Is Atmospheric Superoxide Vitally Necessary? Accelerated Death of Animals in a Quasi-Neutral Electric Atmosphere

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To estimate the necessity of air ions (Als) as a natural source of atmospheric gaseous superoxide for mammalia the effect of air ion deprivation on mice and rats was investigated. Ambient air deionization inside an experimental acrylic glass cage (AGC) was performed by electrostatic field, built up by acrylic glass surfaces. Under these conditions, four hours after the animals were placed into the AGC, the concentration of negative Als was not detectable, the concentration of positive ions was (mean ± SD) 77 ± 18 ionsxcm\textsuperscript{-3}. The negative and positive AI concentrations in identical silicate glass cages (control) were 482 ± 128 ionsxcm\textsuperscript{-3} and 660 ± 148 ionsxcm\textsuperscript{-3} respectively. It was found that the prolonged deficiency of Als in ambient air leads to accelerated death of the animals. The life span of the deprived mice and rats was 16.2 ± 0.9 and 23.0 ± 1.1 days respectively. The pathological symptoms and ultrastructural changes in the adeno- and neurohypophysis in deprived animals observed strongly suggest that animal death is related to disturbances in neurohormonal regulation and pituitary insufficiency. The possible physiological need of Als and atmospheric superoxide, and its role in the development of environmental stress in human beings and in particular in premature infants is discussed.

Introduction

Atmospheric (air) ions accompany terrestrial organisms throughout their lives. Under natural conditions the biologically relevant Als are formed in the lower part of the atmosphere. The principal initial energy sources for the ionisation of gas molecules include cosmic rays and the natural radioactivity of the atmosphere and soil as well as electrical discharges (Chalmers, 1967). According to the certain views the positive ion pool contains initial gaseous ions as N\textsubscript{2}\textsuperscript{+} and O\textsubscript{2}\textsuperscript{+}, and secondary products such as N\textsubscript{2}H\textsuperscript{+}, NO\textsuperscript{+}, H\textsubscript{2}O\textsuperscript{+}. The negative gaseous ions are represented mainly by oxygen ions like O\textsuperscript{–}, O\textsubscript{2}\textsuperscript{–}, and O\textsubscript{3}\textsuperscript{–} as well as oxides as CO\textsubscript{3}\textsuperscript{–}, CO\textsubscript{4}\textsuperscript{–}, NO\textsubscript{3}\textsuperscript{–} and to a lesser extent NO\textsubscript{2}\textsuperscript{–}. Under natural conditions Als occur in a concentration of about 10\textsuperscript{2} – 10\textsuperscript{3} ions x cm\textsuperscript{-3} air. For an additional documentation of atmospheric ion formation, composition and transformation see Pethig (1984) and Dolezalek (1985).

Possibly one of the most important biologically active species of Als is the superoxide radical O\textsubscript{2}\textsuperscript{–} (Goldstein, 1988). This radical is involved in the Als bactericidal effect mechanism (Kellogg et al., 1979). The products formed in the negatively charged oxygen reduce cytochrome c and nitroblue tetrazolium, and lead to the formation of hydrogen peroxide (Goldstein et al., 1992). Inhaled gaseous superoxide decreases the temporal threshold in the spatial synchronisation of the brain biopotentials (Arshavsky et al., 1991), potentiates the antinociceptive effect of analgesic agents, suppresses the MAO-B activity and lipid peroxidation in the basal ganglia of the rats, and facilitates the pathological signs in the patients with parkinsonism (Goldstein and Lewin, 1996; Goldstein et al., 1996; Goldstein et al., 1997). In this connection gaseous superoxide might be an important environmental factor.

However, the biological activity and significance of Als is not universally recognised. According to
Reiter (1992) the question of the biological importance of AIs is meaningless because of the extremely small natural fluctuations in their concentrations. Because of the methodical problems connected with AI investigations, the evidence of their biological activity and significance had been the subject of severe criticism (Charry, 1987). The problems, both technical and conceptual, related to the biological impact of AIs had also been previously discussed in detail (Krueger and Reed, 1976). The major factors contributing to errors of observation were 1) the neglect of by-products such as ozone and/or nitrogen oxides, 2) the failure to monitor and control ion densities, temperature, and humidity, 3) the use of air containing reactive gaseous pollutants, and 4) electrostatic repulsion of AIs by experimental subjects. It is also known that the experimentally applied artificial AI concentrations are about 10 to 1000 times those in natural environments. In addition the artificially produced AI pool does not correspond qualitatively and quantitatively to natural conditions (Kröling, 1985). The importance of AIs and, in particular, gaseous superoxide as an environmental factor remains unknown, too.

Obviously the biological necessity of AIs can be revealed under conditions of their deprivation. However, to the best of our knowledge there are only some reports on this subject. Tchijevsky (1941) was the first to observe impaired behaviour and weight loss in laboratory animals when the animal cages were supplied with deionised air. Moreover, many animals died within five weeks. The pathological causes of the animals' deaths remained unclear. In this context it is interesting that attempts to create a closed ecological system in the USSR in the early sixties as a part of the space research program encountered difficulties because the experimental animals (mice, rats) died within 3 weeks by unclear causes (Rehberg, personal communication). Later it was demonstrated that a partial AI deprivation for one week causes the suppression of the immune system, in particular the weakening of cellular immunity and the lack of blood clotting by inflammation, and the destabilisation of platelets in guinea-pigs (Skards, 1968). Unfortunately all these observations furnish no quantitative data on the concentrations of AIs inside the experimental cages. In addition, one has to keep in mind that the elimination of AIs from the air entering the cage does not exclude the ionisation of gas molecules inside the cage by natural radioactivity. This fact could lead to an unsuccessful experiment with AI deprivation.

As mentioned above recent evidence suggests that the biological activity of negative AIs is attributed to superoxide. Therefore, in this connection the biological effects and vital necessity of atmospheric superoxide are of special interest in relation to the important and in many respects paradoxical role of metabolic superoxide (McCord, 1995). The objective of this work was to study the effect of uninterrupted AI deprivation under controlled conditions on the physiological status and life span of animals. In addition, with relation to the possible involvement of the adeno- and neurohypophysis in the physiological responses to inhaled AIs, a morphological study of these structures was carried out.

Materials and Methods

Experimental cages and air ion measurements

The experiments were performed in similar closed cages (65 × 60 × 54 cm, volume ca 210 l) made of emission-free acrylic glass (AGC, Fig. 1) to create air deionisation conditions, and from a combination of silicate glass and plywood (SGC) for the control animals. The elimination of AIs in AGC was achieved by an electrostatic effect on the inner surfaces of the acrylic glass plate and on the double-walled lid (distance between lid plates was 5 cm). The lid plates were equipped with ventilation openings (inner diameter 20 and 6 mm) and a propeller with acrylic glass blades. The SGC lower part and lid were made of wood and its propeller was made of metal sheet. The flow rate of ventilated air was 1.51 × min⁻¹. All handling was carried out without fresh air entering the cages. Both cages were placed on an earthed surface. The air temperature in the cages was 21 ± 2 °C and the relative humidity 75–80%. Water vapour, CO₂ and NH₃ were removed with the waste-air flow through ventilation slits. Twelve hours after the animals were placed in the cages, the CO₂ concentration was (mean ± SD) 0.16 ± 0.03% by vol. in the AGC and 0.17 ± 0.04% by vol. in the SGC, and the NH₃ concentration, 7.2 ± 0.6 ppm and 7.2 ± 0.7 ppm, respectively. The AIs concentration was measured with an SAI-TGU-66m electromet-
Fig. 1. Experimental acrylate glass cage (schematised): (a) double-walled lid; (b) input ventilation openings; (c) propeller; (d) cleaning and ventilation openings; (e) ventilation output; (f) additional absorber for CO₂ and water vapour; (g) opening for CO₂ and NH₃ concentration measurements; (h) opening for AI concentration measurements; (i) drinking trough; (j) feeding rack; (l) cleaning device.

The study. The mean life span of the animals was determined and the macroscopic and microscopic examination performed. The physiological parameters and macroscopic examination were performed on rats on days 10 and 20 of deprivation and on mice on days 7 and 14 of deprivation. The electron-microscopy study of the rat pituitary was performed on day 10 of deprivation.

Electron-microscopy study

The rats were decapitated under ether anaesthesia. After decapitation the brain area containing the pituitary was fixed in 2.5% phosphate buffered glutaraldehyde (pH = 7.2), postfixed in 2% phosphate buffered osmium tetroxide and contrasted with 1.5% uranyl acetate in 70° ethyl alcohol. After dehydration the tissue was embedded in epon-araldite. Ultrathin sections were additionally contrasted with lead citrate (Reynolds, 1963) and studied under an electron microscope JEM – 100 K (Japan). The percentage of the neurosecretory terminals (NSTs) representing four tentatively differentiated functional stages (Arshavskaya-Popovich et al., 1985) was counted in the postpituitary. More than 200 NST were counted in each animal group investigated. The morphofunctional state of the adenohypophysis was estimated as a proportion of adrenocorticotropic hormone (ACTH), somatotropic hormone (STH), follicle-stimulating hormone (FSH), luteinizing hormone (LH), thyroid-stimulating hormone (TSH) and luteotropic hormone (LTH) producing adenocytes as well as agranular follicle-stellar (AFS) cells and degenerating adenocytes identified according to the integrity of the typical ultrastructural signs (Gordienko and Koziritsky, 1978).

Physiological parameters

Spontaneous vertical activity (VA) and the hole board test (HBT) in animals were investigated with semi-automatic registration. Body and organ weight, food and water intake as well as rectal temperature were measured.

Statistics

The mean values and the standard deviation (mean ± SD) of the physiological parameters and the survival time were determined and calculated.
Table I. Typical parameters of the ambient air in the cages.1

<table>
<thead>
<tr>
<th>Parameter</th>
<th>1th</th>
<th>3th</th>
<th>6th</th>
<th>9th</th>
<th>12th</th>
<th>15th</th>
<th>18th</th>
<th>21th</th>
<th>24th</th>
</tr>
</thead>
<tbody>
<tr>
<td>AI(-), control, ions x cm^-3</td>
<td>471 ± 121</td>
<td>493 ± 120</td>
<td>450 ± 116</td>
<td>444 ± 105</td>
<td>464 ± 121</td>
<td>478 ± 140</td>
<td>452 ± 123</td>
<td>438 ± 113</td>
<td>469 ± 126</td>
</tr>
<tr>
<td>AI(-), depriv., ions x cm^-3</td>
<td>n. d.</td>
<td>n. d.</td>
<td>n. d.</td>
<td>n. d.</td>
<td>n. d.</td>
<td>&lt; 20</td>
<td>&lt; 20</td>
<td>&lt; 20</td>
<td></td>
</tr>
<tr>
<td>AI(+), control, ions x cm^-3</td>
<td>653 ± 138</td>
<td>663 ± 161</td>
<td>640 ± 132</td>
<td>615 ± 134</td>
<td>632 ± 117</td>
<td>696 ± 154</td>
<td>610 ± 119</td>
<td>613 ± 109</td>
<td>650 ± 137</td>
</tr>
<tr>
<td>AI(+), depriv., ions x cm^-3</td>
<td>74 ± 16</td>
<td>81 ± 16</td>
<td>83 ± 18</td>
<td>80 ± 19</td>
<td>75 ± 17</td>
<td>65 ± 17</td>
<td>59 ± 18</td>
<td>48 ± 19</td>
<td>33 ± 18</td>
</tr>
<tr>
<td>CO₂, control, % by vol.</td>
<td>0.17 ± 0.03</td>
<td>0.18 ± 0.05</td>
<td>0.19 ± 0.05</td>
<td>0.17 ± 0.06</td>
<td>0.16 ± 0.08</td>
<td>0.18 ± 0.07</td>
<td>0.17 ± 0.09</td>
<td>0.16 ± 0.07</td>
<td>0.17 ± 0.08</td>
</tr>
<tr>
<td>CO₂, depriv., % by vol.</td>
<td>0.16 ± 0.03</td>
<td>0.16 ± 0.05</td>
<td>0.17 ± 0.07</td>
<td>0.16 ± 0.05</td>
<td>0.15 ± 0.05</td>
<td>0.13 ± 0.04</td>
<td>0.09 ± 0.005</td>
<td>0.06 ± 0.001</td>
<td>0.05 ± 0.001</td>
</tr>
<tr>
<td>NH₃, control, ppm</td>
<td>7.3 ± 0.7</td>
<td>7.8 ± 0.6</td>
<td>7.6 ± 0.6</td>
<td>7.7 ± 0.7</td>
<td>7.4 ± 0.8</td>
<td>7.6 ± 0.7</td>
<td>7.6 ± 0.6</td>
<td>7.8 ± 0.7</td>
<td>7.6 ± 0.5</td>
</tr>
<tr>
<td>NH₃, depriv., ppm</td>
<td>7.1 ± 0.6</td>
<td>7.6 ± 0.7</td>
<td>7.9 ± 0.8</td>
<td>7.1 ± 0.6</td>
<td>6.5 ± 0.7</td>
<td>6.1 ± 0.5</td>
<td>4.5 ± 0.4</td>
<td>&lt; 2</td>
<td>&lt; 2</td>
</tr>
</tbody>
</table>

1 Measurements in the course of the rats incubation during investigation of the survival time. All values = mean of 5–6 measurements ± SD; n. d. = not detectable.

The statistical significance of the difference in values was verified using the Wilcoxon and Mann-Whitney U-Test. All p values were based on two-tailed comparisons. The level of significance was set at p < 0.05. In the electron-microscopic examinations, the percentage (mean ± SD) of the adenocyte types and of over 200 various NSTs cells was calculated from 7 control rats and from 5 of 7 deprived rats (two rats had been taken off because of a suppurative inflammatory process).

**Results**

**Survival time, physiological signs and macroscopic examination**

Under AI deprivation conditions the life span of mice was 16.2 ± 0.9 days and of rats, 23.0 ± 1.1 days. In each deprived group on an average three mice showed reduction in spontaneous motor activity and in orientation reflex, occurring as early as day 3 or 4. These alterations were accompanied by a refusal of food and water intake as well as a severe loss of grooming and of tone in the skeletal musculature. The affected animals died within the 10 days. In other mice these symptoms became apparent after seven days. Their VA and HBT reflexes were reduced to 13.7 ± 3.7% (p < 0.001) and 7.2 ± 3.1% (p < 0.001) of the initial level, respectively. Rectal temperature was reduced by 2.9 ± 0.2 °C (p < 0.01) and body weight by 27.4 ± 3.6% (p < 0.01) by day 14 of deprivation. These alterations were reversible when the animals were replaced into the normal atmosphere by first days of AI deprivation. Moreover, the accelerated death of these replaced animals within three next months was not observed.

In the rats the similar pathologic symptoms were observed after day 10 of deprivation. The VA and HBT reflexes were reduced to 16.2 ± 4.2% (p < 0.001) and 9.5 ± 4.9% (p < 0.001) of the initial values respectively. By day 20 of deprivation, rectal temperature was reduced by 3.0 ± 0.2 °C (p < 0.01) and body weight by 24.6 ± 5.2% (p < 0.01).

Considerable hair loss was observed in all animals deprived. The macroscopic examination of these animals internal organs revealed erosive changes in the gastric mucous membrane as well
as involution of the thymus. In the mice by day 14 and in the rats by day 20 of deprivation, the thymus weight was reduced to 14.1 ± 4.5% ($p < 0.001$) and to 25.1 ± 3.2% ($p < 0.001$) vs. control animals respectively. Other pathologic signs observed were conjunctivitis in 82.6% of mice and in 89.3% of rats, as well as strongly pronounced leukopenia in all deprived animals investigated. Similar abnormalities were not observed in either control group in the SGC.

**Electron-microscopic examinations**

**Adenohypophysis.** Unaltered granular adenocytes represent a minority in the pituitary of deprived rats (Tab. II). In most of the cells the signs of degeneration were very marked. These included in the first instance degenerative pyknotic alterations in the nucleus such a decrease in the volume accompanied by the inflation of the perinuclear cistern, and condensation of chromatin in the margin and central zone (Fig. 2b). The volume of the cells was also decreased. The increases in the lysosomes and in the electronic density of the matrix as well as an evident characteristic split-shaped dilatation of the granular endoplasmic reticulum (GER) channels were strongly pronounced. In the intercellular space numerous secretory granules containing conserved membrane were found. This indirectly indicates disturbances in the permeability of the adenocyte cell membrane exceeding functional range. The altered thyreotropocytes represent the thyroidectomy cells. An increase, a change in the structure of the GER and often the merging of inflated channels into giant cavities, as well as pycnotic alterations in the nucleus, were characteristic for these cells (Fig. 2c).

**Neurohypophysis.** In the neurohypophysis of control animals the NSTs predominated as usual in the phase either of moderate or active excretion of neurohormones into the regional vascular network. In these animals functional exhausted NSTs were rare. The relative proportion of glial and neuronal elements in the various functional phases corresponded to the norm (Fig. 3a and 3a'). In deprived animals more than half of NSTs showed signs of functional exhaustion (Fig. 3b and 3b'). Functionally exhausted NSTs were found both in the proximity of the capillaries and out of them. The remaining NSTs were found in the phase of either active or, more rarely, moderate neurohormonal product excretion into the capillary network. Their neurosecretory granules were often morphologically immature and the electron density of their centre was low. The number of envelopes of emptied granules increased markedly. Swollen mitochondria contained a light matrix and their crystals were rather reduced. Sometimes the degenerated mitochondria transform into lamellar bodies. The extremely numerous pituicytes exhibited degenerative signs.

**Discussion**

The influence of Als on animals has been a controversial subject during this century. The mechanism of physiological activity and the biological role of Als are not fully comprehended, either

Table II. Percentage (mean ± SD) of the various adenocytes types in control and Al deprived rat pituitary (10 days of deprivation).

<table>
<thead>
<tr>
<th>Group of animals</th>
<th>AFS- cells</th>
<th>STH-</th>
<th>ACTH-</th>
<th>TSH-</th>
<th>FSH- &amp; LH-</th>
<th>LTH-</th>
<th>Degenerating cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (1)</td>
<td>5.3 ± 0.7</td>
<td>28.0 ± 3.2</td>
<td>9.7 ± 1.9</td>
<td>24.4 ± 4.1</td>
<td>20.3 ± 3.4</td>
<td>12.0 ± 1.8</td>
<td>0.3 ± 0.1</td>
</tr>
<tr>
<td>(n = 7; 3 male)</td>
<td></td>
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<tr>
<td>Deprivation (2)</td>
<td>12.6 ± 1.8</td>
<td>5.5 ± 0.7</td>
<td>8.2 ± 1.7</td>
<td>9.5 ± 1.1</td>
<td>6.6 ± 0.7</td>
<td>5.2 ± 0.8</td>
<td>52.4 ± 5.3</td>
</tr>
<tr>
<td>(n = 5; 3 male)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>P$_1$ vs. $P_2$</td>
<td>&lt; 0.05</td>
<td>&lt; 0.001</td>
<td>not significant</td>
<td>&lt; 0.001</td>
<td>&lt; 0.01</td>
<td>&lt; 0.05</td>
<td>&lt; 0.001</td>
</tr>
</tbody>
</table>

Abbreviations:
AFS-cells, agranular follicle-stellar adenocytes; STH-, somatotropic hormone producing adenocytes; ACTH-, adrenocorticotropic hormone producing adenocytes; TSH-, thyroid-stimulating hormone producing adenocytes; FSH- & LH-, follicle-stimulating and luteinizing hormone producing adenocytes; LTH-, lactotrophic hormone producing adenocytes.
(Reiter, 1992). However, current knowledge provides evidence that this question could give rise to a new scientific development (Goldstein et al., 1992; 1996). Some unsuccessful experiments with AIs can be explained by spontaneous neutralization of charged particles on the dielectric surfaces of the experimental cages (Krueger and Sigel, 1981). These circumstances were taken into account when developing the design of the cage used. The results described in the study corroborate...
rate the observations made by Tchijevsky (1941) about the vital importance of negative AIs. Our findings lead to the conclusion that causal relationships exist between AI deprivation, disordered neurohumoral regulation and animals death. As it was demonstrated, marked pituitary insufficiency occurs under conditions of uninterrupted AI deprivation. The dysfunction of the gland manifests itself as a reduction in muscle tone, spontaneous animal activity, rectal temperature, feeding motivation and body weight, as well as hair loss. This syndrome is typical of panhypopituitarism (Daniels and Martin, 1987). The histological changes of cells in the rats pituitary described are typical for apoptosis (Schwartz et al., 1993). The increase in thyroidectomy cells observed may be a result either of severe thyroid dysfunction or disordered feed-back mechanisms between the thyroid gland and the pituitary, possibly involving the hypothalamic zone. It is known that the neurosecretory pathways in the neurohypophysis start in the supraoptic and paraventricular nuclei of the hypothalamus. Both centres are sensitive to inhaled artificial AIs (Olivereau, 1976). Thus the involvement of the hypothalamic nuclei in the physiological response to AI deprivation finds plausible arguments. The putative neuronal receptors sensible to AIs are the olfactory receptors and the nerve endings of N. trigeminus (Shandala and Tregubova, 1968). However, more plausible is the involvement of the slow non-myelinated nervous fibres of the vomeronasal organ (Arshavsky et al., 1991) having direct connection to hypothalamic zone (Mendoza, 1992).

Some pathological signs develop in animals under AI deprivation, such as a degenerative change
in the pituitary, erosive alteration in the gastric mucosa, and marked involution of the thymus alone and collectively, representing the consequences of severe stress. The signs of functional exhaustion and the degeneration of secretory elements in the neurohypophysis of deprived animals were similar to the changes found in lemmings before cold-stress induced death (Arshavskaya-Popovich and Polenov, 1989). Long-term AI deprivation can therefore be regarded as a severe stressor whose uninterrupted effect causes the death of the animals. In human beings environment the full AI deprivation seems not to be possible. However, the diminution in AI content and/or ratio in indoor air could lead to marked functional disturbances. It is an opinion known that the lack of negative AIs may be one of possible reasons for the development of sick-building-syndrome (Hawkins, 1981). We assume another possibility of the artificial AI deprivation too, e.g. in the acrylic box for premature infants. The prolonged deprivation stress could to a marked degree influence the functions and post-natal development of hypothalamic-pituitary complex.

The question of whether the animals need gaseous AIs as a carrier of electrical charge, or of any specific ionisation products, requires further argument. The effect of positive AIs is generally regarded as harmful. The physiological mechanisms in the phenomenon involved may be related to disturbances of serotonin metabolism in the brain (Krueger and Sigel, 1981), the inhibition of hypothalamic centres activity (Oliverereau, 1976) and disturbances of hormonal regulation (Möse et al., 1976). Our analysis of the literature shows that the physiological responses to the unipolar positive AIs usually match the responses observed by AI deprivation (Goldstein, unpublished). In addition, the positive ions in the AGC measured are probably heavy pseudo-ions as defined by Tchijevsky (1960). In contrast to positive species, the biological responses stimulated by negative AIs are usually useful and find therapeutic application (Jorde and Schata 1979; Jaskowski, 1985).

The following principal considerations are useful in the discussion of a causal relationship between the biological effects of negative AIs and the superoxide-like reactivity of the negative AIs pool can be advanced. 1) Gaseous superoxide was discussed above as a natural component of the atmosphere. Radioactive radiation emitters, the principal source of natural gaseous superoxide, produce higher quantities of superoxide than under conditions of an electrical corona discharge (Huertas and Fontan, 1983, cyt ed by Dolezalek, 1985). 2) In the corona discharge produced, negative AIs show the superoxide-like chemical reactivity (Goldstein et al., 1992). 3) Air enrichment with artificial negative AIs prevent animal death caused by AI deprivation (Tchijevsky, 1960). 4) Negative AIs (Worden, 1961) and superoxide (Murrell et al., 1989) induce fibroblast and insular beta-cell (Goldstein, unpublished) proliferation in vitro. 5) High concentrations of negative AIs have a bactericidal effect (Kellogg et al., 1979), which may be inhibited by superoxide dismutase, a very specific enzyme for dismutation of \( \text{O}_2^•\) (McCord and Fridovich, 1988). 6) Superoxide dismutase also modifies other physiological responses caused by inhaled superoxide, in particular the potentiating effect of gaseous superoxide on the antinociceptive action of analgesic agents (Goldstein et al., 1996).

Thus the results observed point out the vital necessity of natural atmospheric ions for terrestrial animals and can be explained by the biological importance and physiological role of gaseous superoxide in the ambient air.

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