Hydrogen Peroxide Derived from Cigarette Smoke: "Pardon Impossible, to be Sent to Siberia"?

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Abstract: Hydrogen peroxide (H_2O_2) is one of the most important constituents in the metabolic chain transforming cigarette smoke in the human organism. The present analytical review provides a survey of the state-of-art in the study of H_2O_2 chemistry and biochemistry (both as a reactive and signaling species) in the context of cigarette smoking, taking into account both endogenous and exogenous (environmental, dietary, etc.) sources of H_2O_2. Particular attention is given to the methodological problems of monitoring smoke-associated H_2O_2. Our analysis is of prime interest for understanding the various mechanisms of smoke-induced oxidative stress and for developing rational approaches for diminishing the risk factors for human health associated with cigarette smoking.

Keywords: Hydrogen Peroxide, Reactive Oxygen Species, Free Radicals, Hydrogen Peroxide, Cigarette Smoke.

INTRODUCTION

One of the major undesirable consequences of cigarette smoking is oxidative stress, an imbalance between the production of reactive oxygen species, ROS, and the efficacy of the cell's antioxidant defense system. Oxidative stresss can lead to an altered intracellular redox status causing cellular dysfunction or death [1] and related events, most prominently oxidative DNA damage [2,3], a primary process of carcinogenesis [4-6]. In this context, it is noteworthy that hydrogen peroxide, being a precursor to other ROS, plays a pivotal role in oxygen metabolism [7]. Thus, it comes as no surprise that, in the long run, smoke-associated hydrogen peroxide has come into research fields concerned with smoke chemistry and biological aspects of smoking. Thus, recent studies [8] have revealed the interrelation between the H_2O_2 and free-radical content in the gaseous phase of cigarette smoke, and that incorporation of catalase immobilized on chitosan into the cigarette filter resulted in a 40% decrease of the NO/NO_2 concentration and a diminution of the smoke mutagenicity.

In the present work, we perform a general analysis of the literature on smoke-associated hydrogen peroxide with the major objective to understand H_2O_2 in a tobacco smoke context: Is it merely a signaling agent or indeed a harmful species for human smoking? Such a dilemma is of importance for choosing a harm-reduction strategy, namely to reduce the smoke-associated H_2O_2 or to reduce the peroxide-generating capability of the cigarette smoke. In this context, the “fatal comma” case contained in the title of this review alludes to the problem considered herein. In this famous Russian episode, a warrant signed by Emperor Alexander III read as follows: "Pardon impossible, to be sent to Siberia", which was changed somehow by the kind Queen Maria Fedorovna by shifting the comma: "Pardon, impossible to be sent to Siberia", and the man was set free.

Since the *in-vivo* action of the smoke-associated hydrogen peroxide should be considered against the naturally occurring H_2O_2 background in the human organism, solving the problem of the biological impact of smoke-borne and smoke-induced hydrogen peroxide calls for a multifaceted analysis of the diverse pathways for H_2O_2 generation, including the endogenous and exogenous (most prominently, environmental and dietary) H_2O_2 sources. Such an analysis is given in the subsequent sections of the present review. Since experimental monitoring of smoke-associated H_2O_2 is advantageous for establishing relationships between cigarette-smoke-induced oxidative stress and smoking-related pathologies, we also give considerable attention to the methodology of hydrogen-peroxide assays and analysis.

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BIOLOGICALLY SIGNIFICANT SOURCES OF HYDROGEN PEROXIDE

All H_2O_2 generation pathways may be conveniently divided into two categories, namely, *endogenous* and *exogenous*; the former category pertains to the inherent biochemical processes in the human organism, while the latter one refers to the lifestyle-dependent H_2O_2 sources governed by environmental conditions, diet, smoking, ethanol consumption, etc. In the following subsections, these H_2O_2 sources are sequentially analyzed.

Endogenous H_2O_2 Sources

Generation of hydrogen peroxide proceeds in all known aerobic organisms by cellular metabolism where the take-up of atmospheric oxygen furnishes the external chemical source. In turn, the main part of oxygen is consumed in the mitochondrial system, providing energy to the cells in the form of ATP. Other H_2O_2 sources encompass diverse ways of biosynthesis, metabolic pathways of aromatic compounds, steroids, etc. [7]. For example, oxidation of substrates such as xanthine, hypoxanthine, L- and D-amino acids is promoted by flavoprotein oxidases. With the help of flavin coenzymes, hydrogen transfer to molecular oxygen from these substrates bypasses the cytochrom oxidase-cytochrome system, thereby yielding hydrogen peroxide directly [9]. Another flavin-contained enzyme, NADPH oxidase, is present in polymorphonuclear cells and in the systemic pulmonary vasculature. In resting cells, NADPH oxidase exhibits a low level of activity. Cytokines (as well as some smoke constituents) increase the expression and activity of this enzyme in several cell types resulting in the production of O_2 and H_2O_2 for prolonged periods [10,11].

It should be stressed that the final oxidation product in the above mentioned cases is hydrogen peroxide rather than water, the latter is normally formed in cellular respiration processes. The H_2O_2 formed may be decomposed by catalase or utilized in catalase-mediated reactions. Notably, about a half of patients diagnosed with acatalasia (acatalasemia) exhibit no pathological symptoms [12]. This may imply that catalase alone does not control H_2O_2 regulation in humans. At the same time, it is known that an increased activity of catalase may result in longer lifetimes of living organisms [13]. Enzymes in cellular organelles such as peroxisomes take part in H_2O_2 metabolism. Therefore, simple autoxidizable flavoproteins, e.g. uratoxidase and D-amino acid oxidase, are responsible for the H_2O_2 generation, while catalase (constituting *ca.* 40% of peroxisomal protein in liver cells) along with glutathione peroxidase destroy it [14].

Hydrogen peroxide also forms in reactions involving flavoproteins, such as copper-containing oxidases and molybdenum-containing enzymes (xanthine dehydrogenase, xanthine oxidase, aldehyde oxidase) [15]. The action of monooxygenases (hydroxylases) involves consecutive stages in which the reductant converts

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the flavin into its dihydro form, thereby reducing O_2 to H_2O_2 and subsequently the enzyme-flavin-peroxide complex hydroxylates the substrate. The pertinent monooxygenases are furnished by cytochromes P450. It should be noted that oxygenation is the essential part of the transformation of the metabolic products and other foreign compounds consumed by the organism. Thus, in the course of the metabolism of benzo[a]pyrene, a well-known cigarette-smoke carcinogen, cytochrome P450 serves as activator of this agent with the intermediate formation of the superoxide ion radical and hydrogen peroxide [16,17].

In addition, formation of $O_2^{-\cdot}$ and H_2O_2 takes place by the spontaneous oxidation of hydroquinones, ferredoxins, adrenaline and hemoglobin [18]. ROS are also generated by the interaction of O_2 with FMN and FAD (flavin coenzymes) [15]. In the mitochondrial electron transport chain, different modes of oxygen reduction are possible: in the case of a two electron transfer, H_2O_2 is formed, while a one electron transfer yields the $O_2^{-\cdot}$ species [19]. In the course of normal aerobic metabolism, between 1 and 2 % of all the electrons involved in the mitochondrial respiratory chain yield superoxide or H_2O_2 [20]; the former is formed also in other cellular electron transport systems [21]. The semiquinone radical of coenzyme Q ($CoQ^{\cdot}$), also known as ubiquinone, in the electron transport chain is thought to be the primary source of superoxide in most non-phagocytic cells [22]. In neutrophilic leucocytes and macrophages, ROS are specifically generated for killing microorganisms [23].

Very recent data shed new light upon other important functions of hydrogen peroxide in living organisms. In particular in an elegant experiment, Niethammer, *et al.* [24] succeeded in demonstrating the signaling function of H_2O_2 in the recruitment of leukocytes to sites of injured barrier structures (e.g., epithelia around tissues). They probed the role of hydrogen peroxide during the early events of wound responses in zebra fish larvae, which express a genetically encoded H_2O_2 sensor. Starting approximately 3 min after wounding and peaking at approximately 20 min, a sustained rise in H_2O_2 concentration at the wound margin was observed extending approximately 100-200 μm into the tail-fin epithelium with a decreasing concentration gradient. Using pharmacological and genetic inhibition, the authors showed that this gradient is created by a dual oxidase (Duox) that is required for rapid recruitment of leukocytes to the wound.

Each cell of the human organism generates about 10^{10} molecules (10^{-14} mol) of superoxide during 24 hours [20]. Hence, considering that the number of cells in human body is of the order of 10^{14} [25], one arrives at a value of *ca.* 30 g of H_2O_2 for the estimated amount of hydrogen peroxide produced daily in the whole human body. Thus, the formation of ROS (in particular, H_2O_2) in the human body constitutes a normal physiological phenomenon. Moreover, some microorganisms, most prominently, *Lactobacillus* are known to produce hydrogen peroxide [26]. Finally, the endogenous generation of hydrogen peroxide and other ROS encompasses the following major sources [9-22]: (i) the mitochondrial respiratory chain, (ii) oxidase catalyzed reactions, (iii) microsomal oxidation involving cytochrome P450 in detoxication processes, (iv) spontaneous (nonenzymatic) oxidation of biomolecules (e.g., hemoglobin, ferredoxins, adrenaline).

Exogenous H_2O_2 Sources

Exogenous sources of hydrogen peroxide that include both cigarette smoke as well as environmental and dietary sources furnish the pertinent background for assessing the relative importance of smoking and endogenous H_2O_2 to human health. Since the formation of hydrogen peroxide by combustion [27,28] and gas-phase oxidation [29] are well known processes, it is clear that H_2O_2 that forms during the burning of tobacco is an important source of H_2O_2. However, the methods developed for the H_2O_2 detection in simple inert matrices are not suitable for determining the hydrogen peroxide in cigarette smoke *in situ*, since the latter constitutes an exceedingly complex matrix bearing several thousand constituents [30,31]. Thus far, the direct real-time monitoring of H_2O_2 has been a formidable task, although attempts have been made to determine H_2O_2 in fractionated cigarette smoke or in cigarette smoke extracts in various media. These experimental data are discussed below; for now, it is important to note that hydrogen peroxide exits in cigarette tobacco [32] and can be perhaps formed as a result of vital activity of microorganisms abundant in tobacco [33].

The local atmospheric environment also constitutes a source of hydrogen peroxide that can enter the human organism. Indeed, in ambient air, the amount of radicals, which may yield H_2O_2 in their reactions is considerable. Thus, the concentration of hydroxyl radical can reach 2×10^7 molecules/cm^3 and peroxy radicals may be as high as 2×10^8 molecules/cm^3 [34,35], while the concentration of hydrogen peroxide itself may amount to 5×10^6 molecules/cm^3 [36]. With these data, simple calculations show that an average human consumes up to 20 nmol of hydroxyl radical, 200 nmol of peroxy-radical species and 5 nmol of hydrogen peroxide per 24 hours.

However, the primary H_2O_2 source to humans is of dietary origin. Thus, one cup of coffee (150 ml) may contain from 500-800 μg [37,38] to 2 mg [39] of hydrogen peroxide. In green and black teas, H_2O_2 concentrations can be as high as 1100 and 790 μM respectively [40]. Moreover, evidence has been provided that the cytotoxic effects of tea polyphenols are related to the H_2O_2 generation [41]. Thus, it appears that polyphenolic compounds in beverages may exhibit both pro- and antioxidant properties. However, there exists also an opinion that the prooxidant and mutagenic properties of polyphenols *in vivo* has not been adequately substantiated [42].

CONDITIONS AND DYNAMICS OF HYDROGEN PEROXIDE GENERATION IN HUMANS

The mitochondria in the liver cells of rats generate hydrogen peroxide at the rate of 0.3 to 0.6 nmol/min per 1 mg of protein; adjusting for the mass of the whole organism affords a H_2O_2 generation rate of 2 μmol/min per 100 g of the body mass [20]. Extrapolation of these data to a human with an average weight of 60 kg yields a value of 1.2 mmol/min. This implies that every minute the human body generates about 40 mg of hydrogen peroxide, which is very close to the value we have estimated in the previous section based on the yield of superoxide ion radical. According to other data available in the literature, the rate of H_2O_2 production depends on the rate of oxygen consumption of the particular organism and may range from 50 mmol/min per 1 kg of the body weight at 1 % of the oxygen consumption maximum to 500 mmol/min per 1 kg at 10 % of the maximal O_2 consumption [43].

The steady-state level of H_2O_2 in cells has been estimated to be in the range of $10^{-10} - 10^{-7}$ M [20,44], and it is noteworthy that the norm for H_2O_2 levels is of the order of 10^{-10}–10^{-8} M (1–10 nM), while higher levels are associated with pathology [45]. However, reliable intracellular determination of hydrogen peroxide is problematic. The problem is that the available experimental data were obtained upon diffusion of H_2O_2 from cells into a culture medium. The diffusion coefficient of H_2O_2 is nearly the same as that of H_2O, but more importantly, the permeability coefficients for H_2O and H_2O_2 to cellular membranes are also nearly the same [46]. Moreover, H_2O_2 measurements involve monitoring the change in fluorescence of Amplex red (10-acetyl-3,7-dihydroxyphenoxazine, ADHP) using the commercial Amplex Red Hydrogen Peroxide/Peroxidase Assay Kit [47]. Amplex red is considered to be a sensitive probe for H_2O_2, but it can have interferences that overwhelm the H_2O_2 signal of interest [48]. In the presence of H_2O_2 and horseradish peroxidase (HRP), ADHP generates a fluorescent oxidation product, resorufin, with maximal absorption at approximately 563 nm and maximal emission around 587 nm [49].

In the biomedical context of cigarette smoking, it would be of interest to compare the results of the above-mentioned studies with the data on the H_2O_2 generation in cigarette smoke and formation of H_2O_2 in smokers resulting from the metabolism of smoke-derived chemicals. Indeed, one may presume that there is a harmful role for smoking-associated hydrogen peroxide only if its amount is comparable to the natural H_2O_2 level in the human body, or at least in its most sensitive organs such as the respiratory tract, liver, gastrointestinal tract and the cardiovascular system. In one pertinent study by Smit-deVries *et al.* [50] devoted to the resistance to damage of the lung epithelial cells of smokers, the influence of various doses of hydrogen peroxide was investigated. The authors suggested that the susceptibility of cells to altered function by the action H_2O_2 depends not merely upon the amount of H_2O_2, but also on the time of exposure to this oxidant. Specifically, the authors used the alveolar cell line A549 to study responses of proliferating and quiescent cells in the culture to time- and dose-dependent H_2O_2 challenges. Recovery was monitored after 24 h of incubation in a fresh medium with 10% serum. The adherent cells were counted and the resistance and recovery of the attached cells was assessed by appearance, by measuring the number of viable, apoptotic, and necrotic cells using fluorescent activated cell sorting, and by determining the intracellular free-thiol status. A549 cells recovered from a 1-h challenge with up to 1 mM of H_2O_2 but could not sustain a more prolonged challenge (6 or 24 h) with 0.5 mM or 1.0 mM of H_2O_2. These more severe conditions resulted in the loss of cells by detachment from the plate surface primarily due to necrosis, which reduces the number of viable cells, and causes a strong reduction of the intracellular free thiol content. Quiescent cells have been proved to be more sensitive to oxidative stress than proliferating cells. Intracellular free thiol levels apparently play a critical role in cell survival, preferentially protecting proliferating cells.

In study by Smit-deVries, *et al.* [50], the authors referred to the fact that inflamed lung tissue of smokers was exposed to high H_2O_2 concentrations [51]. The latter contention was based upon the observation that H_2O_2 levels in exhaled breath condensate of smokers or patients with exacerbated chronic obstructive pulmonary disease (COPD is elevated compared to ex-smokers or nonsmokers [51]. Nevertheless, the authors noted that the level of H_2O_2 found either in healthy or in inflamed lung epithelial cells is, in fact, unknown.

Despite numerous sources whereby hydrogen peroxide can enter the human body, H_2O_2 is readily eliminated by excretion through the urine, by exhalation, and perhaps by perspiration. Testing the H_2O_2 content in different organs and biological fluids is sometimes used in laboratory and clinical practice to monitor oxidative stress and the development of diverse pathologies. Attempts have been also made to apply H_2O_2 measurements to assess the physiological changes in smokers after smoking a cigarette. However, the normally high variability of the H_2O_2 level has not yet allowed monitoring H_2O_2 to be developed into a reliable diagnostic methodology. Indeed, the concentration of the hydrogen peroxide changes between 0.05 and more than 100 μM in exhaled breath condensate and in human fluids [52].

METHODOLOGICAL PROBLEMS AND RELEVANCE OF THE H_2O_2 ASSAY

In terms of developing prospective analytical tools for the H_2O_2 monitoring, the works of Pryor and coauthors [53-56] may be considered of the fundamental importance. As it has been already mentioned, no one has succeeded to date in measuring the amount of the hydrogen peroxide in cigarette smoke. Most of the experiments on the H_2O_2 measurements were conducted by bubbling cigarette smoke into an appropriate buffer medium. However, one should keep in mind that in the buffer solutions, the content of H_2O_2 in the reaction mixture may change, for instance, due to superoxide dismutation or alternatively because significant amounts of H_2O_2 can form during the process of bubbling the smoke through the probe buffer. Since cigarette smoke constitutes a heterogeneous system, hydrogen peroxide is usually determined separately in the gaseous and in the particulate phases. In view of insufficient sensitivity and selectivity of the applied experimental tools (polarographic and calorimetric methods, as well as the Clark electrode), hydrogen peroxide has not been detected in the gaseous phase of the smoke [56], while in the whole mainstream smoke of one cigarette, the determined H_2O_2 concentration was reported to be 60 μM [54], or even twice that amount [53]. However, the cited data are difficult to compare with the other results since in these pioneering works the cigarettes (2R1) were smoked under nonstandard conditions and the numbers of puffs as well as the mass of the total particulate matter (TPM) were not reported. Another methodological problem arose from the fact that the research samples were used after natural aging in the probe buffer solutions over several hours and even days. Clearly, it is difficult to compare the H_2O_2 data in aged buffers with that pertained to the real situation of the H_2O_2 transformation in the smoker's respiratory tract.

In more recent studies, more sensitive experimental methods were applied (e.g., fluorescence approach with the use of dyes and peroxidase). For example, in the recent detailed study [57] the authors bubbled five puffs of whole smoke from 1R4F or 2R4F research cigarettes using a five-port smoking machine into a phosphate bufered saline solution (PBS, pH 7.4) containing the fluorescent dye Amplex Red. Concentrations of 1–2 μM of H_2O_2 per cigarette were found in whole smoke bubbled samples before incubation and 7-8 μM after a 150-minute incubation period at 37 °C. For the bubbled gaseous phase smoke, negligible ($<<$ 0.1 μM) H_2O_2 formation was found. Simple calculations applied to the reported data [57] show that one cigarette generates hydrogen peroxide in a buffer medium at a rate of about 40 nM/min, which is by few orders of magnitude less than the natural H_2O_2 formation rate in the human body (*cf.* the estimates at the beginning of this section).

In a methodological context, it is also noteworthy that there exists certain concern over harnessing cell cultures in studies on the biological impact of ROS including also hydrogen peroxide since cells in culture may use ROS-dependent signal transduction pathways that do not operate *in vivo* [57].

In addition, a large amount of H_2O_2 may be ingested by smokers with food and beverages. As mentioned above, the H_2O_2 concentration in a cup of green tea may reach 1 mM (!) [40] *versus* 1-2 μM in the solution of smoke derived from 1 cigarette prior to incubation [58], *ca.* 1000-fold difference between the H_2O_2 contents. However, this fact does not make anyone give up tea drinking. Obviously, to date there exist plenty of unknowns in our understanding on the influence of the hydrogen peroxide in human health. In other words, the very fact that the inhaled smoke bears 1 to 2 μM of H_2O_2 may not have any direct consequences for the health of smokers. However, if the smoke constituents are prone to generating efficiently *secondary* hydrogen peroxide, particularly at sites where the H_2O_2 utilization is slow, it may initiate oxidative damaging processes.

Saliva serves as the first physiological barrier to the cigarette smoke. The most important antioxidant in saliva is uric acid (70 % of the whole antioxidant capacity of saliva) [59]. At the same time, the enzymatic antioxidant defense system, including superoxide dismutase (SOD), catalase and glutathione-peroxidase, are of secondary importance [60]. Oral peroxidase plays a more significant role in the inactivation of H_2O_2. It has been shown that smoking merely one cigarette both by smokers and non-smokers leads to an abrupt decrease in the activity of this enzyme, probably accounted for by the influence of smoke-borne hydroxyl radicals [61]. The change of the antioxidant status of saliva induced by cigarette smoke may have grave consequences for the gastro-intestinal tract,

Table 1. Amounts of Hydrogen Peroxide Derived from Cigarette Smoke

Cigarette	H_2O_2 Assay [a]	H_2O_2 Amount per Cigarette (μg)		H_2O_2 Amount per 1 mg TPM (μg)		Reference
		min [b]	max [c]	min [b]	max [c]	
1R4F	Electrochemical	37.0		2.8-3.5		[53]
1R4F/2RF4	Fluorescence (Amplex Red, HRP)	5.0	22.0	0.4	1.6	[58]
2R1	Clark electrode	2140.0		70.0		[54]
Longfeng	Chemiluminescence (luminol, $K_5[Cu(HIO_6)_2]$)	0.03		0.0015		[69]
3R4F	HPLC	4.0	27.0	0.4	2.7	[68]
Local Brands	Fluorescence ($DCFH_2$, HRP)			0.065-0.17		[32]

[a] Methodology of the H_2O_2 measurement as specified in the cited works; [b] Minimal H_2O_2 data acquired immediately after smoking; [c] Maximal H_2O_2 data in the aged smoke extracts.

which is related to the metabolism of smoke constituents swallowed with the saliva.

In the respiratory tract, both the hydrogen peroxide and the superoxide species are rapidly neutralized by transformation in a defense barrier harnessing catalase, SOD, thioredoxin and other thiolic antioxidants. If the hydrogen peroxide is not destroyed by other enzymes and low-molecular thiols, it can damage SOD. In this case, hydroxyl radicals furnish the pertinent reactive intermediates that affect the enzyme's activity through the inactivation of its functional histidine moiety [62]. It is noteworthy that bicarbonate anion may accelerate inactivation of the enzyme [63].

Transition metals that accumulate in the tar deposition sites may also contribute efficiently to the H_2O_2 decomposition [64,65] with a formation of the highly reactive hydroxyl radical [66], which in turn exerts a damaging impact on mucous membrane and epithelium. The second stage of the smoke metabolism involves enzymatic and nonenzymatic transformation of molecular constituents of the tar followed by H_2O_2 formation. In parallel to these processes, long-lived (up to several hours [46]) semiquinone radicals enter the cell and generate therein superoxide species; the latter undergo dismutation to afford hydrogen peroxide molecules. A portion of the superoxide may be involved into Fe^{3+} reduction in biomolecules followed by iron-ions release. Iron ions (as well as others: Cu^{2+}, Mn^{n+}) catalyze the H_2O_2 decomposition with the formation of hydroxyl radicals cable of damaging DNA [67].

As mentioned above, experimental data point to just a nominal H_2O_2-generation efficiency in the gas phase of the cigarette smoke [56, 57]. In this context, the data reported by the Ma group seem discordant [32]. With the use of dichlorofluorescein along with peroxidase and fluorescence instrumentation, the authors studied different fractions of smoke, both non-filtered and filtered, using carbon-containing and carbon-free filters. Based on the H_2O_2 assay under minimal incubation of the smoke fractions (in fact, determining the hydrogen peroxide immediately after the smoking), they arrived at conclusion that the H_2O_2 concentration in the gaseous phase is 2 to 6 times higher than that in the particulate matter. The amount of hydrogen peroxide in the whole smoke ranged from 45 to 530 nmol per cigarette [32]. The H_2O_2 content renormalized to 1 mg of TPM comprised 2 to 6 nmol depending on cigarette brand, while the maximal H_2O_2 content of tobacco and ash reached 0.074 and 1.74 nmol/mg respectively [32]. Consequently, the hydrogen peroxide appears to derive mainly from tobacco combustion. The authors also concluded that the carbon-containing filter is inefficient towards the ROS scavenging [32].

The use of HPLC with electrochemical detection has shown to have similar accuracy, sensitivity and selectivity in determining hydrogen peroxide comparable to that achieved by the fluorescence method [68]. However, certain differences are noticeable between the results acquired by these two methodologies. With the use of various buffer media, the authors have established a dependence of the H_2O_2 generation rate on the pH of the buffer [68]. For 3R4F reference cigarettes smoked under the ISO procedure [68], the initial H_2O_2 concentration was 4 μg/cigarette. Upon aging at physiological pH, the concentration of the hydrogen peroxide reached its maximum of 27 μg (slightly less than 0.9 μmol) per cigarette after 80 min. As a matter of fact, comparison of data of different investigators reveals a 10000-fold scatter in the H_2O_2 content in cigarette smoke per 1 mg of TPM (Table **1**). Finally, the available literature on the H_2O_2 generation reveals a serious methodological problem of adequate determination of not merely the H_2O_2 amount in cigarette smoke but also its generation efficiency in the presence of different smoke constituents *in vitro*.

HYDROGEN-PEROXIDE GENERATION IN MODEL SYSTEMS

It is evident from the above discussion that the H_2O_2 content alone cannot be considered as a major harmful factor of cigarette smoke. More hazardous are long-lived free radicals capable of penetrating the cell membrane to cause oxidative damage of biomolecules [7]. Some of their reactions yield hydrogen peroxide as a product or reactive intermediate. In this context, studying the generation of H_2O_2 as signaling species is of interest for the assessment of smoking-associated free radical processes responsible for the development of oxidative stress.

The formation and decomposition of the hydrogen peroxide proceed continuously in the reactions of smoke chemicals in a smoke bubbled buffer solutions. Clearly, the rate of the H_2O_2 accumulation in such a reactive medium depends on interaction between its anti-peroxide and properoxide properties. In this context, concentrations of phenolic compounds and their oxidation products are of importance. Phenoxyl radicals are thought to be insensitive towards the reaction with molecular oxygen in contrast to their reaction with superoxide and hydroperoxide radicals [70]; thus, the major contribution to H_2O_2 generation comes from the hydroquinone (H_2Q) content of the reaction mixture [58,71]. In the reaction sequence represented by Eqs. 1-3 [58], the superoxide radical anion furnishes the immediate precursor to H_2O_2

$$H_2Q + O_2 \rightarrow O_2^{-\cdot} + Q^{-\cdot} + 2H^+ \quad (1)$$

$$2\ O_2^{-\cdot} + 2H^+ \rightarrow H_2O_2 + O_2 \quad (2)$$

$$H_2O_2 + e^- \rightarrow HO^- + HO^{\cdot} \quad (3)$$

(Eq. 2), which can then be reduced to the hydroxyl radical by metal ions [58]. The resulting $HO^{\cdot}$ is known for its ability to cause oxidative DNA damage, a primary process of carcinogenesis [4-7]. In

real systems, the reaction mechanisms are not so straightforward. Thus, the triggering process of the H_2Q oxidation (Eq. 1) is rather slow under physiological conditions [72]. However, in the presence of benzoquinone the oxidation of H_2Q may proceed autocatalytically [73]. In addition, there are differences in the oxidation potentials of *p*- and *o*-H_2Q [74,75]. Semiquinone species reacts with molecular oxygen (Eq. 4), and, if $E(Q/Q^{\cdot-}) > 150$ mV, the equilibrium is shifted to the left [76], as shown:

$$O_2^{\cdot-} + O_2 \rightleftharpoons Q + O_2^{\cdot-} \quad (4)$$

In the presence of SOD, the situation may change dramatically after superoxide removal, and this process depends on the substitution of the starting hydroquinone reactant [77]. It is also noteworthy that in a phosphate buffer saline solution, hydroquinone undergoes a slow autoxidation to benzoquinone, which can be accelerated by Cu/Zn-SOD, Mn-SOD, or Fe-SOD with similar efficiency [78]. In contrast, among metals, only Cu^{2+} strongly mediates the oxidation of hydroquinone to benzoquinone. Mn^{2+} exhibits a slight capacity to oxidize hydroquinone, whereas neither Fe^{2+} nor Fe^{3+} is capable of modulating the hydroquinone autoxidation [78].

Unfortunately, no data have been reported to date concerning the influence of SOD on H_2O_2 generation in cigarette smoke extracts. However the very fact that hydrogen peroxide accumulates in smoke extracts upon aging is indicative of the superoxide dismutation processes (either spontaneous or catalytic). Although in protic media, such as aqueous solutions, superoxide undergoes fast dismutation to afford hydroxyl radical. The experimental results acquired by addition of SOD to model solutions containing oxidized hydroquinones [77,78] lead us to hypothesize that the H_2O_2 formation in smoke extracts may be catalyzed by complexes that exhibit SOD-like activity, rather than SOD itself. It is indeed known that some chelated complexes of copper and manganese may possess either catalase- or superoxide dismutase-like activity [79-81]. Of particular interest are metal ion complexes with bicarbonate anion (HCO_3^-) [79,80]. Bicarbonate anion exists in considerable amount (up to 25 mM) in human blood and, thus, the mentioned processes may be indeed of importance in smoking context. Unfortunately, none of the cited works report the influence of this anion on the H_2O_2 generation in cigarette-smoke extracts. One of the possible reasons for this stems from the experimental difficulty of maintaining a stationary bicarbonate concentration in buffer solutions upon their aeration. Another important problem in studying hydrogen peroxide in the context of smoking, which has not been addressed to date in the literature, is the possible role of microorganisms abundant in cigarettes in the pertinent redox processes [33].

As opposed to model experiments with individual reactants, studying the nature of the H_2O_2 generation in cigarette smoke is complicated by the extensively complex composition of the smoke matrix [30, 31]. Nevertheless, much can be gained by monitoring the overall effects derived from such a "black box" of several thousand chemicals.

CONCLUSIONS

How can one solve the dilemma of the "wandering comma" case presented in the title of the current review? The analysis of the literature performed here shows that there exists no unambiguous answer. Clearly, H_2O_2 constitutes a relevant signaling species in the human body. Indeed, the H_2O_2 generation *in vivo* manifests the development of free radical processes that upset the balance between ROS production and the efficiency of the cellular antioxidant defense system. For this reason, monitoring the generation of smoking-associated hydrogen peroxide could advance the diagnostic methodologies for the assessment of oxidative stress, which, in turn, is of prime importance for choosing rational ways of diminishing the undesirable impact of cigarette smoking. In any case, the smoke clearing strategy should be directed towards reducing the H_2O_2 generating ability of smoke constituents under physiological conditions, which would reflect the decrease of the oxidative damaging impact of smoke chemicals in living cells and tissues. As for the primary hydrogen peroxide generated *in situ* in cigarette smoke, comparison of most of the available literature data concerning the amount of hydrogen peroxide consumed by the humans from diverse exogenous H_2O_2 sources (ambient air, food, etc.) and with the H_2O_2 that forms endogenously by metabolic processes in the human body demonstrates that such a smoke-borne hydrogen peroxide does not appear to bring any serious threat for the human health. Nonetheless, there are also data (e.g., reference [8]), which cast doubts on such a contention. And last but not least, the analysis of literature reveals a serious need for the development of modern analytical techniques for the *in-vitro* assay of the smoke-associated hydrogen peroxide with the incentive of acquiring accurate H_2O_2 data.

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