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Assessment of Oxidative Stress in Septic and Obese Patients Using Markers of Oxidation-reduction Potential

YPATIOS SPANIDIS[1], NIKOLAOS GOUTZOURELAS[1], DIMITRIOS STAGOS[1], ANASTASIA S. KOLYVA[2], CHARALAMBOS A. GOGOS[2], DAVID BAR-OR[3,4,5,6] and DIMITRIOS KOURETAS[1]

[1]*Department of Biochemistry and Biotechnology, University of Thessaly, Larissa, Greece;*
[2]*Division of Infectious Diseases, Department of Internal Medicine, Patras University Hospital, Rion-Patras, Greece;*
[3]*Trauma Research Department, St. Anthony Hospital, Lakewood, CO, U.S.A.;*
[4]*Trauma Research Department, Swedish Medical Center, Englewood, CO, U.S.A.;*
[5]*Trauma Research Department, Medical Center of Plano, Plano, TX, U.S.A.;*
[6]*Luoxis Diagnostics, Inc., Englewood, CO, U.S.A.*

Abstract. *Background/Aim: The novel static (sORP) and capacity (cORP) oxidation-reduction potential markers were examined for assessing oxidative stress in plasma of patients with sepsis. Moreover, the possible effect of obesity-induced oxidative stress on patients with sepsis was investigated. Materials and Methods: sORP and cORP markers, as well as the conventional oxidative stress biomarkers total antioxidant capacity (TAC), thiobarbituric acid-reactive substances (TBARS) and protein carbonyls (CARB), were assessed in plasma. Results: sORP marker was increased significantly in the sepsis group, while cORP was significantly lower compared to the control group, indicating oxidative stress. Furthermore, in patients with sepsis, TAC was significantly lower compared to control group. However, obesity had no effect on sORP, cORP and TAC in patients with sepsis, although it increased levels of CARB and TBARS. Conclusion: The present results suggest, for the first time, that ORP markers could be used for assessing oxidative stress in patients with sepsis.*

Reactive oxygen species (ROS) are products of normal metabolism (1). ROS include free radicals such as superoxide radical (O_2•–), hydroxyl radical (OH•), peroxyl radical (RO•$_2$), as well as nonradical species such as hydrogen peroxide (1). Some ROS are necessary for physiological processes such as phagocytosis (2), intracellular signaling (1), cell proliferation, metabolism and apoptosis (3). Endogenous sources of free radicals include the mitochondrial respiratory chain, inflammation, peroxisomes and cytochrome P450 (4). However, excessive production of ROS can lead to oxidative stress, a pathophysiological condition, resulting in oxidative damage of macromolecules (lipids, proteins and DNA) (1, 5). Thus, oxidative stress has been associated with several pathophysiological conditions and diseases (1).

One of the pathological conditions associated with oxidative stress is sepsis, a systemic inflammatory response syndrome attributed to infection (6-8). The infection is most commonly caused by bacteria but can also be caused by fungi, viruses, or parasites (6, 7). Common symptoms of sepsis include fever, increased heart rate, increased breathing rate, and confusion (6, 7). Sepsis leads to collateral damage of normal tissues due to the constant activation of monocytes and endothelial cells that trigger inflammatory and coagulation cascades (9). The severity and outcome of septic syndrome largely depend on the original site and type of infection and the host's inflammatory response, as well as the time of administration of appropriate antimicrobial therapy (10-12).

Another pathological condition that has been associated with oxidative stress is obesity (13,14). Obesity is a chronic metabolic disease in which excess body fat in adipose tissue has accumulated to the extent that it may have a negative effect on health, leading to reduced life expectancy and increased health problems such as heart disease, type 2 diabetes, obstructive sleep apnea, certain types of cancer, and osteoarthritis (14, 15). Since increased oxidative stress is observed in obese individuals, examination of the impact of obesity on sepsis-induced oxidative stress would be interesting (13, 14).

Correspondence to: Dimitrios Kouretas, Department of Biochemistry and Biotechnology, University of Thessaly, Ploutonos 26 & Aiolou, Larissa 41221, Greece. Tel: +30 2410565277, Fax: +30 2410565293, e-mail: dkouret@uth.gr

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A number of biomarkers have been developed for assessing oxidative stress levels in humans. These biomarkers help assess the redox status of a person and consequently predict the possible manifestation of a pathological condition or the progress of an already present disease (16). However, oxidative stress measurement is still incomplete, time consuming and impractical in a clinical setting, and so there is a great need for developing new methods for assessing the redox status in humans (17). A novel method for assessing oxidative stress is the proprietary RedoxSYS Diagnostic System (Luoxis Diagnostics, Inc., Englewood, CO, USA). The RedoxSYS Diagnostic System measures the oxidation-reduction potential (ORP), which is an integrated measure of the balance between total oxidants (*e.g.* oxidized thiols, superoxide radical, hydroxyl radical, hydrogen peroxide, nitric oxide, peroxynitrite and transition metal ions), and total reductants (*e.g.* free thiols, ascorbate, α-tocopherol, β-carotene and uric acid). Thus, ORP is an overall measure of the oxidative stress to which a biological system is subjected (18). The RedoxSYS Diagnostic System enables robust and rapid assessment of oxidative stress in a single drop of plasma *via* measurement within four minutes of two distinct elements to determine ORP, namely the static ORP (sORP) and the capacity ORP (cORP). sORP is the standard potential between a working electrode and a reference electrode with no driving current (or extremely small current) which is proportional to the balance of reductants and oxidants and is what is classically termed ORP (*i.e.* a homeostatic parameter capturing the current balance of oxidants and reductants in a biological specimen). Low sORP values mean that the biological sample is in the normal range of oxidative stress, while higher than normal sORP values means that the biological sample is in a higher state of oxidative stress. cORP is the measure of antioxidant reserve available in the body's system; high capacity values mean that the biological sample has antioxidant reserves in the normal range; lower than normal cORP values means that the biological sample has below normal antioxidant reserves.

In previous studies, we have shown that ORP markers could be used for assessing oxidative stress induced by physiological conditions such as exercise (19-20). In the present study, ORP markers were used for the first time for assessing oxidative stress levels in a pathological condition such as sepsis. The possible effects of obesity-induced oxidative stress on patients with sepsis were also investigated. Apart from ORP markers, conventional oxidative stress biomarkers were used such as total antioxidant capacity (TAC), thiobarbituric acid-reactive substances (TBARS) and protein carbonyls (CARB).

Materials and Methods

Participants. In this prospective study, a total of 42 individuals who were admitted to Patras University General Hospital in Greece were enrolled. According to our protocol, the patients were divided into four groups. The inclusion criteria of each group were: Control group (n=11): individuals with body mass index (BMI) <30 kg/m^2 without clinicolaboratory signs of infection; Obesity group (n=10): individuals with BMI ≥30 kg/m^2 without clinicolaboratory signs of infection; Sepsis group (n=12): individuals with BMI <30 kg/m^2 with sepsis; Sepsis and obesity group (n=9): individuals with BMI ≥30 kg/m^2 with sepsis. Sepsis was defined according to the criteria of the American College of Chest Physicians–Society of Critical Care Medicine Consensus Conference Committee–as the presence of confirmed infection and >2 of the following criteria: (a) a temperature of >38˚C or <36˚C; (b) a heart rate of >90 beats per min; (c) tachypnea, manifested by a respiratory rate of >20 breaths per min or hyperventilation, indicated by a $PaCO_2$ of <32 mm Hg; and (d) an altered white blood cell count of >12,000 or <4,000 cells per mm^3 or the presence of >10% immature forms.

Blood collection and handling. Blood was collected in ethylenediamine tetra-acetic acid tubes for measuring oxidative stress markers. Blood samples were centrifuged immediately at 1,370 × *g* for 10 min at 4˚C and the plasma was collected and used for the measurements of oxidative stress markers. Plasma samples were stored at −80˚C prior to biochemical analyses.

Assessment of sORP and cORP using the the RedoxSYS Diagnostic System. sORP and cORP values were determined using the RedoxSYS Diagnostic System (Luoxis Diagnostics, Inc., Englewood, CO, USA). Twenty microliters of plasma was applied to disposable sensors designed by Luoxis, which were inserted into the RedoxSYS Diagnostic System that measured and reported within four minutes the sORP and cORP values. The RedoxSYS diagnostic system measures the ORP with a three-electrode system: a working electrode, a counter electrode and a reference electrode. Firstly, a negligible amount of current is applied between the working and counter electrodes, and the ORP is measured between them. Once the ORP reading reaches equilibrium, the sORP is established and measured in millivolts . Then a linearly increasing current is applied to the sample, between the counter and working electrodes. The time from the beginning of the current sweep to the maximum rate of change in ORP is referred to as the transition time and the integrated current to this time is the cORP, measured in microcoulombs. Each sample was measured in triplicate.

Assessment of TAC, TBARS and CARB. For TBARS determination, a slightly modified assay of Keles *et al*. was used (21). According to this method, 100 μl of plasma was mixed with 500 μl of 35% trichloroacetic acid (TCA) and 500 μl of tris(hydroxylmethyl) aminomethane hydrochloride (Tris–HCl) (200 mM, pH 7.4) and incubated for 10 min at room temperature. One milliliter of 2 M Na_2SO_4 and 55 mM thiobarbituric acid solution was added and the samples were incubated at 95˚C for 45 min. The samples were cooled on ice for 5 min and were vortexed after adding 1 ml of 70% TCA. The samples were centrifuged at 15,000 × *g* for 3 min and the absorbance of the supernatant was read at 530 nm. A baseline absorbance was taken into account by running a blank along with all samples during the measurement. Calculation of TBARS concentration was based on the molar extinction coefficient of malon dialdehyde.

The determination of CARB was based on the method of Patsoukis *et al*., (22). The derivatization of the carbonyl group with 2,4-dinitrophenylhydrazine (DNPH), which leads to the

formation of a stable 2,4-dinitrophenyl (DNP) hydrazone product, is one of the most widely utilized measurements of protein oxidation (23). It has been shown to be very sensitive, especially for the determination of carbonyl content in purified proteins (23). For this reason, the proteins were precipitated with TCA. In this assay, 50 µl of 20% TCA was added to 50 µl of plasma and this mixture was incubated in an ice-bath for 15 min and centrifuged at 15,000 × *g* for 5 min at 4˚C. The supernatant was discarded and 500 µl of 10 mM DNPH (in 2.5 N HCl) for the sample, or 500 µl of 2.5 N HCl for the blank, was added to the pellet. The samples were incubated in the dark at room temperature for 1 h, with intermittent vortexing every 15 min and were centrifuged at 15,000 × *g* for 5 min at 4˚C. The supernatant was discarded and 1 ml of 10% TCA was added, vortexed and centrifuged at 15,000 × *g* for 5 min at 4˚C. The supernatant was discarded and 1 ml of ethanol–ethyl acetate (1:1 v/v) was added, vortexed and centrifuged at 15,000 × *g* for 5 min at 4˚C. This washing step was repeated twice. The supernatant was discarded and 1 ml of 5 M urea (pH 2.3) was added, vortexed and incubated at 37˚C for 15 min. The samples were centrifuged at 15,000 × *g* for 3 min at 4˚C and the absorbance was read at 375 nm. Calculation of CARB concentration was based on the molar extinction coefficient of DNPH. Total plasma protein was assayed using Bradford reagent from Sigma–Aldrich (Munich, Germany).

The determination of TAC was based on the method of Janaszewska and Bartosz (24). Briefly, 20 µl of plasma were added to 480 µl of 10 mM sodium potassium phosphate (pH 7.4) and 500 µl of 0.1 mM 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical and the samples were incubated in the dark for 30 min at room temperature. The samples were centrifuged for 3 min at 20,000 × *g* and the absorbance was read at 520 nm. TAC is presented as mmol of DPPH reduced to 2,2-diphenyl-1-picrylhydrazine (DPPH:H) by plasma antioxidants.

Statistical analysis. For statistical analysis, data were analyzed by one-way ANOVA followed by Dunnett's test for multiple pair-wise comparisons. The level of statistical significance was set at $p<0.05$. For all statistical analyses, SPSS, version 13.0 (SPSS Inc., Chicago, IL, USA) was used. Data are presented as the mean±SEM.

Results

From the two ORP parameters measured by the RedoxSYS Diagnostic System, the sORP indicating the current redox balance was increased significantly ($p<0.05$) by 9.3% in the sepsis group, and by 6.9% in the sepsis and obesity group compared to the control group (Figure 1A). On the contrary, cORP, reflecting the reserves of antioxidants, was significantly lower by 11.8% in the sepsis group and by 17.7% in the sepsis and obesity group compared to the control group (Figure 1B). Neither of these two markers differed significantly in the obesity group compared to the control group (Figures 1A and B).

Regarding the conventional oxidative stress markers, the plasma levels of CARB indicating protein oxidation were increased significantly ($p<0.05$) by 73.8% in the obesity group and by 167.5% in the sepsis and obesity group compared to the controls, while there was not any significant

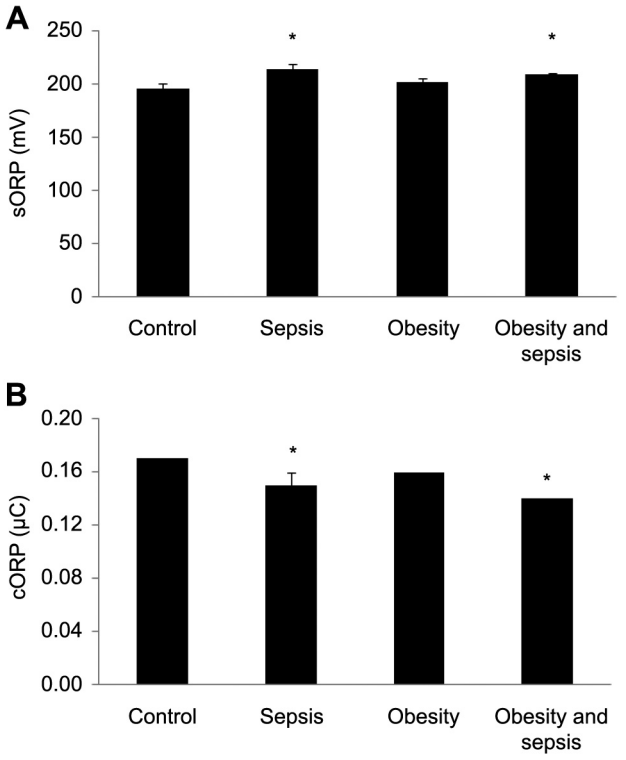

Figure 1. *Oxidation reduction potential (ORP) parameters of the redox status in plasma of control group, sepsis group, obesity group, and obesity and sepsis group as measured by the RedoxSYS Diagnostic System. sORP, Static oxidation-reduction potential; cORP, capacity oxidation-reduction potential. *Significantly different compared to the control group ($p<0.05$).*

change in the sepsis group. Moreover, in the sepsis and obesity group, CARB levels were increased significantly by 130.1% and 53.9% ($p<0.05$) compared to the sepsis group and obesity group, respectively (Figure 2A). TAC was significantly ($p<0.05$) lower by 20.6% in the sepsis group and by 30.8% in the sepsis and obesity group compared to the control group, while there was no significant difference comparing controls with the obesity group (Figure 2B). In the sepsis and sepsis and obesity groups, TAC was significantly ($p<0.05$) lower by 23.4% and 33.3%, respectively, compared to the obesity group (Figure 2B). TBARS, reflecting lipid peroxidation, were significantly ($p<0.05$) higher by 59.1% in the obesity group and by 83.6% in the sepsis and obesity group compared to controls, while there was not any significant difference for the sepsis group (Figure 2C). In the obesity group, and sepsis and obesity group, the levels of TBARS were significantly higher by 42.8% and 64.8%, respectively ($p<0.05$), compared to the sepsis group (Figure 2C).

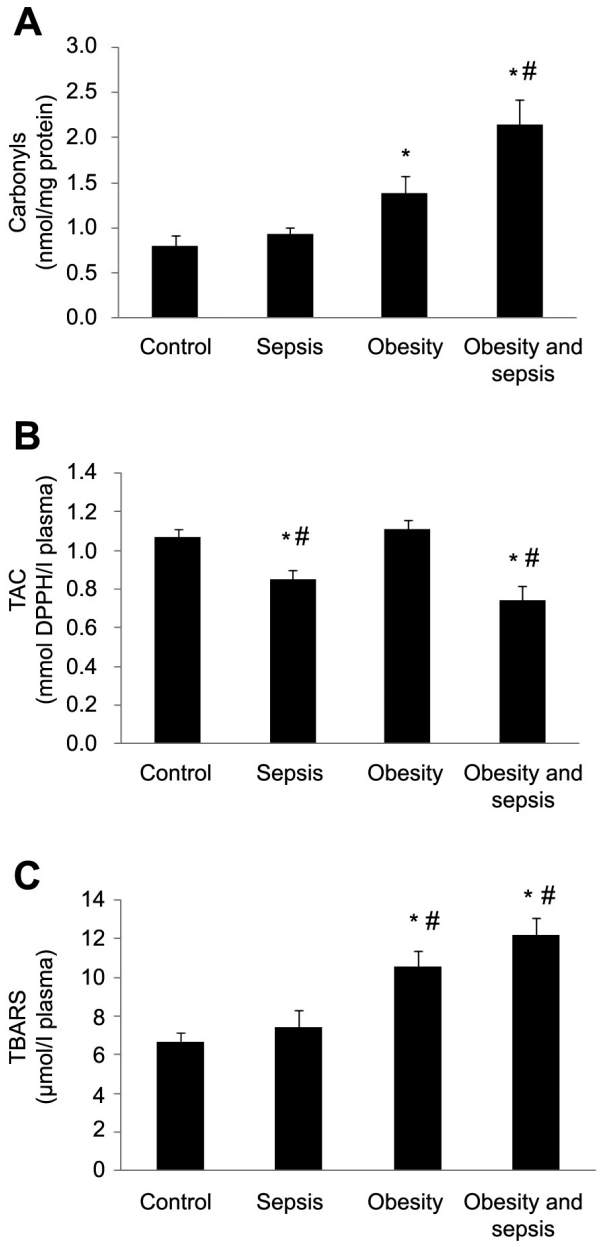

Figure 2. *Conventional parameters of redox status in plasma of the control group, sepsis group, obesity group, and obesity and sepsis group. A: Protein carbonyl levels (CARB); significantly different at p<0.05 compared to *control group, and #sepsis group and obesity group. B: Total antioxidant capacity (TAC); significantly different at p<0.05 compared to *control group, and #obesity group. C: Thiobarbituric acid-reactive substances (TBARS); significantly different at p<0.05 compared to *control group, and #sepsis group.*

Discussion

Sepsis is defined as the systemic inflammatory response syndrome attributed to infection (6, 7). Several studies have reported higher levels of oxidative stress and lower antioxidant potential in patients with sepsis in the first hours or days after ICU admission compared to healthy controls (25, 26). In the present study, we examined if ORP markers as measured by the RedoxSYS Diagnostic System could be used for assessing oxidative stress in patients with sepsis. The results suggested that both sORP and cORP can detect sepsis-induced oxidative stress. Specifically, sepsis increased sORP, reflecting the overall current redox balance, thus higher than normal sORP values means that the biological sample is in a higher state of oxidative stress. Moreover, sepsis reduced cORP indicating a reduced antioxidant reserve. It has been proposed that sepsis-induced oxidative stress is due to the continuous defense and inflammatory response during sepsis that results in a large increase in the levels of free radicals, nitric oxide and inflammatory cytokines (27).

Among the conventional oxidative stress markers, only TAC indicated sepsis-induced oxidative stress, since it was lower in patients with sepsis compared to individuals of the control group. On the contrary, there were no significant changes in CARB and TBARS between patients with sepsis and control individuals. These results for the conventional biomarkers were in agreement with one of our previous studies in which oxidative stress was assessed in patients with sepsis (28). However, in another study in which the variability of oxidative stress during sepsis evolution was investigated, we found that severe sepsis resulting in mortality increased CARB in plasma, while it had no effect on TAC and TBARS levels (29). This latter study demonstrated different effects of sepsis on TAC and TBARS from what was found in the present study. Obviously, an explanation for this inconsistency may be the different severity of the sepsis manifested by the patients of the two studies. On the other hand, we believe that this inconsistency may emphasize the need for finding new markers that would more consistently assess oxidative stress in sepsis and especially would determine an overall redox status of an individual. Most of the current oxidative stress markers assess a single parameter of oxidative damage (*e.g.* lipid peroxidation, protein oxidation) or of antioxidant defense (*e.g.* glutathione GSH levels, catalase activity, superoxide dismutase activity). Consequently, several of these parameters should be measured in order to assess oxidative stress more reliably in an individual. However, the measurement of several biomarkers of oxidative stress is time consuming, and so is impractical in a clinical setting such as in the case of sepsis. Thus, the finding that ORP markers as evaluated by the RedoxSYS Diagnostic System can consistently measure sepsis-induced oxidative stress is important, since they are measured quickly (within four minutes) and easily. Moreover, ORP markers are an integrated oxidative stress measurement, since they assess the total imbalance between oxidants and reductants (*i.e.* sORP) or the total antioxidant defence (*i.e.* cORP). Thus,

the RedoxSYS Diagnostic System could be used at the bedside for monitoring oxidative stress in patients with sepsis similar to the bedside monitoring of heart rate, respiration, *etc*.

ORP markers were used to assess the effects of obesity on sepsis-induced oxidative stress, since obesity alone induces oxidative stress (30). Obesity-induced oxidative stress has been attributed to hyperglycemia, increased muscle activity in order to carry excessive weight, elevated tissue lipid levels and low-grade endotoxemia due to gut barrier dysfunction (30). In a previous study, we have reported that obesity was associated with increased oxidative stress in patients with sepsis (28). The present results from the conventional oxidative stress biomarkers confirmed those of our previous study, that is, obesity increased lipid oxidation (*i.e*. TBARS) and protein oxidation (*i.e*. CARB) in patients with sepsis. These effects were probably due to the combination of the factors inducing oxidative stress in each of the two conditions, sepsis and obesity. However, ORP markers did not detect increased oxidative stress in obese patients with sepsis compared to patients with sepsis alone. Moreover, ORP markers did not reveal increased oxidative stress in obese individuals compared to controls. Like ORP markers, TAC, the other holistic marker measuring total antioxidant defense, did not increase in obese individuals compared to controls, while obese individuals had higher protein oxidation and lipid peroxidation. Other studies have shown that obesity reduced TAC, but these were large studies including more than 1500 individuals (31). Thus, the fact that ORP and TAC markers did not detect differences in oxidative stress between nonobese and obese individuals may be due to the small number of participants in our study. We are currently performing a study including more than 500 individuals in order to examine the assessment of oxidative stress using ORP markers in metabolic syndromes such as obesity.

In conclusion, the present results suggest for the first time that ORP markers as measured by the RedoxSYS Diagnostic System could be used for assessing oxidative stress in patients with sepsis. Currently, we are carrying out a study to examine if ORP markers correlate with injury severity and degree of inflammation in patients with sepsis. The main advantage of using sORP and cORP markers for measuring oxidative stress is their rapid measurement, using a small plasma volume (20 μl), of the total imbalance between oxidants and reductants, and the total antioxidant defense levels respectively. Thus, these markers could be used for alerting a physician to the oxidative stress levels in patients with sepsis so that appropriate intervention (*e.g*. antioxidant administration) can be applied (32). Importantly, studies have shown that the timely administration of antioxidant is more effective in critically ill patients than delayed treatment (33).

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