AGNIESZKA SZUSTER-CIESIELSKA, MARTYNA KANDEFER-SZERSZEŃ

Department of Virology and Immunology
Maria Curie-Skłodowska University, Akademicka 19, 20-033 Lublin, Poland

The influence of sex and age on serum catalase, peroxidase, superoxide dismutase activity and production of \( \text{O}_2^- \) and \( \text{H}_2\text{O}_2 \) by human blood neutrophils

SUMMARY

In this paper we measured spontaneous and in vitro phorbol ester (PMA)-induced production of superoxide anion (\( \text{O}_2^- \)) and hydrogen peroxide (\( \text{H}_2\text{O}_2 \)) in neutrophils isolated from blood of 54 healthy persons, 36 females and 18 males aged 21–81 years. Neutrophils of females, independently of age, produced less intracellular \( \text{O}_2^- \) (measured by NBT method), spontaneously and after PMA induction in comparison to males and also less \( \text{H}_2\text{O}_2 \) PMA-induced. Moreover, neutrophils of females older than 50 (postmenopausal) produced less intracellular \( \text{O}_2^- \) but more extracellular superoxide anion than in the younger women probably because superoxide anions were more effectively released from cells. In contrast to superoxide anion, neutrophils of persons (especially females) 41–50 years old produced relatively high levels of \( \text{H}_2\text{O}_2 \). However, these differences were not statistically significant in Mann-Whitney test because of large between-subject variations.

We also measured serum activity of antioxidant enzymes (AOE) such as catalase (CAT), peroxidase (PER) and superoxide dismutase (SOD). A generally lower CAT activity in sera of females in comparison to males, especially in the age group from 41 to 50 years and a generally higher serum PER activity in females were found. The highest PER activity was detected in females older than 50 and the lowest in those from 31 to 50 years. SOD activity was the highest in men 41–50 years old.

The oxidative stress can be defined as disturbance in the prooxidant-antioxidant balance in favour of the former. As neutrophils of persons 41–50 years old produce relatively high levels of intracellular \( \text{O}_2^- \) and \( \text{H}_2\text{O}_2 \), but serum CAT and PER activity is relatively low, the results suggest that in blood of persons of this age the oxidative stress could be the highest.
STRESZCZENIE

W pracy badano spontaniczne i indukowane estrem forbolu (PMA) wytwarzanie anionorodnika ponadtlenkowego (O$_2^-$) oraz nadtlenku wodoru (H$_2$O$_2$) w neutrofilach izolowanych z krwi 54 zdrowych osób, 36 kobiet, 18 mężczyzn w wieku 21–81 lat. Stwierdzono, że neutrofile kobiet, niezależnie od wieku, wytwarzają mniej O$_2^-$ (mierzonego wewnątrz komórki z użyciem metody NBT) spontanicznie i po indukcji PMA w porównaniu z mężczyznami oraz mniej indukowanego PMA H$_2$O$_2$. Dodatkowo, neutrofile kobiet w wieku powyżej 50 lat (w okresie postmenopausalnym) wytwarzają mniej wewnątrzkomórkowego O$_2^-$, lecz więcej pozakomórkowego anionorodnika ponadtlenkowego niż młode kobiety, co sugeruje różnice w szybkości uwalniania rodnika z komórki. W przeciwieństwie do anionorodnika ponadtlenkowego, neutrofile osób (szczególnie kobiet) w wieku 41–50 lat wytwarzają relatywnie dużo H$_2$O$_2$. Różnice te nie były jednak statystycznie istotne w teście Manna-Whitneya ze względu na duże różnice osobnicze.

Badano także aktywność enzymów antyoksydacyjnych (AOE): katalazy (CAT), peroksydazy (PER) i dysmutazy ponadtlenkowej (SOD) w surowicy. Stwierdzono niższą aktywność CAT w surowicy kobiet w porównaniu z mężczyznami, szczególnie w grupie wiekowej 41–50 lat i wyższą aktywność PER w surowicy kobiet. Najwyższą aktywność surowiczą PER wykryto w surowicy kobiet w wieku powyżej 50 lat, a najniższą u kobiet w wieku 31–50 lat. Aktywność SOD w surowicy była najwyższa u mężczyzn w wieku 41–50 lat.

Stres oksydacyjny jest zaburzeniem równowagi działania czynników prooksydacyjnych i antyoksydacyjnych, z przewagą tych pierwszych. Neutrofile osób w wieku 41–50 lat wytwarzają relatywnie duże ilości wewnątrzkomórkowego O$_2^-$ oraz H$_2$O$_2$, a surowicza aktywność CAT i PER jest stosunkowo niska, dlatego wyniki sugerują, że w krwi osób w wieku 41–50 lat stres oksydacyjny może być największsy.

**Keywords:** aging, reactive oxygen species (ROS), neutrophils, catalase (CAT), peroxidase (PER), superoxide dismutase (SOD).

INTRODUCTION

Neutrophils constitute about 60% of the circulating leukocytes. The phagocytes acts as the first defence line in blood. Along phagocytosis of microorganisms, a number of different cellular processes, including motility, endocytosis, respiratory burst, secretion of cytoplasmic (proteolytic) enzymes and immunoregulatory compounds are initiated (30). The combination of these processes assists in killing and digestion of engulfed bacteria and, if prolonged, the development of local inflammation. The increase of the respiratory burst involves a sudden stimulus-induced non-mitochondrial oxidative metabolism which results in the production of reactive oxygen species (ROS) which, produced in excess, can be harmful for surrounding cells and tissues (25, 34).

It has been suggested that increased incidence of tumors and autoimmune diseases in elderly persons may be related to an impaired immune system (31). Dysfunction of Th1 and Th2 cells with predominating Th2 cells has been detected in elderly subjects. Monocyte and macrophage functions are also affected in aged ones, yielding relatively larger amounts of IL-1, IL-6 and TNF-α as proinflammatory cytokines, while the production of IFN-α as antiviral and immunoregulatory cytokine is reduced (6, 9). Impairment of neutrophil functions as chemotaxis and phagocytosis, resulting in high incidence of serious bacterial infections has also been described (16).

According to the free radical theory of aging proposed by Harman in 1956 (18), oxygen-derived free radicals cause damage to cells which leads to age-associated impairments in cellular
and organic functions. The main source of ROS are mitochondria, but ROS can be released from other sources, for example, neutrophils and macrophages. Moreover, the formation rates of ROS in mitochondria increase with age (15, 36). ROS are highly reactive substances that can cause a wide spectrum of cell damage, including lipid peroxidation, enzymes inactivation and DNA damage (14, 24). According to the new theory of Yu (40) aging is connected with oxidative stress, defined as disturbances in prooxidant-antioxidant balance in favour of the former.

Mammalian cells are protected against ROS by enzymatic and nonenzymatic antioxidant defences. The primary antioxidant enzymes (AOE) are superoxide dismutase (SOD; EC 1.15.1.1) which catalyzes the dismutation of superoxide anions \(O_2^-\) to hydrogen peroxide \(H_2O_2\), catalase (CAT; EC 1.11.1.6) and glutathione peroxidase (GSH-Px; EC 1.11.1.9) which catalyze degradation of \(H_2O_2\) to water and \(O_2\). These enzymes are expressed in different tissues, among other things in blood cells. Complete understanding of the antioxidant system in human blood as a function of sex and age requires measurements not only of the activity of enzymes present in blood cells (erythrocytes, white blood cells) but also that of extracellular AOE present in serum. To assess the oxidative stress in blood of persons at different age and of different sex we measured SOD, CAT and total peroxidase (PER) activity in serum and also ROS production by blood neutrophils.

**MATERIALS AND METHODS**

**Subjects**

We assayed serum activity of SOD, CAT and total peroxidase (PER) and also ROS production by neutrophils isolated from blood of 54 persons (36 females, 18 males) aged 21–81 years. Blood samples were obtained from students and university workers. None of the subjects had any known pathologies at the time of sampling or declared to be a tobacco smoker. Before blood collection the subjects fasted from midnight till the morning. Formal consent was obtained from each person to use their blood for measurements of enzyme activity and ROS production.

**Granulocyte separation**

Blood was taken into heparinized tubes (20 U/ml, Heparinum-Polfa). Granulocytes were separated according to the Sigma procedure: a gradient was formed by layering an equal volume of Histopaque-1077 over Histopaque-1119 (both from Sigma). The whole blood was carefully layered onto the upper Histopaque-1077. The tubes were then centrifuged at 700 × g for 30 min. Granulocytes were isolated from the Histopaque-1077/1119 inter-phase and washed three times with HBSS (Hanks’ Balanced Salt Solution), centrifuged (350 × g for 15 min) and suspended in HBSS.

**Measurement of superoxide production by nitroblue tetrazolium (NBT) reduction assay (32)**

Neutrophils \((4 \times 10^6/ml \text{ HBSS})\) distributed into wells on 96-well microplate (100 µl/well) were covered with 100 µl/well of NBT (Sigma) solution (2 mg/ml) in phenol red-free HBSS containing the stimulants of oxidative burst, phorbol myristate acetate-PMA (final concentration 1 µg/ml, Sigma). Eight vertical wells serves as blank and in these wells the cells were pre-incubated for 10 min at 37°C with 100 µl/well of 10 mM iodoacetamide (Sigma) in HBSS. After incubation the iodoacetamide solution was removed and replaced by a solution containing both 1 mg/ml NBT
and 10 mM iodoacetamide. Samples were incubated at 37°C for 30 min. The amounts of formazan were quantified in a microplate reader (Molecular Devices) at 570 nm. The results were expressed as optical density (OD) per well after blank subtracting.

**Measurement of superoxide anion production**

**by cytochrome c reduction assay** (23)

HBSS (176 μl), 12.5 μl of cytochrome c solution in HBSS (final concentration 75 μM), 5 μl of either SOD solution (final concentration 60 U/ml) or 5 μl of distilled water, and 50 μl of neutrophil suspension (final density of 2.5 × 10⁵ cells/well) were added into each well on 96-well plate. After 3 min of incubation neutrophils were activated with PMA (final concentration 1 μg/ml) in 6.5 μl/well. The microplate was incubated at 37°C for 60 min and transferred to the microplate reader. The absorbance values at 550 nm (differences in OD between samples with and without SOD) were converted to nanomoles of O⁻₂ based on the extinction coefficient of cytochrome c: Δε₅₅₀ = 21 × 10³ M⁻¹ cm⁻¹. The results were expressed as nanomoles of O⁻₂ per 1 × 10⁶ cells per 60 min.

**Measurement of hydrogen peroxide (H₂O₂) production** (32)

The assay was based on horse-radish-dependent peroxidation (HRPO) of phenol red by H₂O₂ leading to the formation of a compound that exhibits absorbance at 600 nm. Neutrophil suspension (4 × 10⁶ cell/ml of HBSS) was distributed into wells (50 μl/well) on 96-well microplate. Cells were covered with 50 μl/well of assay solution. The assay solution was prepared on the day of experiment and consisted of HBSS, phenol red (Sigma, final concentration 0.56 mM), HRPO (Serva, final concentration 20 U/ml) and PMA (Sigma, final concentration 1 μg/ml). To control wells an assay solution without PMA was added. The plate was incubated at 37°C for 60 min and then the reaction was stopped by adding 10 μl/well of 1 N NaOH. After 3 min incubation the plates was read at 600 nm in the microplate reader.

The results were expressed as nanomoles of H₂O₂ per 10⁶ cells per 60 min based on the phenol red extinction coefficient (Δε₆₀₀ = 19.8 × 10³ M⁻¹ cm⁻¹).

**Serum catalase (CAT) activity assay**

Serum was separated by centrifugation of unheparinized blood and kept at −20°C before measuring the enzyme activity. The assay was performed according to the method described by Pifferi (33) in Nowak modification (28). The reaction mixture prepared in Eppendorf tube consisted of 500 μl of 0.05 M phosphate buffer pH 7.0, 300 μl of distilled water, 50 μl of 1.1 mM H₂O₂ in distilled water and 50 μl of serum sample (or 50 μl of distilled water as blank). After 5 min of incubation at 25°C 100 μl of 50% trichloroacetic acid (TCA, Sigma) was added into each tube and they were centrifuged (1000 × g for 5 min). Next, to each tube 10 μl of titanium (IV) reagent (27) was added and 200 μl of supernatant was transferred into wells on 96-well microplate. The absorbance was read at 405 nm in microplate reader. The results were expressed as catalase activity in U/ml of serum after comparison with the standard curve prepared by plotting the absorbance (OD) at 405 nm (ordinate) as a function of standard CAT (Sigma) concentration (abscissa) between 0 and 33 U/ml. One unit of CAT decomposed 1.0 μmole of H₂O₂ per min at pH 7.0 and 25°C.
**Serum peroxidase (PER) activity assay** (10)

The reaction mixture contained 0.1 ml of 1% o-dianisidin (Fluka) in methanol with 9.9 ml of 0.003% H$_2$O$_2$ in 0.05 M phosphate buffer pH 7.0. The serum samples in 96-well plate (10 μl/well) were mixed with 190 μl/well of the reaction mixture. The extinction was read in microplate reader at 450 nm after 60 sec. incubation at 25°C. The results were expressed as whole peroxidase activity in U/ml of serum after comparison with the standard curve, which was prepared in the same way as described for CAT, in the range value of standard peroxidase activity (HRPO, Serva) between 0–2.5 U/ml of phosphate buffer. One unit of peroxidase was defined as enzymatic activity which catalyzed the decomposition of 1 μmol of H$_2$O$_2$ per minute at 25°C, pH 7.0.

**Serum superoxide dismutase (SOD) activity assay**

To measure the SOD activity we modified the method described by Oberley and Spitz (29). Briefly, to each well on 96-well microplate 160 μl of the reaction mixture consisting of DETAPAC (diethylenetriamine pentaacetic acid, Sigma, final concentration 1 mM in 0.05 M phosphate buffer, pH 7.8), MTT (tiaryl blue, Sigma, final concentration 1 mg/ml in phosphate buffer) and xanthine (Sigma, 1.8 mM in buffer) were added. 20 μl of serum or 20 μl of phosphate buffer (blank) was added to the wells. Reaction was started by adding 20 μl of xanthine oxidase (final concentration 0.01 U/ml of phosphate buffer). Microplate was incubated at 25°C for 60 min. and the absorbance of samples and blank was read at 570 nm in microplate reader. The results were expressed in U of SOD/ml of serum after comparison with standard curve which was prepared by diluting standard SOD (Sigma) in the range from 0 to 0.935 U/ml of phosphate buffer. One unit of SOD inhibited the rate of MTT reduction by 50% generated by xanthine/xanthine oxidase system at 25°C, pH 7.8.

**Statistics**

Data received were analysed in Mann-Whitney test, using software "Statistica".

**RESULTS AND DISCUSSION**

Cells of the immune system utilize reactive oxygen intermediates as the first defence line against bacterial/viral pathogens. Large amounts of ROS are produced by activated macrophages and neutrophils that participate in immune and inflammatory responses. While ROS generated by phagocytes help to combat in invading organisms they may be deleterious to surrounding host cells and tissues (16, 25, 34).

In our experiments we examined the production of superoxide anion (O$_2^-$) and hydrogen peroxide (H$_2$O$_2$) by neutrophils isolated from blood of clinically healthy men and women of different age. We analysed the influence of sex independently of age and also the influence of both sex and age on ROS production. Females in comparison to males independently of age produced less intracellular ROS spontaneously and after induction with phorbol ester (PMA) and less PMA-induced hydrogen peroxide (Table 1). Moreover, elderly females produced less
intracellular ROS (measured by NBT method) probably because ROS were quickly released from cells and were detected by the method with cytochrome c (compare Fig. 1A and B) and both males and females especially at the age of 41–50 produced less extracellular ROS in comparison to younger and older persons. However, these differences were not statistically significant because of the large between-subject variations. Especially high production of superoxide anion was detected in women with postmenopausal status (older than 50 years). These results confirmed observations of other authors, who detected that the level of lipid peroxide (LPO) as a marker of oxidative stress was the highest in erythrocytes of postmenopausal women (4).

The data from the literature are controversial. When nonstimulated and polystyrene beads stimulated ROS production in neutrophils was measured by the chemiluminescence assay it was detected that neutrophils of aged subjects produced more ROS in comparison to young men and women despite the fact that killing activity of neutrophils was lower in aged persons (11, 20). No significant differences between males and females were detected. When PMA was used as neutrophil stimulator a significant reduction of chemiluminescence (ROS production) was observed in persons over 75 years old (7, 8). It seems likely that the results depended on the method used for ROS detection, on the inducer used for neutrophil activation, on the age and sex of the persons examined. More experiments are needed to explain these discrepancies.

In the recent years the activity of antioxidant enzymes (AOE) was measured not only in blood cells (erythrocytes or leukocytes) but also in plasma and serum. It was detected that the plasma levels of CAT, GSH-Px and SOD were lower than

<table>
<thead>
<tr>
<th>Variables</th>
<th>NBT-PMA</th>
<th>Cyt-PMA</th>
<th>PMA</th>
<th>H_2O_2</th>
<th>SOD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Females</td>
<td>0.46</td>
<td>0.29</td>
<td>5.84</td>
<td>1.72</td>
<td>16.35</td>
</tr>
<tr>
<td>Males</td>
<td>0.71</td>
<td>0.42</td>
<td>5.78</td>
<td>1.62</td>
<td>23.3</td>
</tr>
<tr>
<td>Whole group</td>
<td>0.55</td>
<td>0.34</td>
<td>5.82</td>
<td>1.69</td>
<td>18.67</td>
</tr>
</tbody>
</table>

1 Control (spontaneous) ROS release without induction.
2 O_2^- production phorbol ester (PMA)-induced, measured by the NBT method.
3 O_2^- production PMA-induced, measured by the cytochrome c method.
4 H_2O_2 production PMA-induced, measured by the phenol red method.
Fig. 1. Age dependence of intracellular $O_2^-$ production in PMA stimulated blood neutrophils measured by the NBT method (A), extracellular $O_2^-$ measured by the cytochrome c method (B) and $H_2O_2$ production measured by the phenol red method (C)
in red blood cells and several other tissues, though detectable. Moreover, they were found to be very important in the regulation of plasma levels of ROS and in protection of plasma compounds against ROS. Imbalance in the activity of extracellular AOE and plasma levels of free radical species was detected, for example, in chronic renal failure (26), after smoke exposure (21), in cigarette smokers (1) and psychiatric patients (19), but there is lack of publications concerning the influence of age on the serum activity of AOE.

It has been detected (5) that AOE activity in human blood cells exhibits large between-subject variations, but generally females have a higher SOD and CAT activity in erythrocytes and a lower GSH-Px activity than males. In our experiments when serum activity of AOE was measured we also detected big individual differences (high standard deviation) in serum activity of AOE, but females had a little lower activity of CAT than males. However, these differences were not statistically significant (Table 1, Fig. 2A). The lowest CAT activity was detected especially in females of group A (20–30 years old) and C (41 to 50 years old).

Several types of GSH-Px have been identified in blood, two are classical cellular enzymes found in red blood cells and one extracellular enzyme (eGSH-Px) found in plasma. Cellular enzymes are selenium-dependent, each containing Se atom in the active site in the form of selenocysteine and exist as tetramers of identical subunits (37). Plasma GSH-Px differs distinctly from cellular GSH-Px in substrate specificity, gene structure and gene localization. This enzyme is actively secreted into plasma by kidney proximal tubules, by liver, heart, lungs and skeletal muscles (3, 12). eGSH-Px plays an important role in protection of extracellular fluid components against peroxide-mediated damage (27, 35, 39). Since o-dianisidine was shown to be able to detect several glutathione peroxidase activities (22), we used it to detect total peroxidase (PER) activity present in serum.

Total peroxidase (PER) activity in sera of females was comparable to that of males (Table 1). When we analysed the activity in different age groups the lowest activity was detected both in females and males at the age from 31 to 50 years and the highest in persons older than 50. It should be noted that according to other authors GSH-Px activity (2, 5) in human erythrocytes increases with the age. Moreover, the increase in PER serum activity in elderly people observed by us was in agreement with the observations that rats at the age corresponding to approximately 60–70-year-old men showed significantly higher GSH-Px activity in erythrocytes than young rats (13, 17).

In contrast to CAT and PER serum activity, SOD activity was in our experiments relatively high in group C (41–50 years) and low in older females. No significant differences in serum SOD activity between males and females was detected. These results differed in comparison to those of other authors, who detected that SOD activity in erythrocytes of females is higher than that of males
Fig. 2. Age dependence of catalase (A), total peroxidase (B) and superoxide dismutase (C) serum activity. Activity of enzymes was measured by the spectrophotometric methods as described in Materials and Methods.
and that this activity decreases with age (5). As we examined serum activity of SOD which, as it is known from the literature, is connected with the expression and release of extracellular SOD 3 (38), we can only speculate that the differences between our results and those of other authors are caused by differences in the regulation of the expression of the activity of cytosolic SOD 1, mitochondrial SOD 2 and extracellular SOD 3 in the same age group of persons.

The oxidative stress can be defined as a disturbance in the prooxidant-antioxidant balance in favour of the former. As the production of intracellular ROS, induced by PMA (measured by NBT method) and also induced by PMA hydrogen peroxide was relatively higher in persons 41–50 years old, we can suggest that in this group the oxidative stress is the highest. It can also be speculated that not only ROS produced in mitochondria but also ROS produced by neutrophils can participate in many age-related diseases such as cancers, diabetes, atherosclerosis and of the impairment immune system, especially when age-related decrease in the plasma activity of AOE occurs.

REFERENCES