 1021–1027.
6. Müller A. and Sies, H. Assay of ethane and pentane from isolated organs and cells. *Meth. Enzymol.*, **105,** 311–319, (1984).
7. Ishikawa, T. and Sies, H. Cardiac transport of glutathione disulfide and S-conjugate. *J. Biol. Chem.*, **259,** 3838–3843, (1984).
8. Tietze, F. (1969) Enzymatic method for quantitative determination of nanogram amounts of total and oxidized glutathione: application to mammalian blood and other tissues. *J. Anal. Biochem.*, **27,** 502–522, (1969).
9. Nishiki, K., Jamieson, D., Oshino, N. and Chance, B. Oxygen toxicity in the perfused rat liver and lung under hyperbaric conditions. *Biochem. J.*, **160,** 343–355, (1976).
10. Seeger, W., Remy, N. and Neuhof, H. A highly sensitive gas chromatographic method does not detect exhalation of volatile hydrocarbons from ventilated lungs under massive peroxidative stress. *Exp. Lung Res.*, **14,** 387–401, (1988).

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