in vivo *25*: 1013-1018 (2011)

Italian Air Force Acrobatic Pilots Are Protected against Flight-induced Oxidative Stress

GIADA DOGLIOTTI[1], ELENA DOZIO[1], MARCO AGRIFOGLIO[2], ELENA COSTA[3], GUIDO BROICH[4], ALEXIS E. MALAVAZOS[5], FABRIZIO PALUMBO[6] and MASSIMILIANO M. CORSI[1,3]

[1]*Department of Human Morphology and Biomedical Sciences Città Studi, University of Milan, Milan, Italy;*
[2]*Department of Cardiovascular Sciences, University of Milan, Milan, Italy;*
[3]*Clinical Pathology Operative Unit, Department of Health Services Diagnosis and Treatment, Laboratory Medicine IRCCS Policlinico San Donato, Milan, Italy;*
[4]*Policlinico di Monza, Monza, Italy;*
[5]*Operative Unit of Diabetology and Metabolic Diseases, IRCCS Policlinico San Donato, Milan, Italy;*
[6]*A. Mosso Legal Medicine Institute, Italian Air Force, Milan, Italy*

Abstract. *Background/Aim: The purpose of this study was to assess the oxidative stress of aircraft pilots by evaluating different markers of oxidative stress and any imbalance between free radicals and antioxidants in plasma. Patients and Methods: A group of 13 supersonic aircraft pilots, following regular exercise and personalized diet, were compared with a group of 40 healthy controls. Oxidative stress indicators, such as reactive oxidative metabolites, carbonyl proteins, 8-hydroxy-2-deoxyguanosine and total antioxidant status, were evaluated after three months of intense flight. Results: Reactive oxygen metabolites, carbonyl protein and 8-hydroxy-2-deoxyguanosine plasma levels did not differ in supersonic aircraft pilots and healthy controls. The two groups also had similar total antioxidant status levels. Conclusion: We suggest that supersonic aircraft pilots working at high altitude, even if exposed to physiological stresses, can, with proper diet, regular exercise and periodical medical examinations, maintain a healthy balance between oxidant and antioxidant status.*

Several environmental challenges are present, when working at high altitudes. Supersonic aircraft pilots usually suffer from physiological stress, mainly related to working at high altitude, from hypoxia, wind, solar UV rays and dehydration which can all have negative effects on physical and mental performance (1). At high altitude, the low pressure of atmospheric oxygen limits energy generation when it is most needed and oxygen availability decreases as a result of reduced partial oxygen pressure, thus accentuating hypoxemia (2). In particular, an increased production of free radicals has been described for humans exposed to hypoxia and altitude (3), causing changes in physiological and cognitive functions that can persist even for some time after returning to sea level (4). Free radicals have the potential to attack any organic molecule, generating reactive oxygen metabolites (ROMs) which can oxidize proteins and DNA, causing cellular and tissue damage (5). Protein oxidation results in the formation of new functional groups, such as carbonyl or hydroxyl groups, which may lead to protein fragmentation, formation of protein–protein cross-linkages, disruption of the tertiary structure and loss of functional activity (6, 7). Oxygen free radicals can damage DNA in different ways, both causing DNA single- and double-strand breaks, and base modifications (8). Hydroxylation of deoxyguanosine residues in DNA, mainly *via* hydroxyl radicals, results in the formation of 2,6-diamino-4-hydroxy-5-formamidopyrimidine (8-OH-dG) (9), which is also the most utilized marker of oxidative damage of DNA, both in physiological and pathological conditions (10, 11).

The aim of this study was to assess whether daily exposure for three months to environmental stress due to flights at high altitude may affect the oxidant/antioxidant balance of supersonic aircraft pilots from the Italian Air Force, thus increasing the risk for these individuals to develop future pathologies related to the increased oxidative stress, such as atherosclerosis and cardiovascular diseases. In particular, the following oxidative stress indicators were measured: dROMs, carbonyl proteins, 8-OH-dG. The total antioxidant status (TAS) was also evaluated; this is an integrated parameter that considers the cumulative status of

Correspondence to: Giada Dogliotti, via Mangiagalli 31, 20133 Milano, Italy. Tel: +39 0250315342, Fax: +39 0250315338, e-mail: giada.dogliotti@unimi.it

Key Words: Oxidative stress, carbonyl proteins, reactive oxygen metabolites, 8-hydroxy-2-deoxyguanosine.

all the antioxidants present in plasma and providing information on the delicate *in vivo* balance between oxidants and antioxidants (12-14).

Patients and Methods

Study design and population. Two groups of subjects were studied. The first included 13 male supersonic aircraft pilots, aged 27-38 years (mean±standard deviation (SD) 32.2±3.6 years), clinically healthy, as confirmed by biochemical and clinical tests. Physical examination, electrocardiogram, echocardiogram, and chest radiogram (15) (periodically performed at the Central Military Aero-medical Board) confirmed that none had cardiovascular problems. All pilots practiced sports, followed a balanced diet, and their mean body mass index (BMI) was 24.4±1.28. None had any allergies. The second group comprised 40 healthy adult male volunteer blood donors, aged 30-49 years (35.8±4,9 years), from the Italian Association of Voluntary Blood Donors (AVIS) in Milan, Italy, living at sea level (122±41m). Their mean BMI was 23.8±2.4. All controls gave informed consent for blood sampling and the project was approved by the University of Milan Ethics Committee. All subjects were normotensive [systolic (SBP) and diastolic blood pressure (DBP) <130 and <80 mmHg, respectively], normoglycemic (fasting glucose <110 mg/dl), normolipidemic (total cholesterol <220 mg/dl, high densitive lipoproteine (HDL)-cholesterol >50 mg/dl, triglycerides <150 mg/dl) and drug free.

The mean total flight time was 970.76±334.07 h/year and 4.41±1.51 h/day and the mean number of daily flights was 5.88±2.02. All pilots routinely used oxygen masks and wore patented suits to protect the normal physiological functioning of the body.

Plasma was prepared from heparinized venous blood, collected after an overnight fasting. After collection, bood samples were immediately centrifuged for 15 min at 3000 rpm and plasma was drawn off and stored at –20˚C until assay. For pilots, plasma was taken before starting the seasonal working activity and after three months of intense flight.

Measurements. To measure ROM, d-ROMs test (Diacron srl, Grosseto, Italy) was used in which the hydroperoxides of a biological sample are subjected to Fenton's reaction to generate alkoxyl and peroxyl radicals *in vitro*. Briefly, a small amount of plasma is diluted in an acidic buffered solution (pH 4.8). Under these conditions, Fe^{++} bound to plasma proteins catalyzes the breakdown of blood hydroperoxides to alkoxyl and peroxyl radicals. Chromogenic compound (*N*,*N*,-diethylparaphenylen-diamine) was then added to the solution and oxidization by hydroperoxyl and alkoxyl radicals was quantified photometrically at a wavelength of 505 nm. The d-ROMs test results are expressed as arbitrary units called Carratelli units (Carr U), where 1 Carr U corresponds to 0.08 mg/100 ml H_2O_2 (16). Reference values for healthy individuals are between 250 and 300 Carr U. Reported maximum intra- and inter-assay CVs were 2.9% and 4.7% respectively.

Carbonyl protein was detected by an immune-enzymatic assay (Immun-Diagnostik AG, Stubenwald-Allee, Bensheim) (17, 18). Samples containing protein were derivatized with 80 μl of dinitrophenylhydrazine (DNPH); then the non-protein constituents and unconjugated DNPH were separated by three ultra-centrifugations at 11000 rpm for 15 min each. The proteins were left overnight to coat an ELISA plate then incubated with 200 μl of anti-DNPH antibody, followed by 200 μl of antibody-linked horseradish peroxidase. Absorbance at 450 nm was related to a standard curve prepared with oxidized serum albumin. The carbonyl protein content was calculated from the estimated carbonyl concentration and the total protein content of the sample. Reference values for healthy individuals in EDTA-plasma are between 75-200 pmol/mg. Reported maximum intra- and inter-assay CVs were 8.11% and 7.66%, respectively. DNA oxidation was evaluated by the 8-OH-dG test (OXIS Health Products Inc., Portland, OR, USA) (19), as previously reported by Looi *et al*. (12).

This test is based on competition of 8-OH-dG in the sample with 8-OH-dG bound on a precoated plate for 8-OH-dG monoclonal antibody binding sites using a chromogen (3,3',5,5'-tetramethylbenzidine) which results in the development of color in proportion to the amount of antibody bound to the plate, measured at 450 nm (20, 21). Reference values for healthy individuals are between 10-50 ng/ml. Reported maximum intra- and inter-assay CVs were 6.65% and 5.82%, respectively.

TAS (Randox Laboratories Ltd., Crumlin, U.K.) was evaluated spectrophotometrically by redox of methyglobin as previously described by Lockington (13). TAS results are expressed as mmol/l (mean±SD), with a normal value of 1.728±0.004 mmol/l. RX Monza software was used to calculates the TAS concentration. Reported maximum intra- and inter-assay CVs were 3.08% and 3.75%, respectively.

Statistical analysis. The data are expressed as mean±standard deviation (SD). Significant differences between the means for multiple phases were evaluated using one-way analysis of variance (ANOVA). $P<0.05$ was considered statistically significant.

Results

Participant characteristics. Some clinical characteristics of supersonic aircraft pilots and control individuals are reported in Table I. As shown, no statistically significant differences in anthropometric and clinical parameters were observed between the two groups, nor for pilots between parameters before (T0) and after three months of intensive flights (T1).

Oxidant and antioxidant balance. Oxidative stress analysis revealed that at plasma levels dROMS in supersonic aircraft pilots (17.6±1.22 mg/100 ml of H_2O_2) were not statistically different from those of healthy controls (15.95±3.96 mg/100 ml of H_2O_2, $p=0.164$) (Figure 1A). Similarly, carbonyl proteins (201.77±44.95 *vs*. 213.45±34.83 pmol/mg, $p=0.466$) (Figure 1B), 8-OH-dG (16.42±3.47 *vs*. 15.10±1.52 ng/ml, $p=0.221$) (Figure 1C) and TAS (1.62±0.26 *vs*. 1.58±0.26 mmol/l, $p=0.71$) (Figure 1D) values were similar in supersonic aircraft pilots and controls. The same analyses were also performed after three months of intensive flights (T1) and revealed that dROMS (18.036±1.22 mg/100 ml of H_2O_2; $p=0.371$ *vs*. pilots at T0 and $p=0.082$ *vs*. controls) (Figure 1A), carbonyl proteins (219.94±49.90 pmol/mg; $p=0.339$ *vs*. pilots at T0 and $p=0.704$ *vs*. controls) (Figure 1B), 8-OH-dG (17.07±3.65 ng/ml; $p=0.645$ *vs*. pilots at T0 and $p=0.085$ *vs*. controls) (Figure 1C) and TAS levels

Table I. *Anthropometric and clinical parameters*.

Parameters		Pilots		*p*-Value		
	Control group (A)	T0 (B)	T1 (C)	A *vs*. B	A *vs*. C	B *vs*. C
Weight (kg)	68±9	71.5±13	NR	0.43	NR	NR
Height (m)	1.68±12	1.71±7	NR	0.99	NR	NR
BMI	23.8±2.4	24.4±1.28	NR	0.42	NR	NR
Age (years)	35.8±4.9	32.2±3.6	NR	0.13	NR	NR
Leukocyte (n)	5125±1925	5835±1354	6032±1135	0.28	0.16	0.69
Glucose (mg/dl)	62.1±3.01	60.85±1.89	61.02±1.35	0.21	0.25	0.79
Triglycerides (mg/dl)	80.12±15.2	72.9 ±24.2	74.12±17.3	0.37	0.36	0.88
Cholesterol, total (mg/100 ml)	180.2±12.5	171±21.2	173.5±16.2	0.20	0.26	0.73
HDL (mg/100 ml)	58.2±8.3	61.6±6.1	63.8±5.9	0.25	0.06	0.36
Systolic blood pressure (mmHg)	131.5 ±1.9	132.6±1.85	133.1±2.21	0.15	0.06	0.54
Diastolic blood pressure (mmHg)	70.3±3.1	69.8±2.3	70.1±2.5	0.64	0.86	0.18

NR: Not relevant; BMI: body mass index; HDL: high-density lipoproteins.

(1.56±0.21 mmol/l; p=0.524 *vs*. pilots at T0 p=0.83 *vs*. controls) (Figure 1D) were almost the same as at T0 and as in controls.

Correlation between hours of flying and molecules of interest. Linear regression analysis performed to evaluate a possible correlation between the change (ΔT=T1-T0) of the levels of each parameter and the hours of flight suggested that in these pilots, increasing flight time was not related to variation of their oxidant status: d-ROMs: r^2=0.02 and p=0.67 (Figure 2A); carbonyl proteins: r^2=0.12 and p=0.24 (Figure 2B); 8-OH-dG: r^2=0.11 and p=0.25 (Figure 2C); TAS: r^2=0.22 and p=0.11 (Figure 2D).

Discussion

Physiological stresses from hypoxia at altitude, caused by the low atmospheric pressure, cold, wind, solar UV rays and dehydration can all have negative effects on physical and mental performance and, in particular, can affect, the cardiovascular system (22). A considerable body of literature documents an increased production of indicators of oxidative stress both in laboratory rats and humans exposed to hypoxia and high altitude (23); moreover, the oxidative theory of atherosclerosis emphasizes the role of ROS in the initiation of endothelial damage and in the development of atherosclerotic plaque (2).

In our study, we selected a peculiar group of flying personnel, supersonic aircraft pilots from the Italian Air Force. Since these individuals are clinically healthy and their lipid concentrations are in the expected normal ranges, they represent a distinctive and homogeneous population in which to study the effect of environment stress on the oxidant/antioxidant status which, in addition to other well-known risk factors, such as cholesterol and triglycerides, may also play an important role in the risk of future development of cardiovascular diseases. By evaluating some markers of oxidative stress, dROMs, carbonyl proteins, 8OH-dG and TAS, we did not observe any substantial increase in oxidative stress or an impaired oxidant/antioxidant balance in pilots compared to healthy matched controls; this is still true for these pilots after three months of intensive flight. In addition, pilots were periodically monitored using other instrumental analyses, such as electrocardiogram, echocardiogram, and chest radiogram, which confirmed a good health status, especially of the cardiac system.

Our observations thus suggest that although these individuals are continuously exposed to risk factors which may induce an imbalance in their oxidant/antioxidant status, with possible deleterious effects on their cardiovascular system, they are able to counteract the negative effects of such hostile working conditions at high altitude. Previous studies suggested that space flight and aviation seem to cause consistent oxidative stress, mainly indicated depletion levels of plasma and white blood cell vitamin E, reduced coenzyme Q_{10} (ubiquinol) and total coenzyme Q_{10}, probably reflecting a high metabolic requirement for ubiquinol under stressful circumstances (24). There is in fact evidence that antioxidant defense mechanisms may be overwhelmed during work at high altitude (2, 25). This seems not to be the case for our study subjects, who did not display any significative sign of increased oxidant status. The main reasons for this may be sought in the lifestyle of these individuals. In particular, moderate physical activity and a good daily supply of 'sacrificial' antioxidants seem to be two good ways utilized to counteract any oxidant/antioxidant imbalance. Oxidative stress can usually be offset by a strong antioxidant enzyme system coupled

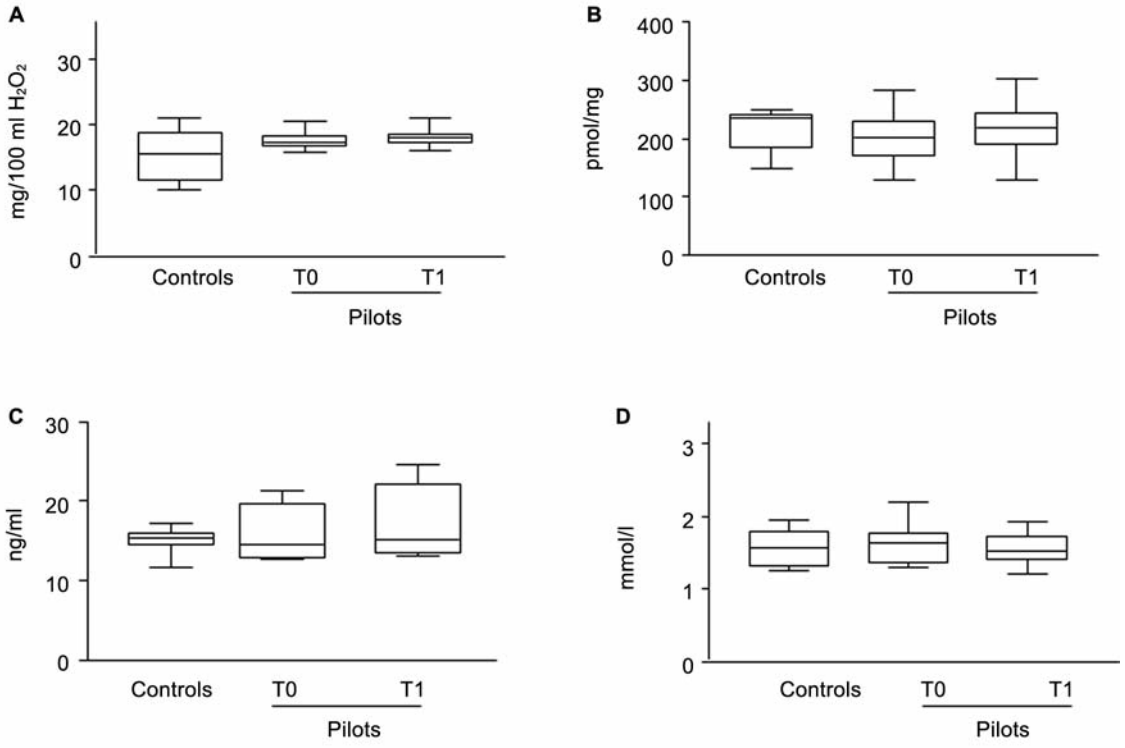

Figure 1. *Plasma levels of A: dROMs, B: carbonyl protein, C: 8-OH-dG and D: TAS in supersonic aircraft pilots before (T0) and after (T1) intensive flight compared to healthy controls.*

with a good daily supply of 'sacrificial' antioxidants, such as vitamin C, β-carotene, vitamin E and other antioxidant nutrients found in fruit and vegetables. However, there is a delicate balance between oxidants and antioxidants (26). In fact, if the regular intake of dietary antioxidants by people working at high altitude is not optimal, oxidants may outweigh the antioxidants. Our study subjects followed a diet that consisted of fish, not processed meat, less carbohydrate compared to standard Mediterranean diet, fruits and vegetables that had an oxygen radical absorbance capacity (ORAC) of 500 units, such as orange, kiwi, avocado, peppers, all rich in vitamin C, β-carotene, vitamin E and other antioxidant nutrients. In addition to their diet, the pilots also performed moderate physical activity, which has previously been shown to increase the activity of antioxidative defense enzymes and to preserve antioxidant capacity, thus allowing the conclusion that proper physical training has a highly beneficial influence on mechanisms of defense against the deleterious influence of ROS (7, 27, 28). Endurance sports, such as distance running, middle distance running, swimming and cycling, are recommended since the hypoxia they induce is similar to that experienced in flight particularly under conditions of high acceleration and physical or emotional stress, and thus allow the body to adapt to this situation by optimizing the processes of oxygen exchange between air, blood and tissues, resulting in improved respiratory and cardiovascular functions. Taking into account the above factors, our pilots were subjected to exercise such us, diving, trampolining, gymnastics and rotator exercises which also allowed them to overcome conditions of psychological stress (fear, anxiety, emotional tension) often caused by vestibular disorders, frequently related to spatial disorientation.

To date, it is not clear whether hypoxia itself or oxygen-reperfusion after hypoxia, or both, may also cause increased stress, which in turn is responsible for most cellular damage. In fact, it is possible that when the availability of oxygen goes up after a period of hypoxia, similarly to reperfusion after ischemia, the shift between low to high oxygen concentration may produce a stress condition characterized by increased ROMs which result in cellular damage.

In conclusion, since the biomarkers of oxidative stress remained unchanged and a good health status, also at the cardiac level, was monitored in our study subjects, medical advice to pilots should probably highlight the importance of a balanced diet and adequate physical activity to prevent the action of oxidants and maintain the normal physiological balance between oxidant and antioxidant levels.

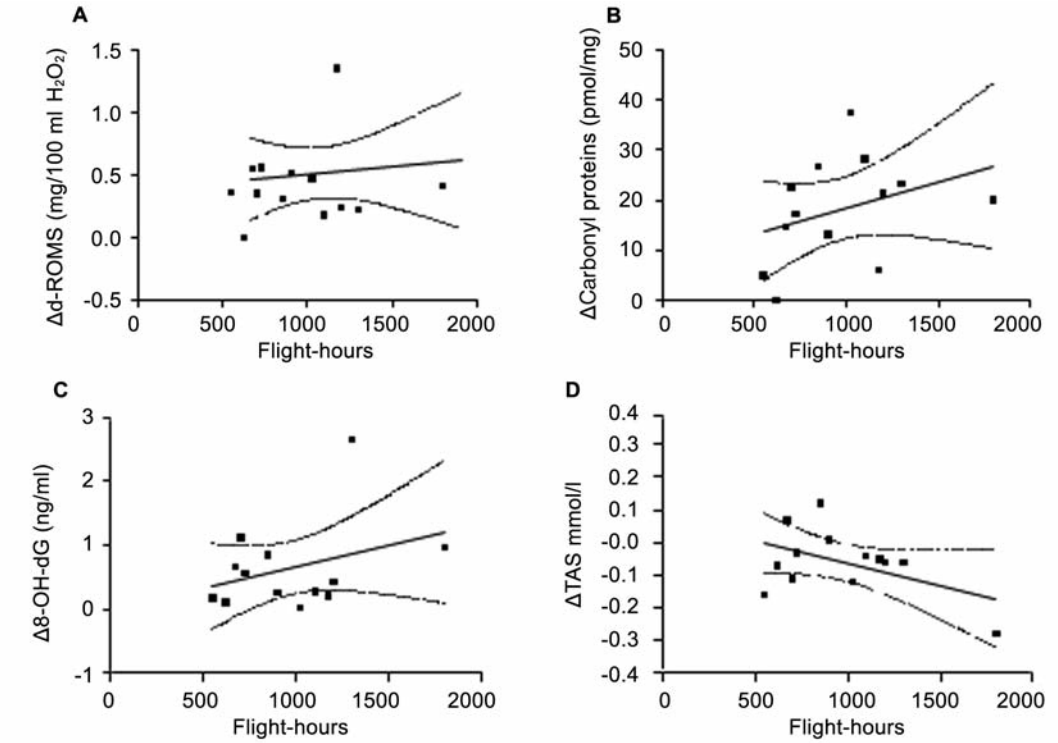

Figure 2. *Curves of linear regression between changes (T1-T0) in plasma levels of A: dROMs, B: carbonyl protein, C: 8-OH-dG and D: TAS and hours of flight.*

Acknowledgements

The Authors are grateful to Judith Baggott for the English revision.

References

1 Corsi MM, Massaccesi L, Dogliotti G and Goi G: *O*-Beta-*N*-acetyl-D-glucosaminidase in erythrocytes of Italian Air Force acrobatic pilots. Clin Chem Lab Med *48*: 213-216, 2010.

2 Askew EW: Work at high altitude and oxidative stress: antioxidant nutrients. Toxicology *180*: 107-119, 2002.

3 Tahawi Z, Orolinova N, Joshua IG, Bader M and Fletcher EC: Altered vascular reactivity in arterioles of chronic intermittent hypoxic rats. J Appl Physiol *90*: 2007-2013; discussion 2000, 2001.

4 Neubauer JA: Invited review: Physiological and pathophysiological responses to intermittent hypoxia. J Appl Physiol *90*: 1593-1599, 2001.

5 Berlett BS and Stadtman ER: Protein oxidation in aging, disease, and oxidative stress. J Biol Chem *272*: 20313-20316, 1997.

6 Simonelli F, Zarrilli F and Mazzeo S: Serum oxidative and antioxidant parameters in a group of Italian patients with age-related maculopathy. Clin Chim Acta *320*: 111-115, 2002.

7 Zawadzka-Bartczak E: Activities of red blood cell antioxidative enzymes (SOD, GPx) and total antioxidative capacity of serum (TAS) in men with coronary atherosclerosis and in healthy pilots. Med Sci Monit *11*: CR440-444, 2005.

8 Banfi G, Malavazos A, Iorio E, Dolci A, Doneda L and Verna R: Plasma oxidative stress biomarkers, nitric oxide and heat-shock protein 70 in trained elite soccer players. Eur J Appl Physiol *96*: 483-486, 2006.

9 Buss H, Chan TP, Sluis KB, Domigan NM and Winterbourn CC: Protein carbonyl measurement by a sensitive ELISA method. Free Radic Biol Med *23*: 361-366, 1997.

10 Winterbourn CC and Buss IH: Protein carbonyl measurement by enzyme-linked immunosorbent assay. Methods Enzymol *300*: 106-111, 1999.

11 Chiou CC, Chang PY, Chan EC, Wu TL, Tsao KC and Wu JT: Urinary 8-hydroxydeoxyguanosine and its analogs as DNA markers of oxidative stress: development of an ELISA and measurement in both bladder and prostate cancers. Clin Chim Acta *334*: 87-94, 2003.

12 Looi ML, Mohd Dali AZ, Md Ali SA, Wan Ngah WZ and Mohd Yusof YA: Oxidative damage and antioxidant status in patients with cervical intraepithelial neoplasia and carcinoma of the cervix. Eur J Cancer Prev *17(6)*: 555-560, 2008.

13 Lockington D, Macdonald ECA, Young D, Stewart P, Caslake M and Ramaesh K: Presence of free radicals in intracameral agents commonly used during cataract surgery. Br J Ophthalmol *94(12)*: 1674-1677, 2010.

14 Askew EW: Environmental and physical stress and nutrient requirements. Am J Clin Nutr *61*: 631S-637S, 1995.

15 West JB: The physiologic basis of high-altitude diseases. Ann Intern Med *141*: 789-800, 2004.

16 Dalle-Donne I, Giustarini D, Colombo R, Rossi R and Milzani A: Protein carbonylation in human diseases. Trends Mol Med *9*: 169-176, 2003.
17 Dalle-Donne I, Rossi R, Giustarini D, Milzani A and Colombo R: Protein carbonyl groups as biomarkers of oxidative stress. Clin Chim Acta *329*: 23-38, 2003.
18 Su M, Yang Y and Yang G: Quantitative measurement of hydroxyl radical induced DNA double-strand breaks and the effect of *N*-acetyl-L-cysteine. FEBS Lett *580*: 4136-4142, 2006.
19 Pilger A and Rudiger HW: 8-Hydroxy-2'-deoxyguanosine as a marker of oxidative DNA related to occupational and environmental exposures. Int Arch Occup Environ Health *80*: 1-15, 2006.
20 Spencer JP, Jenner A and Chimel K: DNA strand breakage and base modification induced by hydrogen peroxide treatment of human respiratory tract epithelial cells. FEBS Lett *374*: 233-236, 1995.
21 Wu LL, Chiou CC, Chang PY and Wu JT: Urinary 8-OH-dG: a marker of oxidative stress to DNA and a risk factor for cancer, atherosclerosis and diabetics. Clin Chim Acta *339*: 1-9, 2004.
22 Magalhaes J, Ascensao A and Marques F: Effect of a high-altitude expedition to a Himalayan peak (Pumori, 7,161 m) on plasma and erythrocyte antioxidant profiles. Eur J Appl Physiol *93*: 726-732, 2005.
23 Ghiselli A, Serafini M, Natella F and Scaccini C: Total antioxidant capacity as a tool to assess redox status: critical view and experimental data. Free Radic Biol Med *29*: 1106-1114, 2000.
24 Zawadzka-Bartczak E, Kopka L and Gancarz A: Antioxidative enzyme profiles in fighter pilots. Aviat Space Environ Med *74*: 654-658, 2003.
25 Carr AC and Frei B: Toward a new recommended dietary allowance for vitamin C based on antioxidant and health effects in humans. Am J Clin Nutr *69(6)*: 1086-107, 1999.
26 De Luca C, Deeva I, Mariani S, Maiani G, Stancato A and Korkina L: Monitoring antioxidant defenses and free radical production in space-flight, aviation and railway engine operators, for the prevention and treatment of oxidative stress, immunological impairment, and pre-mature cell aging. Toxicol Ind Health *25*: 259-267, 2009.
27 Packer L: Oxidants, antioxidant nutrients and the athlete. J Sports Sci *15*: 353-363, 1997.
28 Bailey DM and Davies B: Acute mountain sickness; prophylactic benefits of antioxidant vitamin supplementation at high altitude. High Alt Med Biol *2*: 21-29, 2001.

Received May 27, 2011
Revised July 6, 2011
Accepted July 7, 2011