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

Yuwadee Puttivisit, B.Sc., Ratana Banjerdpongchai, Ph.D.,
Nattakarn Leelarungrayab, M.Sc., Viboon Rattanapanone, Ph.D.

Department of Biochemistry, Faculty of Medicine, Chiang Mai University

Abstract
Hydrogen peroxide (H$_2$O$_2$) 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$_2$O$_2$ 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$_2$O$_2$ 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$_2$O$_2$ in non-smokers (n=20) and smokers (n=20) by FOX assay, the mean H$_2$O$_2$ levels were 0.04±0.12 and 0.76±0.90 µM, respectively. By adding H$_2$O$_2$ 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$_2$O$_2$ 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$_2$O$_2$ level in non-smokers rose 63.75-fold compared to a control (before H$_2$O$_2$ 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$_2$O$_2$. Chiang Mai Med Bull 2005;44(4):129-135.

Keywords: hydrogen peroxide (H$_2$O$_2$), 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$_2$O$_2$ concentration (oxidative stress) in the airways. H$_2$O$_2$ 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
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**

Tetramethylebenzidine (TMB), horseradish peroxidase (HRP), guanidine hydrochloride, xylenol orange [XO; 3-cresolsulfonaphthalein-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\(^{3+}\) 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\(^{2-}\)). The TMB acted as a chromogen and reacted with O\(^{2-}\) to develop a blue color. The reaction was stopped with acid, which then became a yellow color. Finally, the optical density was
Comparison of serum hydrogen peroxide levels

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**

H₂O₂ 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 H₂O₂ 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 H₂O₂ 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 H₂O₂ in non-smokers (n=20) and smokers (n=20) by TMB assay, the mean H₂O₂ levels were 0.50±0.36 and 1.16±0.58 µM, respectively (Table 1). When adding H₂O₂ to the sera of non-smokers, H₂O₂ levels were remeasured to see the antioxidant or scavenging capacity of the sera. It was found that H₂O₂ levels were decreased until hardly determined by the TMB method, as shown in Fig. 2 and Table 1. Whereas, the serum H₂O₂ 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 H₂O₂ level was lowered in non-smokers by 94% after adding extra H₂O₂ (0.06 µM) and low-
erated 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$_2$O$_2$ levels in non-smokers and smokers were 0.04±0.12 and 0.76±0.90 µM, respectively. The mean serum H$_2$O$_2$ level in non-smokers was very low compared to that of smokers, as shown in Fig. 3 and Table 2, but when adding H$_2$O$_2$ to create the status of oxidative stress in the sera of both kinds of subjects, the levels of H$_2$O$_2$ were increased from the control, as shown in Fig. 3.

When detected by the FOX assay, the mean H$_2$O$_2$ level after adding extra H$_2$O$_2$ (0.06 µM) rose 63.75-fold in non-smokers compared to a control. Meanwhile, the mean H$_2$O$_2$ level (after adding extra H$_2$O$_2$ 0.06 µM) in the smokers rose 1.79-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.

**Discussion**

Urinary and serum H$_2$O$_2$ 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$_2$O$_2$ in exhaled air,$^{(12)}$ and plasma nitric oxide.$^{(13)}$ There are two kinds of indices, i.e. one from local milieu (breath con-
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densate or bronchoalveolar lavage) and the other a systemic marker as urine or blood.

First, the two methods were validated in measuring H$_2$O$_2$ by using urinary H$_2$O$_2$ as a target. It was found by both methods that the mean levels of H$_2$O$_2$ in the urine from the normal subjects were not significantly different ($p>0.05$).

The different pattern of H$_2$O$_2$ 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$_2$O$_2$ levels in non-smokers to decrease progressively (Fig. 2). Whereas, the H$_2$O$_2$ 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$_2$O$_2$ added ex vivo.

Meanwhile, when measuring the H$_2$O$_2$ by FOX assay, the mean level of H$_2$O$_2$ (before H$_2$O$_2$ addition) in non-smokers was 12.5-fold lower compared to that measured by the TMB method. When adding H$_2$O$_2$, the remeasured mean H$_2$O$_2$ 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.

The difference between these two methods of detecting the levels of H$_2$O$_2$ was the ability of the FOX assay to measure hydroperoxides (ROOH), lipid hydroperoxides and protein hydroperoxides together with H$_2$O$_2$ in the sample, which gave the result as H$_2$O$_2$ amount. This may cause a high level of H$_2$O$_2$ when H$_2$O$_2$ is added, and produce lipid hydro-

Figure 3. The H$_2$O$_2$ concentrations measured by FOX method of non-smokers (n=20), and smokers (n=20). The sera were remeasured for H$_2$O$_2$ after adding H$_2$O$_2$ 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 ($p<0.001$).
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 characteristics in measuring $H_2O_2$ levels.

**References**

Comparison of serum hydrogen peroxide levels among smokers and non-smokers.


การเปรียบเทียบระดับไฮโดรเจนเปอร์ออกไซด์ในชีวิตประจำวันคนที่ไม่สูบบุหรี่และคนสูบบุหรี่โดยการวัดสองวิธี

ยุวดี พุทธิวิศิษฏ์, วท.บ., รัตน บริสินพงศ์ชัย, Ph.D., นัฏฐกาล ลีลารุ่งระยับ, วท.ม., วิบูลย์ รัตนาปนนท์, Ph.D.

บทคัดย่อ ไฮโดรเจนเปอร์ออกไซด์เป็นสารผลิตภัณฑ์ในขบวนการเมแทบอลิสมที่มีองค์ประกอบของออกซิเจนและมีความไวในการเกิดปฏิกิริยาซึ่งเป็นตัวควบคุมสำคัญในการเร่งของออกซิเดชัน วัตถุประสงค์ของการศึกษานี้เพื่อวิเคราะห์ระดับรังสีรังสีในคนไม่สูบบุหรี่และคนสูบบุหรี่โดยวิธีเตตราเมธิลีนเบนซิดีน (ทีเอ็มบีหรือ TMB) และวิธีฟอกซ์(FOX) ในคนปกติจำนวน 20 คน ระดับไฮโดรเจนเปอร์ออกไซด์เฉลี่ยในปัสสาวะเมื่อวัดโดยวิธีทีเอ็มบีและฟอกซ์คือ 0.78±0.35 และ 0.82±0.59 ไมโครโมลาร์ตามลำดับซึ่งไม่มีความแตกต่างกันอย่างมีนัยสำคัญทางสถิติ (p>0.05) เมื่อวัดระดับรังสีรังสีในชีวิตประจำวันคนสูบบุหรี่ (จำนวน 20 คน) และคนไม่สูบบุหรี่ (20 คน) โดยวิธีฟอกซ์พบว่าระดับเฉลี่ยเท่ากับ 0.04±0.12 และ 0.76±0.90 ไมโครโมลาร์ตามลำดับเมื่อวัดโดยวิธีฟอกซ์คือระดับสูงกว่าในคนที่ไม่สูบบุหรี่ (p<0.05) เมื่อเทียบกับคนไม่สูบบุหรี่ในกลุ่มที่มีความแตกต่างอย่างมีนัยสำคัญทางสถิติ (p<0.05) เนื่องจากการประหยัดของความชื้นทางشيخวัดในชีวิตประจำวัน ค่าที่เห็นในกลุ่มคนสูบบุหรี่มีแนวโน้มที่ลดลงค่อนข้างมากเมื่อเทียบกับคนไม่สูบบุหรี่ (p<0.05) ทำให้การชัยเอกซ์รังสีรังสีได้ใช้ในการตรวจวัดระดับไฮโดรเจนเปอร์ออกไซด์ เชิงบวก

คำสำคัญ: ไฮโดรเจนเปอร์ออกไซด์ วิธีทีเอ็มบี วิธีฟอกซ์ คนสูบบุหรี่ คนไม่สูบบุหรี่