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Original article

COMPARISON OF SERUM HYDROGEN PEROXIDE LEVELS BETWEEN NON-SMOKERS AND SMOKERS BY TWO METHODS

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Abstract Hydrogen peroxide (H_2O_2) is a reactive oxygen metabolic by-product that serves as a key regulator for a number of oxidative stress-related states. The aim of this study was to compare serum H_2O_2 levels between non-smokers and smokers by two methods, i.e. the tetramethylenebenzidine (TMB) method and ferrous ion oxidation xylenol orange (FOX) assay. In normal subjects (n=20), the mean H_2O_2 levels in urine when measured by the TMB and FOX method were 0.78±0.35 and 0.82±0.59 μM, respectively, which were not significantly different (p>0.05). When measuring the levels of serum H_2O_2 in non-smokers (n=20) and smokers (n=20) by FOX assay, the mean H_2O_2 levels were 0.04±0.12 and 0.76±0.90 μM, respectively. By adding H_2O_2 in different concentrations (0.01, 0.02, 0.03, 0.04 and 0.06 μM), the scavenging capacity of sera in both groups was compared. By the TMB method, the mean H_2O_2 level in non-smoking subjects decreased by 94% and in smoking subjects it decreased by 16.38%. However, for the FOX assay, the mean H_2O_2 level in non-smokers rose 63.75-fold compared to a control (before H_2O_2 addition), whereas, in smokers it rose 1.79-fold compared to a control. In conclusion, the sera of smokers had significantly (p<0.05) lower antioxidant capacity than non-smokers. Both methods had different characteristics in measuring H_2O_2. **Chiang Mai Med Bull 2005;44(4):129-135.**

Keywords: hydrogen peroxide (H_2O_2), tetramethylenebenzidine (TMB) method, ferrous ion oxidation xylenol orange (FOX assay), smokers, non-smokers

The massive health problem associated with cigarette smoking is exacerbated by the addictive properties of tobacco smoke and limited success of the current approach to cessation of smoking. Cigarette smoking is associated with increased nitric oxide (NO) production and it increases in H_2O_2 concentration (oxidative stress) in the airways. H_2O_2 is increased in an exhaled breath condensate of asthmatic subjects and may be used as a non-invasive marker of oxidative stress.[1] Cigarette smoking is the most commonly identified correlate with chronic obstructive pulmonary diseases (COPD).[2] Many studies clearly indicate the

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important role of tobacco smoking in reducing the body's antioxidant content and they emphasize the protective effect of antioxidant nutrients.[3-5]

Reactive oxygen species (ROS) or free radicals are atoms or molecules with unpaired electrons or molecules with a very short half-life. Such molecules are first found in the body endogenously in the electron transport chain of the mitochondria, e.g. superoxide anions, hydroxyl radicals, and hydrogen peroxide. Even though hydrogen peroxide does not have unpaired electrons in its molecule, it is very reactive and able to change to water when it is catalyzed by catalase.[6]

ROS causes damage to macromolecules in the human body. It oxidizes lipids to become lipid peroxide, destroys proteins and induces protein cross-linking, and it also causes catastrophe in deoxyribonucleic acid (DNA) and mutation. Diseases related to ROS such as cancer, atherosclerosis, heart diseases, stroke, emphysema, diabetes mellitus, rheumatoid arthritis, ulcer, and cataracts, are well documented. It also causes degenerative diseases and aging.[7]

Hydrogen peroxide alone is lower in reactivity, but it can change into a hydroxyl radical when exposed to ultraviolet or catalyze by ferrous or cuprous ion. Hydrogen peroxide has a role in the kidneys, since it acts as an antibacterial in urine. The high levels of hydrogen peroxide in urine indicate the status of oxidative stress and renal or urinary tract infection.[8]

The aim of this study was to compare serum H_2O_2 levels in smokers and non-smokers by two different methods, i.e. the TMB and FOX methods.

Materials and methods

Tetramethylenebenzidine (TMB), horseradish peroxidase (HRP), guanidine hydrochloride, xylenol orange [XO; *o*-cresosulfonaphthalein-3, 3-bis (sodium methyliminodiacetate)] and ferrous sulfate were obtained from Sigma (St. Louis, MO, USA). Perchloric acid and hydrogen peroxide were obtained from Aldrich Chemical (Milwaukee, WI, USA). Other chemicals were obtained from Merck (Darmstadt, Germany).

The blood was collected from twenty normal volunteers, who were non-smokers, and twenty age-matched smokers who had smoked 5-10 cigarettes per day for more than a year.

Ten milliliters of whole blood were collected, and the serum was stored in 1.5-mL aliquots at -80 degrees Celsius if not used immediately.

FOX assay[9]

The principle of the FOX method was to change H_2O_2 oxidized ferrous ion (Fe^{2+}) into ferric ion (Fe^{3+}), which then reacted with xylenol orange to develop color. It could then be measured for absorbance at 560 nanometers.

Seven hundred microliters of sera were mixed with 6 mM of guanidine hydrochloride (100 μL), 0.5 M of perchloric acid (40 μL), 5 mM of XO (25 μL) and 5 mM of ferrous sulfate (10 μL). The mixture was incubated at room temperature for 30 min and then measured for absorbance at 560 nm, with XO/Fe^{2+} as a blank.

TMB method[10]

Regarding the TMB assay, horseradish peroxidase (HRP) reacted with H_2O_2 and developed a superoxide anion radical ($O_2^{\cdot-}$). The TMB acted as a chromogen and reacted with $O_2^{\cdot-}$ to develop a blue color. The reaction was stopped with acid, which then became a yellow color. Finally, the optical density was

measured at 450 nanometer.

Two hundred microliters of sera were mixed with phosphate buffer saline (250 μL), 420 μM of TMB (250 μL) and 52.2 U/mL of HRP (30 μL). Then, the solution was incubated at room temperature for 20 min. One molar of sulfuric acid (40 μL) was added to stop the reaction and then the absorbance was determined at 450 nm, with TMB/HRP as a blank.

Scavenging capacity test

H_2O_2 was added into the solution (according to FOX and TMB methods) to the final concentrations of 0.01, 0.02, 0.03, 0.04 and 0.06 μM before incubation. The solution was then processed according to each method.

Statistical analysis

The data were shown as mean±SEM and analyzed by using the Student t-test and two-way ANOVA. The p value was significant at <0.05.

Results

When comparing urinary H_2O_2 levels in the normal healthy subjects, measured by the FOX assay and TMB method, found the mean levels were 0.82±0.59 and 0.78±0.35 μM, respectively (as shown in Fig. 1). There was no significant difference (p>0.05) between these two methods.

The mean serum level of H_2O_2 in non-smokers was lower when compared to that of smokers (detected by both methods), which was significantly different (p<0.05). When measuring the levels of serum H_2O_2 in non-smokers (n=20) and smokers (n=20) by TMB assay, the mean H_2O_2 levels were 0.50±0.36 and 1.16±0.58 μM, respectively (Table 1). When adding H_2O_2 to the sera of non-smokers, H_2O_2 levels were remeasured to see the

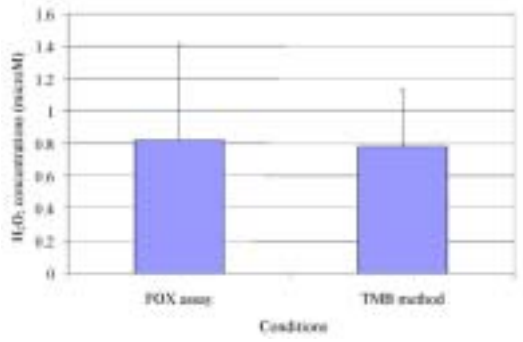

Figure 1. Urinary hydrogen peroxide levels of normal healthy subjects (n=20) using FOX and TMB methods. The value represented as mean±SEM. It was **not** significantly different (p>0.05).

Table 1. The serum H_2O_2 concentrations measured by TMB method of non-smokers (n=20), and smokers (n=20). The sera were remeasured for H_2O_2 after adding H_2O_2 at 0 (1*), 0.01 (2*), 0.02 (3*), 0.03 (4*), 0.04 (5*) and 0.06 (6*) μM as final concentrations. The value represented as mean±SEM.

Conditions	Non-smokers (μM)	Smokers (μM)
1*	0.50±0.36	1.16±0.58
2*	0.11±0.25	0.99±0.62
3*	0.04±0.19	0.99±0.61
4*	0.04±0.15	1.00±0.67
5*	0.04±0.17	1.00±0.66
6*	0.03±0.15	0.97±0.65

antioxidant or scavenging capacity of the sera. It was found that H_2O_2 levels were decreased until hardly determined by the TMB method, as shown in Fig. 2 and Table 1. Whereas, the serum H_2O_2 levels in smokers detected by the same method were not scavenged by the antioxidants in the sera, as shown in Fig. 2. Thus, this way could be used to determine the scavenging capacity of the blood compared between smokers and non-smokers which was also statistically significant (p<0.001).

With TMB method, the mean serum H_2O_2 level was lowered in non-smokers by 94% after adding extra H_2O_2 (0.06 μM) and low-

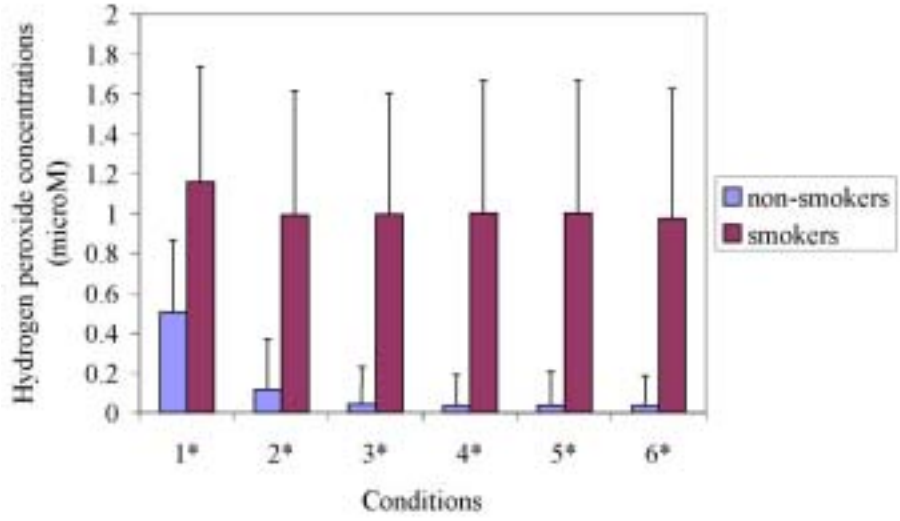

Figure 2. The serum H_2O_2 concentrations measured by TMB method of non-smokers (n=20), and smokers (n=20). The sera were remeasured for H_2O_2 after adding H_2O_2 at 0 (1*), 0.01 (2*), 0.02 (3*), 0.03 (4*), 0.04 (5*) and 0.06 (6*) μM as final concentrations. The value represented as mean+SEM. It was significantly different in each condition between smokers and non-smokers by two-way ANOVA (p<0.001).

ered by 16.38% in smokers. This meant that non-smokers had a good antioxidant system, which could buffer the oxidants added *in vitro*.

By using the FOX assay, the mean serum H_2O_2 levels in non-smokers and smokers were 0.04±0.12 and 0.76±0.90 μM, respectively. The mean serum H_2O_2 level in non-smokers was very low compared to that of smokers, as shown in Fig. 3 and Table 2, but when adding H_2O_2 to create the status of oxidative stress in the sera of both kinds of subjects, the levels of H_2O_2 were increased from the control, as shown in Fig. 3.

When detected by the FOX assay, the mean H_2O_2 level after adding extra H_2O_2 (0.06 μM) rose 63.75-fold in non-smokers compared to a control. Meanwhile, the mean H_2O_2 level (after adding extra H_2O_2 0.06 μM) in the smokers rose1.79-fold compared to a control, which was statistically significant (p<0.001). This phenomenon was the opposite to that which occurred when measuring by the TMB method.

Table 2. The serum H_2O_2 concentrations measured by FOX method of non-smokers (n=20), and smokers (n=20). The sera were remeasured for H_2O_2 after adding H_2O_2 at 0 (1*), 0.02 (2*), 0.03 (3*), 0.04 (4*), and 0.06 (5*) μM as final concentrations. The value represented as mean±SEM.

Conditions	Non-smokers (μM)	Smokers (μM)
1*	0.04±0.12	0.76±0.90
2*	1.91±0.70	0.98±1.44
3*	2.35±0.60	1.09±1.57
4*	2.21±0.45	1.14±1.53
5*	2.55±0.53	1.36±1.62

Discussion

Urinary and serum H_2O_2 could be used as systemic markers for oxidative stress. There are many kinds of biomarkers that are indices of oxidative stress status that contains high levels of oxidants and low levels of antioxidants, e.g. urinary 8-hydroxydeoxyguanosine (8-OHdG),[11] H_2O_2 in exhaled air,[12] and plasma nitric oxide.[13] There are two kinds of indices, i.e. one from local milieu (breath con-

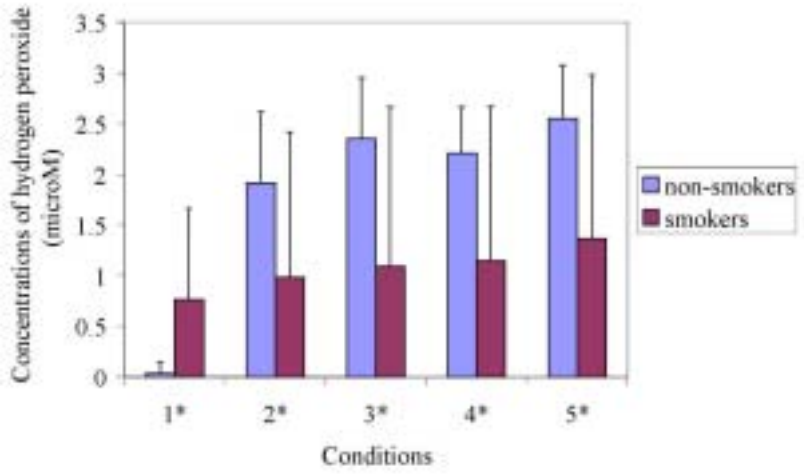

Figure 3. The H_2O_2 concentrations measured by FOX method of non-smokers (n=20), and smokers (n=20). The sera were remeasured for H_2O_2 after adding H_2O_2 at 0 (1*), 0.02 (2*), 0.03 (3*), 0.04 (4*), and 0.06 (5*) μM as final concentrations. The value represented as mean+SEM. It was significantly different in each condition between smokers and non-smokers by two-way ANOVA (p<0.001).

densate or bronchoalveolar lavage) and the other a systemic marker as urine or blood.

First, the two methods were validated in measuring H_2O_2 by using urinary H_2O_2 as a target. It was found by both methods that the mean levels of H_2O_2 in the urine from the normal subjects were not significantly different (p>0.05).

The different pattern of H_2O_2 levels found between non-smokers and smokers by the TMB method might come from the antioxidant levels in the sera, e.g. ascorbic acid, glutathione, alpha-tocopherol, catalase and superoxide dismutase, which caused the H_2O_2 levels in non-smokers to decrease progressively (Fig. 2). Whereas, the H_2O_2 levels of the smokers detected by the TMB method did not decrease abruptly, as shown in Fig. 2. This might be due to the lower levels of antioxidants in the smokers' blood. The antioxidants might have been used up due to the oxidative stress produced by the H_2O_2 added *ex vivo*.

Meanwhile, when measuring the H_2O_2 by FOX assay, the mean level of H_2O_2 (before H_2O_2 addition) in non-smokers was 12.5-fold lower compared to that measured by the TMB method. When adding H_2O_2, the remeasured mean H_2O_2 levels of non-smokers were higher, because they were not measured in the same way as the TMB method. The mechanism or reason that explains this phenomenon is still to be clarified. The subjects collected were of the same group as those determined by the TMB method. Further experiments are needed to demonstrate and elucidate this phenomenon as to whether it would be specific or not. However, oxidative stress may also develop when serum is exposed to air or ultraviolet radiation.[8]

The difference between these two methods of detecting the levels of H_2O_2 was the ability of the FOX assay to measure hydroperoxides (ROOH), lipid hydroperoxides and protein hydroperoxides together with H_2O_2 in the sample, which gave the result as H_2O_2 amount.[9] This may cause a high level of H_2O_2 when H_2O_2 is added, and produce lipid hydro-

peroxides and protein hydroperoxides. Whereas, the TMB method requires the specificity of HRP to react with H_2O_2 and develop a superoxide anion radical, which then reacts with TMB (a chromogen) to produce color change. The oxidation of ferrous to ferric ions was not involved in the reaction. This characteristic of the TMB method is the specificity of the assay, as it is an enzymatic reaction. Various endogenous and exogenous factors exert control over cellular protection against reactive oxygen species, and it was reported that vitamin E supplementation was a factor that reduced oxidative stress of erythrocytes in smokers and non-smokers.[14]

Non-smokers seem to have a high scavenging capacity, which reduced oxidants generated from smoke. Smoking is an essential factor that creates oxidative stress, and its cessation should be recommended to everyone in order to prevent development and progression of COPD, neoplastic disorders, atherosclerosis, degenerative diseases and retinal diseases.[7] Serum and urinary H_2O_2 may be used as markers for early diagnosis and might also provide information for the prognosis of such diseases. Antioxidant supplementation by nutritional means should be advised to non-smokers and especially smokers in order to prevent and inhibit disease progression and development.

Conclusion

TMB and Fox methods could be used to measure serum and urinary H_2O_2 in smokers and non-smokers. The mean H_2O_2 level of non-smokers was lower than that of smokers by both methods. Non-smokers' sera had a better scavenging capacity than that of smokers. However, when adding H_2O_2 *ex vivo*, its mean level increased when measured by the FOX method and decreased by the TMB method. This could be due to the development of lipid hydroperoxides and protein hydroperoxides, which might react with FOX reagent(s) but not TMB one(s). The TMB method is specific for H_2O_2 measurement, since it is an enzymatic reaction. Each method had unique characterstics in measuring H_2O_2 levels.

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การเปรียบเทียบระดับไฮโดรเจนเปอร์ออกไซด์ในซีรั่มระหว่างคนไม่สูบบุหรี่และคนสูบบุหรี่โดยการวัดสองวิธี

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ภาควิชาชีวเคมี คณะแพทยศาสตร์ มหาวิทยาลัยเชียงใหม่

บทคัดย่อ ไฮโดรเจนเปอร์ออกไซด์เป็นสารผลิตภัณฑ์ในขบวนการเมแทบอลิสม์ที่มีองค์ประกอบของออกซิเจนและมีความไวในการเกิดปฏิกิริยา ซึ่งเป็นตัวควบคุมสำคัญในภาวะเครียดออกซิเดชัน วัตถุประสงค์ของการศึกษานี้เพื่อจะเปรียบเทียบระดับซีรั่มไฮโดรเจนเปอร์ออกไซด์ในคนไม่สูบบุหรี่และคนสูบบุหรี่โดยวิธีเตตราเมธิลีนเบนซิดีน (ทีเอ็มบี หรือ TMB) และวิธีฟอกซ์ (FOX) ในคนปกติจำนวน 20 คน ระดับไฮโดรเจนเปอร์ออกไซด์เฉลี่ยในปัสสาวะเมื่อวัดโดยวิธีทีเอ็มบีและฟอกซ์มีค่าเท่ากับ 0.78±0.35 และ 0.82±0.59 ไมโครโมลาร์ตามลำดับซึ่งไม่มีความแตกต่างกันอย่างมีนัยสำคัญทางสถิติ (p>0.05) เมื่อวัดระดับซีรั่มไฮโดรเจนเปอร์ออกไซด์ในคนไม่สูบบุหรี่ (จำนวน 20 คน) และคนสูบบุหรี่ (20 คน) โดยวิธีฟอกซ์พบว่าค่าเฉลี่ยเท่ากับ 0.04±0.12 และ 0.76±0.90 ไมโครโมลาร์ตามลำดับ เมื่อเติมไฮโดรเจนเปอร์ออกไซด์ให้มีความเข้มข้นต่างๆ (0.01, 0.02, 0.03, 0.04 และ 0.06 ไมโครโมลาร์) เปรียบเทียบความสามารถในการทำลายออกซิแดนท์ของซีรั่มในคนทั้งสองกลุ่ม โดยวิธีทีเอ็มบีระดับเฉลี่ยของไฮโดรเจนเปอร์ออกไซด์ในคนไม่สูบบุหรี่ลดลงร้อยละ 94 และในคนสูบบุหรี่ลดลงร้อยละ 16.38 แต่โดยวิธีฟอกซ์ระดับเฉลี่ยในคนไม่สูบบุหรี่เพิ่มขึ้น 63.75 เท่าเปรียบเทียบกับก่อนเติมไฮโดรเจนเปอร์ออกไซด์ ขณะที่ในคนสูบบุหรี่มีระดับเพิ่มขึ้น 1.79 เท่า โดยสรุปซีรั่มของคนสูบบุหรี่มีความสามารถในการทำลายออกซิแดนท์ลดลงเมื่อเทียบกับคน ไม่สูบบุหรี่อย่างมีนัยสำคัญ (p<0.05) ทั้งสองวิธีมีลักษณะที่แตกต่างกันในการตรวจวัดระดับ ไฮโดรเจนเปอร์ออกไซด์ **เชียงใหม่เวชสาร 2548;44(4):129-135.**

คำสำคัญ: ไฮโดรเจนเปอร์ออกไซด์ วิธีทีเอ็มบี วิธีฟอกซ์ คนสูบบุหรี่ คนไม่สูบบุหรี่