Journal of the Korean Society of
Tobacco Science. Vol.12. No.1(1990)
Printed in Republic of Korea.

Evaluation of Cigarette Quality by Measurement of Oxygen Free Radicals in Smoke

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(Received for publication, Auqust 10, 1990)

담배 연기 중 산소 자유 라디칼 측정에 의한 품질 평가

임흥빈, 김숙영, 문자영, 이동욱, 김용태

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초 록

지금까지 연기중의 Nicotine, CO등 몇몇 화합물의 함량 측정과 관능 검사등을 통하여 담배의 품질을 평가해 왔다. 그러나 담배 연기중의 수 많은 화학 성분들을 고려할 때 전연기 성분이 생체에 미치는 영향을 측정함으로써 그 품질을 평가할수 있는 새로운 방법이 요구된다. 담배연기에 의한 생체 손상 중 가장 일차적이고 영향이 큰 것으로 알려진 활성 산소종 (H_2O_2, O_2^-, $\cdot OH$)은 그 반응성이 크고 life time이 짧기 때문에 측정이 어렵다. 저자들은 이들을 분해하는 효소를 이용하여 연기중에서 생성되는 이들 산소 자유 라디칼을 측정하였으며 그 결과 담배의 종류에 따라 이들의 생성이 현저한 차이를 보여 일반적으로 고급 담배로 알려진 것들은 낮았고 저급 담배일 수록 높았다. 따라서 이들 산소 자유 라디칼의 측정은 흡연과 건강이라는 측면에서 매우 유용한 담배의 품질 평가 방법으로 이용될수 있을 것으로 생각된다.

ABSTRACT

To evaluate tobacco quality, several mathods including sensory test, or measurement of some toxic compounds such as tar, nicotine and carbon monoxide in cigarette smoke have been used. However, many detrimental effects of smoking on the physiological functions including respiratory system reported were turned out to be the action of reactive oxygen species. Therefore, the amounts of oxygen free radicals such as superoxide, hydroxyl radical, even hydrogen peroxide in the cigarette smoke are thought the very important factors.

In the present study, we have determined the generation of superoxide and the content of hydrogen peroxide using superoxide dismutase and catalase in the gas and particulate phases obtained from cigarette smoke, respectively. In the aqueous extracts of total particulate materials, suproxide and hydrogen peroxide were detected, and there was an excellent correlation between oxygen ent of oxygen free radicals in cigarette smoke may be a useful index for evaluation of cigarette quality in the aspect of smoking and health.

Introduction

Cigarette is one of the most popular luxuries, and about half of the world populations enjoy with various types of cigarettes. Although the several detrimental effects of smoking on the human health have been known, its consumption is gradually increased. Recently, however, many smokers have been concerned at their health about the risk of smoking because physicians and epidemiologists have reported that smoking is a major cause of human respiratory or many other diseases(5, 12, 13). Therefore the manufacture of less hazardous cigarettes is one of the important tasks to the tobacco scientists.

In order to evaluate the cigarette quality, so far several methods including sensory test, or measurement of some undesirable compounds such as tar, nicotine or carbon monoxide in cigarette smoke have been used. Each method for the evaluation of cigarette quality has a special purpose and characteristics. Sweetness, aroma and order of the cigarette are very important factors which decide the quality. Evaluation of the content of some hazardous chemicals is one of the general index of cigarette quality. However, when considering that cigarette smoke is a complex mixture containing thousands of compounds, the methods which are able to evaluate whole smoke or a closer system to the

human body are urgently required.

Since Nakayama(8) had detected superoxide in the cigarette smoke in 1984, the reactive oxygen species including hydrogen peroxide and hydroxyl radical have arisen as an important issue. A recent evidence points out the involvement of reactive oxygen species in variety of pathological events, namely of the chronic diseases such as amphysema, cancer and diabetes are related with the reactive oxygen free radicals(3, 10). Therefore, the estimation of the oxygen free radicals in smoke is able to be utilized as another method for evaluating of cigarette quality. Church and pryor(4) have intensively studied free radical formation and its toxicological implications in cigarette smoke. Especially, DNA damage(1,2) and inactivation of alpha 1-antitrypsin or other thiol dependent enzymes(11) by oxygen free radicals indicate that these radicals in cigaretts smoke are very consequent factors. Indeed, the single-strand breaks of DNA by tar extract are inhibited by catalase and superoxide dismutase(1,2,7). Therefore, we attempted to determine some oxygen free radicals in the cigarette smoke.

In this communication, we established the methods for hydrogen peroxide and superoxide in the cigarette smoke, and discussed the possibility as a new evaluation method for cigarette quality.

Materials and Methods

The filter-cigaretts containing low (less than 6.0mg/cigarette), midddle (6.0 to 15mg/cigarette) or high(over 15mg/cigarette) tar were purchased from commercial sources in May 1990 in Taejon, Korea. These samples were used immediately after opening the sealed packages. These included three imported(middle tar), and three domestic(low, middle and high tar) brands were used. Details are as follows ;

Samples	Brands	Length
A	imported	100 mm
B	imported	100 mm
C	imported	85 mm
D	Domestic	85 mm
E	Domestic	100 mm
F	Domestic	85 mm

Catalse, superoxide dismutase, ferricytochrome c, potassium ferricyanide weer obtained from Sigma Chemicals Co. Deionized water(18mega ohm) was used for the preparation of reagent or samples.

Preparation of samples

Cigaretts were smoked under the CORESTA standard condition(one puff /min, 2sec/puff, 35ml/puff). The total particulate materials(TPM) from one cigarette were collected on a Cambridge filter. The filter was soaked in 20 ml of distilled water and sonic-

ated for one minute in a sonication bath, and then the solution was filtered. This procedure was repeated twice under the same condition, and the filtrates were combined. On the other hand, the gas phase passed out through Cambridge filter was bubbled into the 20ml of distilled water. Both TPM extracts and gas bubbled solutions were used as samples for the determination of reactive oxygen species.

Determination of hydrogen peroxide

To determine hydrogen peroxide formed in samples, 50ul of catalase(140 units) was added into the reaction mixture contained 200ul of sample and 750ul of distilled water. For reference, equal amount of inactivated catalase which was heated for one minute in boiling water bath was added instead of catalase. The reaction mixture was incubated in a water bath at 37° C for 15min. One ml of 1.2M trichloroacetic acid was added into both sample and reference tubes. The protein precipitated were removed by centrifugation at 3000rpm for 10minutes, and one ml of resulting supernatant was mixed with 0.2ml of 10mM ferrous ammonium sulfate and 2.5M potassium ferricyanide to develop the color. The absorbance of the red ferrithiocyanate complex formed by hydrogen peroxide was read at 480nm after standing at room temperature for 10minutes.

Determination of superoxide

The formation of superoxide was assayed by monitoring the reduction of ferricytochrome c by superoxide and its inhibition by superoxide dismutase. The reaction mixture contained 150ul of 0.2mM ferricytochrome c and 350ul of sample in 0.5M potassium phosphate buffer(pH 7.7). The final volume of the reaction medium was 1.0ml, and the reaction was initiated by the addition of sample. The absorbance change of the solution was read at 550 nm for 2 or 3min, and read continuously after the addition of 10ul of superoxide dismutase(200units). Superoxide formed by the sample was calculated by using molar extinction coefficient of ferricytochrome c(9).

Results and Discussion

Cigarette smoke can be classified by two major fractions based on the use of filter. One is the particulate phase and the other is the gas phase. Namely, the particulate phase is defined as the materials that are trapped when the smoke stream is passed through a Cambridge filter that retains 99.9% of all particular matter with size greater than 0.1um. The gas phase is the material that passes through the filter.

The authors have analyzed the hydrogen peroxide and superoxide formed in both particulate and gas phases using

their scavenging enzymes. Nakayama et al(8) determined hydrogen peroxide with horse radish peroxidase and 2.2-azinodi(3-ethylbenzothiazoline-6-sulfonate)(ABTS) in cigarette smoke. However, they did not use the smoke puffed by standard smoking condition. This method was also not easy to use, and a fault had been found out on it.

In this study, a new sensitive and convenient method was established. Moreover, our standard method was confirmed through preliminary experiments such as incubation time, the condition of sonication, and the amount of catalase to convert hydrogen peroxide to oxygen and water, even though dose dependent. Also it was confirmed by the effect of aging of the solutions after preparation. The major defect of ABTS method developed by Nakayama et al(6) was the immediate reduction of ABTS by some components in the TPM extracts. However, our method using catalase could rule out such a problem, and showed an excellent reproducibility Fig.1. shows the change in amount of hydrogen peroxide formed during the standing at room temperature. These results indicate that the formation of hydrogen peroxide in tar extracts was linearly increased until at least 6hrs after preparation. Therefore, the selection of a specific time for its accurate measurement is repuired. Accordingly this oxygen species was measured at 4hrs post standing exactly, since this time was selected by considering the sample number and the formation velocity of it.

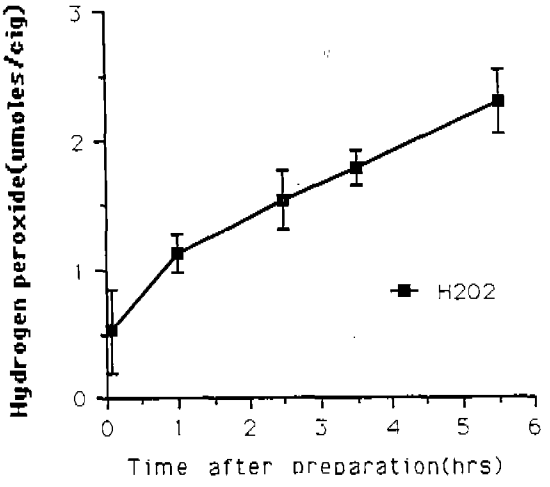

Fig.1. Effect of aging of TPM extracts on hydrogen peroxide formation. TPM extracts of middle tar cigarette were used, and detail procedures are described in "Materials and Methods"

It was attempted to detect the hydrogen peroxide in the gas phase un-

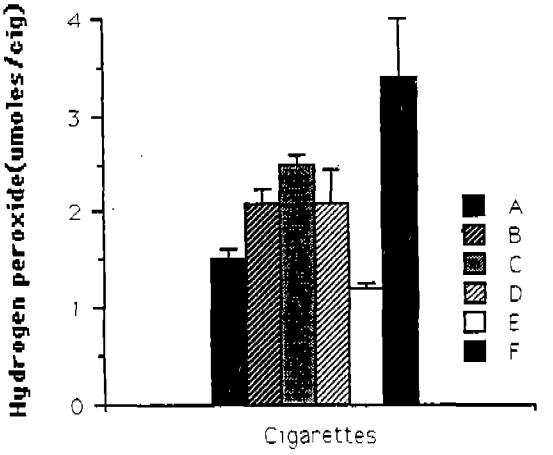

Fig.2. Formation pf hydrogen peroxide in TPM extracts from smoke of several commercial cigarettes

der various conditions. Hydrogen peroxide in TPM extacts of the cigaretts obtained from commercial sources has been shown in Fig.2.
This oxygen species formed in TPM extracts obtained from low tar cigarette of domestic brands(Sample E)was 1.2±0.2 umole/cigarette, which was much less than that of high tar cigarette F(3.4±0.6 umole/cigarette). Other cigarettes(Sample A,B,C and D) which contained middle tar resulted levels of 1.5±0.1 to 2.5±0.1 umole/cigarette. The pattern of hydrogen peroxide formation in TPM extracts from the cigarettes was similar to that of tar content which was determined by CORESTA standard method(Table 1).

Table 1. The content of tar, and the formation of hydrogen peroxide and superoxide in TPM extracts from smoke of commercial cigarettes

Cigarettes	Brands	Tar (mg/cig)	H_2O_2 (μmole/cig)	O_2^- (μmole/cig)
A	Imported	10.6	1.5±0.1	0.29±0.05
B	Imported	13.2	2.1±0.1	0.29±0.04
C	Imported	11.6	2.5±0.1	0.23±0.02
D	Domestic	5.7	1.2±0.1	0.25±0.05
E	Domestic	9.3	2.1±0.4	0.24±0.06
F	Domestic	18.5	3.4±0.6	0.47±0.08

Now the question arises which of the components in cigarette smoke is responsible for the generation of the active oxygen species. First, the autoxidation of phenols such as catechols or hydroquinones should be supposed. But, our interest is that the reactive oxygen species are generated in the physiolgical conditions by cigarette smoke, thus we consider that this is a more physiologically seriousness depending on smokers. Because it is reasonable to expect that the same reaction occurs in the pulmonary cells after inhalation and absorption of the cigarette smoke.

Superoxides as hydrogen peroxide and hydroxyl radical are also very important oxygen free radical in the cells. Moreover, superoxide itself does not have any toxicity on the biological materials, and is readily converted to hydrogen peroxide by spontaneous dismutation or superoxide dismutase. However superoxide produces hydroxyl radical which is the ultimate reactive oxygen species and hydrogen peroxide through well known reactions such as Harber Weiss or dismutation reactions. So far, many investigators have used nitro blue tetrazolium(NBT) to determine superoxide, which was also developed by Nakayama(8). However, it was very difficult to determine the superoxide in cigarette smoke because of its low sensitivity.

We measured superoxide formation using ferricytochrome c and superoxide dismutase, and could determine this free radical in the picomole levels. The superoxide formation also had affected aging as shown in Fig.3. Therefore, it was estimated at 30min after the preparation Fig.4. indicates

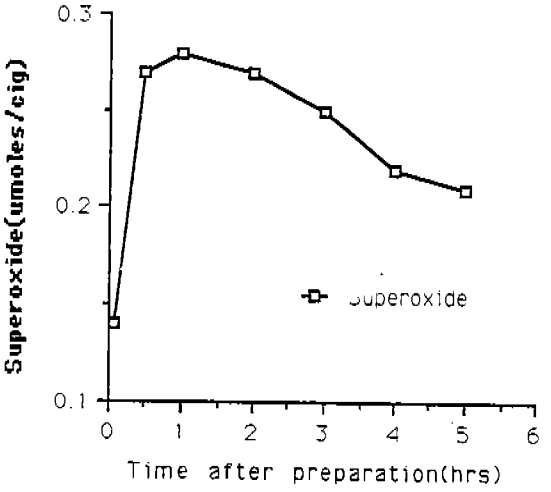

Fig.3. Effect of aging of TPM extracts on superoxde formation. The same sample as in Fig.1 was used.

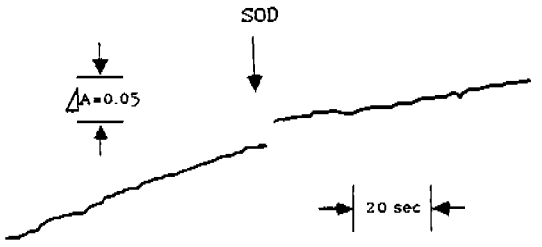

Fig.4. Determination of superoxide. The rate of ferricytochrome c reduction by superoxide was monitored at 550 nm.

the typical spectrophotometrical profile for assay of superoxide in TPM extracts.

The amount of superoxide formed or inhited by the addition of superoxide dismutase was calculated from the absorbance change of ferricytochrome c at 550nm. As shown in Fig.5, trace amount of this oxygen species was detected in the gas phase of low

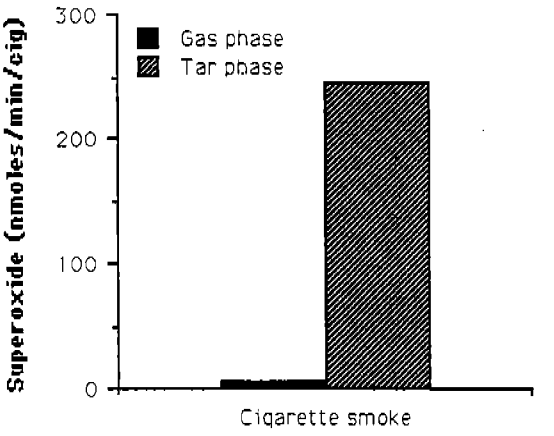

Fig.5. Formation of superoxide by gas phase and TPM extracts from the smoke of low tar cigaretts

tar cigarette, however this was relatively very low when compared with that of tar extracts. Interestingly, the formation of superoxide in TPM extracts of cigarettes did not well correlated with their tar contents. Table 1 shows the relationships between

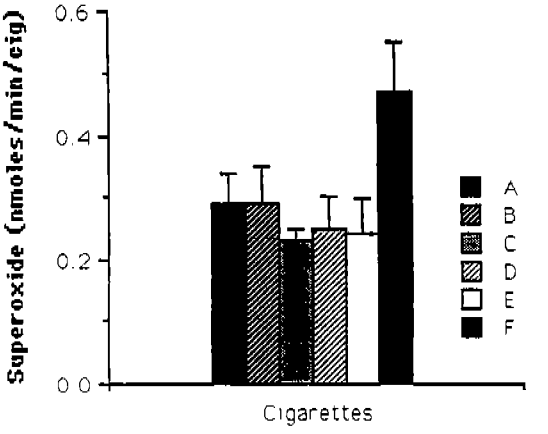

Fig.6. Superoxide production in the TPM extracts from smoke of commercial cigarettes

tar content, hydrogen peroxide and superoxide formed in cigarette smoke. The suproxide production in smoke from middle tar cigarettes showed slightly different pattern when compared with those of hydrogen peroxide as given in Fig.6. When considering the possible mechanisms of superoxide and hydrogen peroxide formation in TPM extracts, it is suggested that the composition of some compounds participated in the reaction to generate these oxygen species should be different.

On the other hand, the low tar cigaretts(Sample E) generated 0.25 ± 0.05 umoles/cigarette/min of superoxide in its TPM extracts, while high tar cigarette formed 0.47+0.08 umole of this oxygen free radical under the same condition. These results indicate that there was a correlation between the velocity of superoxide formation and the sort of cigarettes.

As already mentioned above, cigarette smoke is a very complicate and complex chemical system. therefore it can be expected the existence of many potential pathways for these species to interact with one another and with biological materials in smoker's lung or other organs. Considering current data and the physiological consequence of the oxygen free radicals in the cigarette smoke, it is concluded that quantitation of these radicals may be an another useful index for evaluation of cigarette quality in the phase of smoking and health with other estimating methods of sensory test or determination of the contents of several constituents of cigarette smoke.

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