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Improved antioxidative protection in winter swimmers

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Summary

Adaptation to oxidative stress is an improved ability to resist the damaging effects of reactive oxygen species, resulting from pre-exposure to a lower dose. Changes in uric acid and glutathione levels during ice-bathing suggest that the intensive voluntary short-term cold exposure of winter swimming produces oxidative stress. We investigated whether the repeated oxidative stress in winter swimmers results in improved antioxidative adaptation. We obtained venous blood samples from winter swimmers and determined important components of the antioxidative defense system in the erythrocytes or blood plasma: reduced and oxidized glutathione (GSH and GSSG), and the activities of superoxide dismutase (SOD), glutathione peroxidase (GPx) and catalase (Cat). The control group consisted of healthy people who had never participated in winter swimming. The baseline concentration of GSH and the activities of erythrocytic SOD and Cat, were higher in winter swimmers. We interpret this as an adaptative response to repeated oxidative stress, and postulate it as a new basic molecular mechanism of increased tolerance to environmental stress.

Introduction

Life in modern Western society is characterized by a deficiency of natural stimulating factors such as physical exercise and thermal stimuli. Humans are protected against thermal and climatic influences from the environment, with cold and heat stresses avoided as often as possible. This deficiency of natural stimulating factors has been suggested to contribute to increased morbidity, e.g. in degenerative diseases of the skeletal and muscle system, heart diseases, and infectious diseases. It has also been suggested as a factor for 'labilities' of the central nervous system. The negative results of the deficiency of natural stimuli can be partly prevented by exercise and body hardening, which play an important role in physical therapy and rehabilitation medicine. One traditional aspect of body-hardening therapies is cold exposure. Many people are convinced that daily cold showers, swimming in cold water, or the cold diving bath after sauna bathing, are all useful for the prevention of diseases. Exposure to intensive short-term cold stimuli such as swimming in the cold water of lakes or rivers during the winter time has been used as one form of body hardening for many centuries. Hardening means exposure against a natural (e.g. thermal) stimulus, resulting in an increased resistance to diseases, especially to acute respiratory diseases.

In previous investigations, a markedly decreased frequency of infectious diseases was observed,[1,2] with the annual number of infections of the upper

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respiratory tract in winter swimmers decreased by more than 40% in comparison with a control population. The decreased number of infectious diseases may be partially due to an improved acral circulation and its influence on the circulation in the nose and throat region.[3] However there has been a lack of studies on the biochemical mechanisms contributing to hardening by repeated cold exposure. Our contact with clubs of winter swimmers, including medical advice, offers a convenient possibility for studying biochemical changes in people who expose themselves to a repeated intense cold. Winter swimming or ice-bathing is an extreme kind of body hardening.[1] In Germany about 3000 people are members of winter-swimming sport-clubs, and some thousands more are accustomed to winter swimming. We investigated members of a Berlin winter swimmer club.

A previous study found changes in blood low-molecular-mass antioxidant uric acid[4–7] which could not be explained by changes of purine metabolism or by uric acid excretion in 10 club members, and concluded that the intensive voluntary short-term cold exposure during ice-bathing leads to an oxidative stress.[8] The aim of the present study was to investigate whether the repeated oxidative stress in winter-swimmers results in an improved antioxidative adaptation which could lead to clinical benefit.[9] Therefore, the initial values of the glutathione system (reduced glutathione—GSH and oxidized glutathione—GSSG) and activities of superoxide dismutase (SOD), glutathione peroxidase (GPx), and catalase (Cat) in the blood of winter swimmers were compared with those in healthy controls.

Methods

The Berlin winter swimmer club has been flourishing for several years. The members are not selected in any way, and all swim regularly outdoors in ice-cold water. Their activities start in the fall, and members swim at least once per week during the whole cold season for about 5 min—never longer than 10 min—in ice-cold water. Sometimes, ice on the lake has to be removed to allow them to get into the water. Blood samples were taken from 36 winter swimmers (23 men, 13 women) for measurements of GSH, GSSG, 4-hydroxynonenal, and other biochemical measurements. The subjects investigated have been winter swimmers for at least 2 years (2–11 years). Their mean age was 36.1 ± 11.8 years (mean $\pm$ SE). The control group consisted of 40 healthy people (28 men, 12 women) who had never participated in winter swimming or other cold-exposure hardening procedures. The mean age of the control group was 36.3 ± 10.4 years (mean $\pm$ SE).

Venous blood samples for the winter swimmers were taken before and after the winter-swimming. Between the first blood drawing and 1 h after swimming, the subjects did not eat or drink. The swimming was done in the morning following a short running exercise for warming up. The water temperature at experimental days was between 1 and 4 °C, the air temperature was between -1 and 5 °C, respectively. The blood of the control group was taken under equivalent conditions to those for the winter swimmers.

Sodium citrate was added to the blood samples to prevent coagulation. Plasma was separated from blood cells by centrifugation for analysis of uric acid and HNE. Uric acid was measured enzymically with urate oxidase.

For analysis of GSH and GSSG concentrations, samples were drawn and precipitated with ice-cold metaphosphoric acid and kept at 4 °C. Samples were centrifuged for 10 min at 1200 **g**. Supernatants were collected and separated into two aliquots for the GSH and GSSG measurements. GSH was assayed by means of Ellman's reagent (DTNB).[10] GSSG was determined fluorimetrically after addition of o-phthaldialdehyde.[11] GSH autoxidation was prevented by addition of 50 mM N-ethylmaleimide (NEM).

For the interpretation of uric acid changes in the plasma, the uric acid concentration was additionally measured in the urine of winter-swimmers. The urinary bladder was evacuated 2 h before the whole body cold exposure. Immediately before the cold exposure, urine was collected, and volume and uric acid concentration were measured. From urine volume and uric acid concentration in urine, the renal excretion rate was calculated in mmol/h. Two hours after winter-swimming, urine was collected again for volume and uric acid concentration measurements, to establish the renal excretion of uric acid during and following the cold exposure.

L-Ascorbic acid (L-ascorbate/vitamin C) was measured by means of reduction of the tetrazolium salt MTT [3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide] in the presence of the electron carrier PMS (5-methylphenazinium methyl sulphate) at pH 3.5 to a formazan. For the specific determination of L-ascorbate, in a sample blank determination only the L-ascorbate fraction as part of all reducing substances present in the sample is oxidatively removed by ascorbate oxidase (AAO) in the presence of oxygen. The dehydroascorbate formed does not react with MTT/PMS. The absorbance difference of the sample minus the absorbance difference of the sample blank is equivalent to the quantity of L-ascorbate in the sample. The MTT-formazan is the measuring parameter and is determined by absorbance in the visible range at 578 nm.

Test kit no. 409677 (Boehringer Mannheim) was used.

The measurement of HNE was carried out by modifying this aldehyde with dinitrophenylhydrazine, TLC separation of dinitrophenylhydrazones into three groups and finally the isocratic separation of dinitrophenylhydrazine-derivatives of 4-hydroxy-alkenals.[12–14] For HPLC analysis, a methanol : water mixture (4 : 1, v : v) was used as eluent. The detection wavelength was 378 nm. HNE standard was prepared from the diacetal, which was stored as a solution in chloroform at −20 °C. The diacetal was kindly supplied by the laboratory of H. Esterbauer/H. Zollner at the Institute of Biochemistry, University of Graz, Austria.

The separated red blood cells after washing were used for the determination of enzyme activities (SOD, GPx, Cat). The superoxide dismutase activity was measured by a colorimetric method.[15] The haemolysate was mixed with ice-cold 0.05 M phosphate buffer containing 1 mM EDTA. The SOD was extracted from the supernatant by chloroform-ethanol and measured by a method which uses the ability of the enzyme to inhibit the reduction of nitroblue tetrazolium (NBT) by superoxide (generated by the reaction of photoreduced riboflavin and oxygen). For each sample to be essayed, six tubes were set up containing 10, 20, 40, 60, 80, 500 µl of red blood cell extract plus 0.2 ml of EDTA/NaCN, 0.1 ml NBT, 0.05 ml riboflavin, and phosphate buffer to give a total volume of 3 ml. A tube containing no red blood cell extract was included in each run. After mixing, the tubes received uniform illumination for 20 min. Optical densities were measured at room temperature, at 560 nm. Results were given as units of superoxide dismutase per gram of haemoglobin. One unit is defined as the amount of enzyme causing 50% of the maximum inhibition of NBT reduction.

Catalase activity was detected by a modified UV assay.[16] A 1 : 500 dilution of the concentrated haemolysate was prepared with a phosphate buffer (50 mM, pH 7.0) immediately before the assay. The reaction was started by the addition of 1 ml H_2O_2 (10 mM) to a final volume of 3 ml. Absorbance was measured at room temperature, at 240 nm; the decrease in H_2O_2 extinction was recorded for 30 s. The change in the absorbance of a reference reaction with enzyme source but phosphate buffer instead of H_2O_2 was subtracted from each assay. Catalase activity was calculated, and the results were expressed in units/mg Hb.[16]

Glutathione peroxidase activity was measured according to reference 17. The extraction mixture consisted of 50 mM potassium phosphate buffer (pH 7), 1 mM EDTA, 1 mM NaN_3, 0.2 mM $NADPH_2$, 1 EU/ml GSSG reductase, 1 mM GSH, 1.5 mM cumene hydroperoxide or 0.25 mM H_2O_2 in a total volume of 1 ml. Red-cell lysate was added to 0.8 ml of the above mixture and allowed to incubate 5 min at room temperature before initiation of the reaction by the addition of 0.1 ml peroxide solution. Absorbance at 340 nm was recorded for 5 min and the activity was calculated from the slope of these lines as mol NADPH oxidized per minute. Blank reactions with enzyme source replaced by distilled water were substracted from each assay.

After the normality of data distribution had been determined, differences between winter-swimmer and control groups were tested by analysis of variance. Pairwise comparisons were carried out by the independent two-sided t-test, p values below 0.05 being regarded as significant.

Results

Figure 1 shows the differences in erythrocytic glutathione between winter swimmers and controls. The GSH in red blood cells of winter swimmers was 2.85 ± 0.41 mmol/l ($n = 36$, mean ± SE) cells in comparison with 2.32 ± 0.41 mmol/l cells ($n = 40$) in controls. The erythrocytic GSSG level of winter swimmers is lower than that in the control group (Figure 1).

The changes in the major cytotoxic aldehydic lipid peroxidation product HNE, in GSH, ascorbic acid and uric acid as water-soluble antioxidants, and in other routine biochemical parameters were measured before, and 1 h after ice-bathing in 10 winter-swimmers, and in selected cases also immediately after the ice-bathing (see measurements of HNE and of ascorbic acid 15 min after finishing the winter-swimming) (Table 1). GSH, ascorbic acid, and uric acid were decreased after the whole-body cold exposure. The plasma uric acid decrease could not

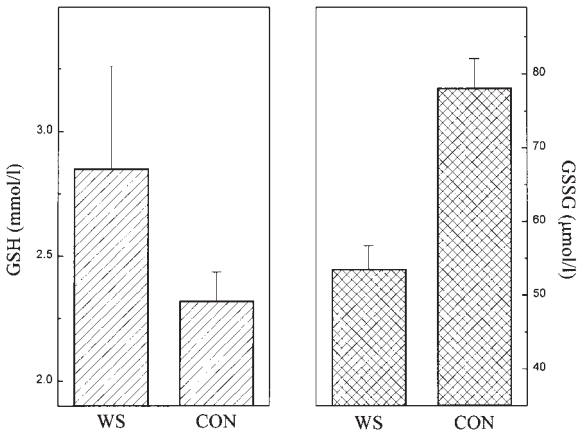

Figure 1. Glutathione status in the erythrocytes of winter-swimmers (WS; $n = 36$) and healthy controls (CON; $n = 40$). Values are means ± SE.

Table 1 Biochemical parameters in winter swimmers before and after ice-bathing

Parameter	Units	Before (0 min)	1 h	*n*	*p*
GSH, reduced glutathione	mmol/l cells	2.79 ± 0.42	2.46 ± 0.41	20	< 0.05
GSSG, oxidized glutathione	µmol/l cells	59.1 ± 13.9	81.9 ± 22.1	20	< 0.01
Uric acid	µmol/l	354 ± 99	298 ± 120	36	NS
HNE, 4-hydroxynonenal	µmol/l serum	0.077 ± 0.013	0.097 ± 0.121[1]	10	< 0.01 (15 vs. 0)
Ascorbic acid, Vit C	µmol/l serum	49.0 ± 13.2	30.9 ± 10.7[2]	10	< 0.01 (1 h vs. 0) < 0.01 (15 vs. 0)
ASAT, aspartate aminotransferase	µkatal/l	0.31 ± 0.14	0.30 ± 0.14	36	NS
AP, alkaline phosphatase	µkatal/l	2.286 ± 0.734	0.2448 ± 0.805	10	NS
γGT, γ-glutamyl transaferase	µkatal/l	0.49 ± 0.25	0.46 ± 0.25	36	NS
CK, creatine kinase	µkatal/l	2.25 ± 1.29	2.23 ± 1.23	20	NS
Plasma protein concentration	g/dl	78.7 ± 2.9	80.3 ± 4.4	36	NS
K^+, potassium ions	mval/l	4.65 ± 0.33	4.69 ± 0.37	36	NS
Glucose	mmol/l	4.38 ± 0.43	4.36 ± 0.62	20	NS

The time interval after ice-bathing was 1 h except for HNE and ascorbic acid, which were additionally measured 15 min after ice-bathing. *n*, number of winter swimmers; results as means ± SE. [1] 15 min after ice-bathing: 0.145 ± 0.035 µmol/l. [2] 15 min after ice-bathing: 29.5 ± 14.5 µmol/l.

be explained by an increased uric acid excretion into the urine; urine volume of winter swimmers before cold exposure was 135 ± 122 ml, and after cold exposure it was 174 ± 116 ml. The uric acid concentrations were 2.148 ± 0.415 and 1.736 ± 0.379 mmol/l, respectively. Based on these findings, the uric acid excretion was 0.145 ± 0.028 mmol/h before cold exposure and 0.151 ± 0.033 mmol/h after cold exposure.

HNE (one of the main lipid peroxidation products and therefore an indicator of oxidative tissue damage and oxygen radical production) was measured also 15 min after ice-bathing because it is known to be rapidly metabolised. HNE 1 h after the ice-bathing was similar to the HNE value before cold exposure, but it was increased 15 min after ice-bathing. The rapid changes in HNE and ascorbic acid blood plasma levels show the highly dynamic nature of free radical formation and lipid peroxidation.

The stability of most biochemical routine parameters shows that there were no signs of tissue or organ damages during the intensive short-term cold exposure.

Figure 2 compares the activities of erythrocytic SOD, GPx and Cat in winter-swimmers to those in controls. SOD and Cat activities were significantly increased in winter-swimmers in comparison to controls ($p < 0.01$ each).

Discussion

The changes in uric acid, GSH, GSSG, and HNE during winter-swimming demonstrate that the intensive short-term whole-body cold exposure induces an oxidative stress. Findings of oxidative stress from cold exposure in animals were published by others, e.g. findings of a glutathione depression in mice,[18] changes of antioxidant defense systems in rats[19] and in the ground squirrel.[20] Here we describe oxidative stress by cold exposure in humans. In the humans and animals, long-term and extreme cold exposure leads to organ damage and death[21] involving free-radical-mediated injury,[22] but short-term intensive or mild cold exposure has been used by physicians in physical therapy or by people themselves for body hardening.[1]

Winter swimming represents an intensive but short-term cold exposure. The accelerated free-radical-initiated lipid peroxidation during winter swimming does not lead to extensive tissue or organ damage, as demonstrated by the approximately constant plasma enzyme activities of ALAT, ASAT, creatine kinase, etc. (Table 1). As regards the main radical sources for the oxidative stress in winter swimming, a partial uncoupling of the respiratory chain of the mitochondria of the skeleton muscles (during muscle shivering?), accelerated catecholamine autoxidation, and a partial activation of leukocytes have been suggested, and all three processes are connected with increased free radical formation and accelerated lipid peroxidation.[23–25] An almost fourfold increase of norepinephrine concentration was measured after whole body immersion in cold water.[26]

If one compares some aspects of the antioxidative defense system of winter swimmers with those of healthy controls, there appears to be improved antioxidative protection in the form of higher initial GSH concentration and SOD and Cat activities in the erythrocytes of winter swimmers. We postulate that this improvement in antioxidative protection

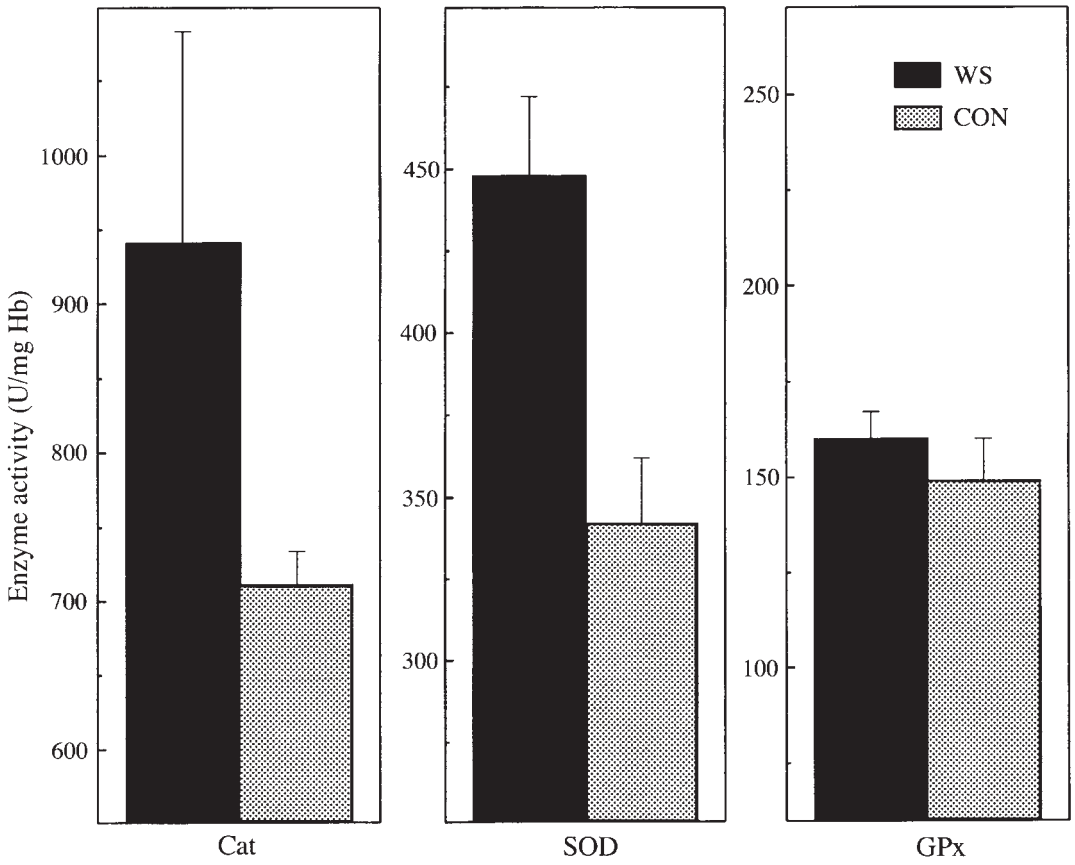

Figure 2. Erythrocytic activities of important antioxidative enzymes: superoxide dismutase (SOD), glutathione peroxidase (GPx), and catalase (Cat). The enzyme activities of 18 winter swimmers (WS) were compared with 18 healthy controls (CON). Values are means ± SE, units/mg haemoglobin.

results from the repetition of a non-damaging mild oxidative stress. It is known from other experimental models (in isolated cells, animals, plants, and humans) that cells or organisms often better resist damaging effects of toxic agents when first pre-exposed to a lower dose.[9] Many different types of damaging agents, including alkylating agents, heat stress, oxidative stress, radiation, and heavy metals have been reported to induce an adaptive response. For example, it has been shown that exercise training, which involves an oxidative stress,[27] leads to a reduction in the amount of lipid peroxidation produced during acute exercise.[28] Although most of our understanding of adaptive response to oxidative stress comes from studies of bacteria,[29–31] such responses may well prove to be of clinical benefit. For example, ischaemic reperfusion injury, which is known to have a significant oxidative damage component,[32,33] can be decreased by ischaemic preconditioning.[34]

With the winter swimmers as model for whole-body cold-exposure-induced oxidative stress, we have found another example for improved antioxidative protection as the response to preconditioning by repeated mild oxidative stress.

References

1. Brenke R. Winter-swimming—an extreme form of body hardening. *Therapeutikon* 1990; **4**:466–72.
2. Brenke R, Siems W. *Das Buch vom Winterschwimmen. Gesund und fit durch Abhärtung*. Husum, Husum-Verlag, 1996.
3. Brenke R, Siems W, Maass R. Body hardening by exposition to cold stimuli of different intensity: Changes of purine and oxygen radical metabolism. *Wiener Med Wschr* 1994; **144**:66–8.
4. Becker BF, Reinholz N, Oezcelik T, Leipert B, Gerlach E. Uric acid as radical scavenger and antioxidant in the heart. *Pfluegers Arch* 1982; **415**:127–35.
5. Simic MG, Jovanovic S. Antioxidation mechanisms of uric acid. *J Am Chem Soc* 1989; **111**:5778–82.
6. Kaur H, Halliwell B. Action of biologically-relevant oxidizing species upon uric acid. Identification of uric acid oxidation products. *Chem Biol Interaction* 1990; **73**:235–48.
7. Sevanian A, Davies KJA, Hochstein P. Serum urate as an antioxidant for ascorbic acid. *Am J Clin Nutr* 1991; **54**:1129S–34S.
8. Siems WG, van Kuijk FJGM, Maass R, Brenke R. Uric acid and glutathione levels during short-term whole body cold exposure. *Free Rad Biol Med* 1994; **16**:299–305.

9. Crawford DR, Davies KJA. Adaptive response and oxidative stress. *Environ Health Perspect* 1994; **102**(Suppl. 10):25–8.
10. Beutler E, Duron O, Kelly BM. Improved method for the determination of blood glutathione. *J Lab Clin Med* 1963; **61**:882–8.
11. Hissin PJ, Hilf R. A fluorimetric method for determination of oxidized and reduced glutathione in tissues. *Anal Biochem* 1976; **74**:371–7.
12. Sommerburg O, Grune T, Klee S, Ungemach FR, Siems WG. Formation of 4-hydroxynonenal and further aldehydic mediators of inflammation during bromotrichloromethane treatment of rat liver cells. *Mediators Inflammation* 1993; **2**:27–31.
13. Esterbauer H, Cheeseman KH, Dianzani MU, Poli G, Slater TF. Separation and characterization of the aldehydic products of lipid peroxidation stimulated by ADP-Fe^{2+} in rat liver microsomes. *J Biochem* 1982; **208**:129–40.
14. Grune T, Siems WG, Zollner H, Esterbauer H. Metabolism of 4-hydroxynonenal, a cytotoxic lipid peroxidation product, in Ehrlich mouse ascites cells at different proliferation stages. *Cancer Res* 1994; **54**:5231–5.
15. Winterburn CC, Hawkins RE, Brian M, Carrell R. The estimation of red cell superoxide dismutase activity. *J Lab Clin Med* 1975; **85**:337–41.
16. Aebi HE. Catalase. In: Bergmeyer J, Grassl M, eds. *Methods of Enzymatic Analysis*. Weinheim, Verlag Chemie, 1983:273–5.
17. Lawrence RA, Burk RF. Glutathione peroxidase activity in selenium-deficient rat liver. *Biochem Biophys Res Comm* 1976; **71**:952–8.
18. Simmons HF, James RC, Harbison RD, Roberts SM. Depression of glutathione by cold-restraint in mice. *Toxicology* 1990; **61**:59–71.
19. Spasic MB, Saicic ZS, Buzadzic B, Korac B, Blagojevic D, Petrovic VM. Effect of long-term exposure to cold on the antioxidant defense system in the rat. *Free Rad Biol Med* 1993; **15**:291–9.
20. Buzadzic B, Spasisc MB, Saicic ZS, Radojicic R, Petrovic VM. Seasonal dependence of the activity of antioxidant defence enzymes in the ground squirrel (*Citellus citellus*): the effect of cold. *Comp Biochem Physiol B* 1992; **101**:547–51.
21. MacKenzie MA. Cold exposure and winter mortality in Europe [letter; comment]. *Lancet* 1997; **350**:590–2.
22. Manson PN, Jesudass R, Marzella L, Bulkley GB, Im MJ, Narayan KK. Evidence for an early free radical-mediated reperfusion injury in frostbite. *Free Rad Biol Med* 1991; **10**:7–11.
23. Gutteridge JMC, Westermarck T, Halliwell B. Oxygen radical damage in biological systems. In: Johnson JE, Walford R, Harman D, Miquel J, eds. *Free Radicals, Aging, and Degenerative Diseases*. New York, Alan R. Liss, 1985;99–139.
24. Kalyanaraman B, Felix CC, Sealy RC. Electron spin resonance-spin stabilization of semiquinones produced during oxidation of epinephrine and ist analogues. *J Biol Chem* 1994; **259**:354–8.
25. Rosen GM, Pou S, Ramos CL, Cohen MS, Britigan BE. Free radicals and phagocytic cells. *FASEB J* 1995; **9**:200–9.
26. Smith DJ, Deuster PA, Ryan CJ, Doubt TJ. Prolonged whole body immersion in cold water: hormonal and metabolic changes. *Undersea Biomed Res* 1990; **17**:139–47.
27. Davies KJA, Quintanilha AT, Brooks GA, Packer L. Free radicals and tissue damage produced by exercise. *Biochem Biophys Res Comm* 1982; **107**:1198–205.
28. Alessio HM, Goldfarb AH. Lipid peroxidation and scavenger enzymes during exercise: adaptive response to training. *J Appl Physiol* 1988; **64**:1333–6.
29. Demple B, Halbrook J. Inducible repair of oxidative DNA damage in *Escherichia coli*. Nature 1983; **304**:466–8.
30. Christman MF, Morgan RW, Jacobson FS, Ames BN. Positive control of a regulon for a defense against oxidative stress and heat shock proteins in *Salmonella typhimurium*. *Cell* 1985; **41**:753–62.
31. Nunoshiba T, Hashimoto M, Nishioka H. Cross-adaptive response in *Escherichia coli* caused by pretreatment with hydrogen peroxide against formaldehyde and other aldehyde compounds. *Mutat Res* 1991; **255**:265–71.
32. Hernandez LA, Grisham MB, Twohig B, Arfors KE, Harlan JM, Granger DN. Role of neutrophils in ischemia-reperfusion-induced microvascular injury. *Am J Physiol* 1987; **253** (HCP22):H699–H703.
33. Siems WG, Grune T, Esterbauer H. 4-Hydroxynonenal formation during ischemia and reperfusion of rat small intestine. *Life Sci* 1995; **57**:785–9.
34. Murray CE, Richard VJ, Reimer KA, Jenning RB. Ischemic preconditioning slows energy metabolism and delays ultrastructural damage during a sustained ischemic episode. *Circ Res* 1990; **66**:913–31.