Original Article

SOD2 mRNA as a potential biomarker for exercise: interventional and cross-sectional research in healthy subjects

Shihona Ogino,[1,2] Noriyoshi Ogino,[1,3,*] Kotomi Tomizuka,[1] Masamitsu Eitoku,[1] Yosuke Okada,[2] Yoshiya Tanaka,[2] Narufumi Suganuma,[1] and Keiki Ogino[1]

[1]Department of Environmental Medicine, Kochi Medical School, Nankoku, Kochi 783-8505, Japan
[2]First Department of Internal Medicine, and [3]Third Department of Internal Medicine, School of Medicine, University of Occupational and Environmental Health, Kitakyushu, Fukuoka 807-8555, Japan

(Received 20 February, 2021; Accepted 15 March, 2021; Published online 8 May, 2021)

The health-promoting effects of exercise are explained by the biological adaptation to oxidative stress via maintenance of mitochondrial function especially in muscles. Although the induction of antioxidant enzymes in muscle is a useful indicator of exercise, it is not widely used due to the invasiveness of muscle biopsies. To explore more suitable biomarkers for exercise, we examined mRNA levels of antioxidant enzymes in peripheral blood mononuclear cells of 14 volunteers in an exercise intervention study. These results were validated in a cross-sectional study of 392 healthy individuals, and we investigated the association between exercise habits, smoking, alcohol consumption, mitochondrial DNA, malondialdehyde, and various clinical features. The 2-week exercise increased superoxide dismutase 1 at the end of exercise and superoxide dismutase 2 from week 4 onwards. In the cross-sectional study, superoxide dismutase 2 correlated positively with exercise habits and number of mitochondrial DNA, and negatively with malondialdehyde levels. Multivariate binominal regression analysis showed that superoxide dismutase 2 was positively associated with exercise habits in nonsmoking individuals. These results suggest that mRNA levels of superoxide dismutase 2 in blood might be a potentially useful biomarker for exercise in healthy individuals. This study was registered with University Hospital Medical Information Network (No: 000038034).

***Key Words*: oxidative stress, exercise, mitochondria, biomarker, lifestyles**

Increasing physical activity, such as through exercise, can prevent various diseases and extend life expectancy.[1–4] It is thought that exercise training improves health by maintaining mitochondrial function, especially in muscles.[5–7] Exercise training involves repeated exposure of skeletal muscle to an acute increase in various stresses, including oxidative stress.[8] During exercise, oxygen is utilized in the muscle mitochondria for substrate metabolism and ATP production, generating reactive oxygen species (ROS).[9,10] Relatively low concentrations of ROS are produced in muscle cells during exercise, which stimulate the expression of various antioxidant enzymes, such as catalase (*CAT*), glutathione peroxidase 1 (*GPX1*), and superoxide dismutase (*SOD*) via its related transcription factors. These adaptive responses are involved in increasing mitochondrial biogenesis and anti-oxidative capacity.[11–16]

Although establishing an exercise routine is a preventative measure for many lifestyle diseases, many people fail to participate in it.[17] To motivate people to start and continue their exercise habits, we searched for biomarkers that confirm the effects of short-term exercise, compared to existing long-term markers, such as HbA1c and blood pressure. We found previously that mRNA levels of several antioxidant enzymes were significantly increased in peripheral blood mononuclear cells (PBMC) after relatively intense aerobic exercise for 1 h per day over 4 weeks.[16] Furthermore, it has been reported that exercise-induced changes in muscle mitochondria also occur in PBMCs.[11,18] However, there is little information on blood biomarkers for the effects of mild and short exercise training. In this study, we evaluated antioxidant enzymes in PBMCs after exercise by comparing mRNA levels before and after 2-week daily 30-min runs (5 days a week). In addition, we examined the relationship between exercise habits among different lifestyles using blood tests and relative mRNA levels of antioxidant enzymes in the peripheral blood of 392 workers who underwent health examinations in a cross-sectional study.

Materials and Methods

Study design. The study consisted of two arms, an intervention study on 14 participants and a cross-sectional validation study of 392 subjects. In the intervention study, exercise training was conducted at Kochi University, Japan. The study period was from November 2019 to January 2020. Exercise training continued at 5 times per week for 2 weeks. Blood samples were withdrawn for analysis before and after the intervention. The Human Ethics Committee of Kochi University approved the study (#31-120), and all subjects provided written informed consent. This study was registered with UMIN (UMIN 000038034).

A cross-sectional study was conducted to explore the relationship between mRNA levels of antioxidant enzymes in the blood and oxidative stress-related parameters with regard to exercise training habits. The participants were healthy workers ($n = 392$) who underwent comprehensive medical examination at Junpukai Health Maintenance Center in Okayama Prefecture from December 2019 to March 2020 without comorbidities nor medical treatments. This study was approved by the Human Ethics Committee of Okayama University (#1903-027) and Kochi University (#2020-49) after the authors moved their research base university.

Exercise training. Exercise training for the intervention study consisted of jogging for 30 min 5 days a week for 2 weeks,

*To whom correspondence should be addressed.
E-mail: n-ogino@med. uoeh-u.ac.jp

doi: 10.3164/jcbn.21-24

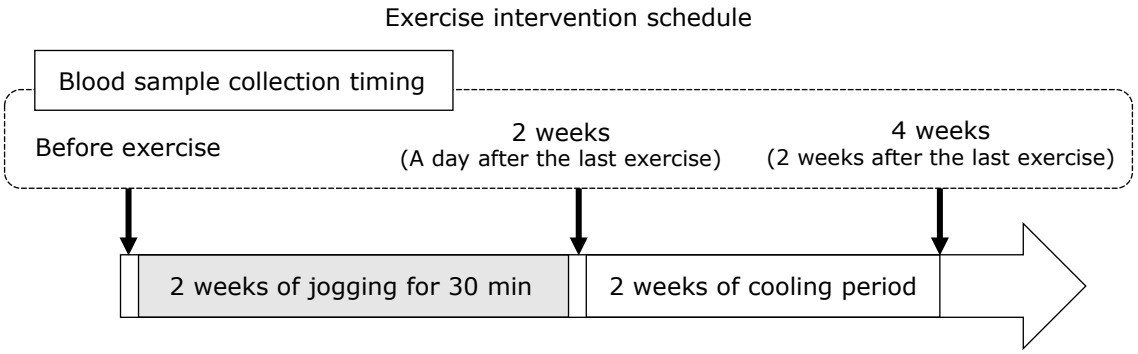

Fig. 1. Scheme of intervention study. Blood examination was performed three times: before exercise, at the end of 2 weeks of physical exercise intervention, and 2 weeks after the final exercise session.

at the university campus (Fig. 1). For the cross-sectional study, the information about the extent of exercise training per week was obtained from the data of self-reported questionnaires performed by medical staffs in Junpukai Health Maintenance Center.

Blood tests and medical measurements. For intervention study, a blood sample (10 ml) was collected at three different times (First, before the start of exercise; Second, after the day of the final exercise; Third, after 2 weeks of the final exercise session. Fig. 1). Isolation of the peripheral blood mononuclear cells from whole blood was conducted using Lymphoprep™ (STEMCELL Technologies, Vancouver, Canada), according to manufacturer's protocol. The supernatant was also collected for analysis of malondialdehyde (MDA).

For the cross-sectional study, venous blood samples were obtained from the participants with overnight fasting state by medical staffs in Junpukai Health Maintenance Center. The whole blood with the anticoagulant was used for measurement of HbA1c, relative expression of anti-oxidant enzymes mRNA and mitochondria DNA. Plasma or serum isolated from the blood was used for the measurement of alanine aminotransferase (ALT), triglyceride (TG), low-density lipoprotein-cholesterol (LDL-c), uric acid, and fasting plasma glucose (FPG), using the automated XE-2100 (Sysmex, Kobe, Japan) and H7700 (Hitachi High-Technologies, Tokyo, Japan) analyzers.

For assessment of oxidative stress, we measured the plasma or serum level of MDA using the method described previously.[19] Briefly, 10 µl of plasma, diluted with 90 µl of PBS (−), was added to 200 µl of 1% phosphoric acid and reacted with 0.67% 2-thiobarbituric acid (TBA) stock solution. The samples were boiled at 95°C for 30 min, centrifuged at 800 × *g* for 5 min, and the supernatant was measured for the fluorescence detector set at 535 nm excitation and 585 nm emission.

Body mass index (BMI) and conventional blood pressure were measured by trained medical staffs. Information of age, sex, and lifestyle factors were obtained using self-reported questionnaires, including cigarette smoking on scales of dichotomized [no smoking (no smoking + past smoking) or present smoking] or trichotomized (no smoking, past smoking, or present smoking), and alcohol consumption (dichotomized). Exercise habits were collected as described above.

mRNA levels of antioxidant enzymes. Total RNA was purified from PBMC or whole blood using ISOGEN (NIPPON GENE, Tokyo, Japan) in combination with High-Salt Precipitation Solution (NIPPON GENE). RNA concentration was measured using a NanoDrop 1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE). Complementary DNA (cDNA) was synthesized from 1 µg of total RNA in a 20 µl reaction volume using the ReverTra Ace qPCR RT kit (Toyobo, Osaka, Japan) according to the manufacturer's protocol. An aliquot of cDNA was used as a template for quantitative PCR using Thunderbird SYBR qPCR Mix (Toyobo, Osaka, Japan) on the StepOne Plus Real-time PCR System (Applied Biosystems, Foster City, CA) operated in the relative gene expression mode using the ROX dye as a passive reference (see Supplemental Table 1* for the primers). Primers were chosen from PrimerBank or qPrimerDepot, such that each amplicon spanned at least one intron. The relative expression levels were calculated using the ΔΔCt method with *GAPDH* as the endogenous control. For example, relative *SOD1* gene expression analysis was performed using the eq. $2^{-\Delta Ct}$ [ΔCt = Ct (*SOD1*) − Ct (*GAPDH*)]. For the intervention study, the mean relative expression level of post-intervention to pre-intervention is presented.

Measurements of mitochondrial DNA. Total DNA was extracted as described in detail previously.[20] Briefly, 200 µl of a whole blood was diluted in alkaline lysis reagent (100 µl of 25 mM NaOH and 100 µl of 0.2 mM disodium EDTA; pH was adjusted to 12). Samples were heated at 95°C for 10 min and were added neutralizing reagent (200 µl of 40 mM Tris-HCl at pH of 5), then centrifuged at 1,500 rpm for 5 min. In the next step, 1 µl of supernatant was used for measurement of relative mitochondrial DNA (mtDNA) copy number normalized to the expression level of nuclear β-globin. The calculation method using RT-qPCR and the primers were according to the protocol previously described.[21] Forward and reverse primers for the β-globin gene were 5'-GAAGAGCCAAGGACAGGTAC-3' and 5'-CAACTTCATCCACGTTCACC-3', respectively. Forward and reverse primers for the mitochondrial *ND1* gene were 5'-AACATACCCATGGCCAACCT-3' and 5'-AGCGAAGGG TTGTAGTAGCCC-3', respectively. Briefly, after denaturation at 95°C for 5 min, the samples were subjected to 40 cycles of incubation at 95°C for 0.1 s, 58°C for 6 s, and 72°C for 18 s. The mtDNA copy number was calculated using the following equation: relative copy number = $2^{\Delta Ct}$ [ΔCt = Ct (β-globin) − Ct (*ND1*)].

Statistical analysis. Data are expressed as mean ± SEM. Differences in mean values of several clinical parameters were analyzed according to age, sex, exercise habits, alcohol consumption, and smoking habits using the Mann–Whitney *U* test or unpaired *t* test, and ANOVA or Kruskal–Wallis test. We performed logistic regression analysis to investigate the association of antioxidant enzymes mRNA with clinical parameters and lifestyles. The covariates for adjustment were age, systolic blood pressure (SBP), diastolic blood pressure (DBP), LDL-c, uric acid, log-transformed values of BMI, WBC count, ALT, fasting plasma glucose (FPG), HbA1c, mRNA levels antioxidant enzymes (*SOD1*, *SOD2*, GPX, and *CAT*), mtDNA, and three-part scale variables of smoking (ANOVA), dichotomized or trichotomized variables of smoking, and dichotomized scale variables of exercise habit and alcohol consumption. All probability

*See online. https://doi.org/10.3164/jcbn.21-24

doi: 10.3164/jcbn.21-24

values for $p<0.05$ were regarded as statistically significant. All statistical analyses were performed using GraphPad Prism 5.0 for Mac (GraphPad Software, Inc., San Diego, CA) and PASW Statistics 18 for Mac.

Results

Characteristics of subjects of the two studies. The characteristics of the intervention study are shown in Table 1. This population consisted of healthy individuals who were non-smokers and non-regular alcohol drinkers. About the half of the participants had an exercise routine.

The characteristics of the cross-sectional study are shown in Table 2 and 3. The values of BMI, blood pressures, ALT, triglyceride, uric acid, FPG, MDA, mRNA levels of *SOD2*, *GPX1*, and mtDNA copy numbers were higher in males than in females. Age had no significant effect on the mRNA levels of antioxidant enzymes. When the degree of smoking was trichotomized (no smoking, past smoking, and present smoking), the values of mtDNA and WBC increased in a stepwise manner according to non-smoking, past smoking and present smoking groups. When the degree of exercise was dichotomized (no exercise and exercise training habits groups), the values of *SOD2* mRNA and mtDNA were significantly higher in the exercise group (Table 3). Finally, *SOD2* and *GPX1* mRNA levels were significantly higher in the regular alcohol consumption group (Table 3).

mRNA levels of antioxidant enzymes in the intervention study. Compared to the mRNA levels before exercise, those of *SOD1* increased significantly at the end of two weeks of intervention, and the mRNA levels of *SOD1* and *SOD2* were significantly upregulated two weeks after the end of the intervention period. MDA decreased slightly immediately after the exercise and increased significantly two weeks after the exercise (Fig. 2). The increase in *SOD2* was independent of prior exercise habits (Fig. 3A and B). Before the exercise intervention at baseline, *SOD2* was significantly higher in individuals with exercise habits compared to those without (Fig. 3C).

Antioxidant enzymes, clinical parameters and lifestyle in the cross-sectional study. Among the antioxidant enzymes (*SOD1*, *SOD2*, *CAT*, and *GPX1*), only *SOD2* correlated significantly and positively with exercise habits (Table 4). *SOD2* also had positive correlations with *GPX1*, *CAT*, mtDNA, and alcohol consumption, and a negative correlation with *SOD1* expression. Copy numbers of mtDNA correlated negatively with WBC count and positively with *SOD1* and *SOD2* levels. MDA correlated negatively with *SOD2*, *GPX1*, and *CAT*, and positively with mtDNA (Table 4). Stratification according to sex and smoking habits showed a significant correlation between *SOD2* mRNA levels and exercise habits in non-smoking group (Table 5).

Table 1. Characteristics of participants in the intervention study

Variables			%
Age		23.4 ± 0.6	
Body mass index (kg/m^2)		22.4 ± 0.4	
Total		14	
Sex	Male	11	79
	Female	3	21
Smoking		0	0
Alcohol consumption	No	9	64
(units/week)	<10	5	36
Exercise	No	7	50
(days/week)	1–5	4	29
(days/week)	>6	3	21

Table 2. Characteristics of the participants of the validation study stratified by sex and age

Variables	Total	Male	Female	*p*	Age <46	Age ≥47	*p*
	(*n* = 392)	(*n* = 221)	(*n* = 171)		(*n* = 205)	(*n* = 187)	
Age	46.71 ± 7.61	46.95 ± 7.98	46.41 ± 7.10	0.61	40.81 ± 4.02	53.18 ± 4.85	
Sex (male/female)	221/171				113/92	108/79	<0.01
BMI	23.16 ± 4.64	25.58 ± 4.11	20.04 ± 3.22	<0.0001	23.13 ± 4.75	23.19 ± 4.53	0.663
Systolic blood pressure	113.4 ± 17.16	117.40 ± 15.82	108.2 ± 17.47	<0.0001	111.0 ± 15.8	116.00 ± 18.21	0.006
Diastolic blood pressure	72.48 ± 11.76	74.68 ± 11.08	69.64 ± 12.04	<0.0001	69.96 ± 10.47	72.25 ± 12.49	<0.001
WBC	5,199 ± 1,506	5,298 ± 1,528	5,071 ± 1,472	0.067	5,209 ± 1,537	5,187 ± 1,475	0.520
ALT (U/L)	22.87 ± 15.42	27.84 ± 17.71	16.44 ± 8.21	<0.0001	23.22 ± 18.02	22.49 ± 11.98	0.433
TG (mg/dl)	96.63 ± 73.58	115.40 ± 87.50	72.27 ± 38.47	<0.0001	87.96 ± 58.84	106.10 ± 86.08	0.008
LDL-c (mg/dl)	131.4 ± 32.95	133.1 ± 30.26	129.10 ± 36.10	0.057	127.07 ± 34.70	136.20 ± 30.28	0.0012
Uric acid (mg/dl)	5.42 ± 1.41	6.21 ± 1.18	4.41 ± 0.97	<0.0001	5.29 ± 1.47	5.56 ± 1.32	0.060
HbA1c (%)	5.66 ± 0.39	5.67 ± 0.45	5.63 ± 0.29	0.673	5.57 ± 0.31	5.74 ± 0.44	<0.001
FPG (mg/dl)	99.65 ± 12.42	102.8 ± 14.22	95.56 ± 7.96	<0.0001	98.50 ± 13.39	100.90 ± 11.16	0.002
MDA (μM)	0.23 ± 0.06	0.24 ± 0.06	0.22 ± 0.05	<0.001	0.230 ± 0.06	0.234 ± 0.06	0.205
SOD1	1.68 ± 3.94	1.67 ± 2.84	1.68 ± 5.02	0.069	1.74 ± 4.86	1.61 ± 2.58	0.474
SOD2	0.56 ± 0.55	0.65 ± 0.67	0.44 ± 0.30	<0.0001	0.57 ± 0.63	0.55 ± 0.45	0.257
GPX1	0.48 ± 0.55	0.57 ± 0.67	0.37 ± 0.29	<0.0001	0.49 ± 0.60	0.48 ± 0.48	0.386
CAT	0.44 ± 0.50	0.47 ± 0.58	0.39 ± 0.36	0.069	0.46 ± 0.58	0.41 ± 0.39	0.568
mtDNA	2.93 ± 1.14	3.14 ± 1.26	2.67 ± 0.90	<0.0001	2.90 ± 1.15	2.98 ± 1.13	0.711
Smoking habits (–/+)	292/100	134/87	158/13	<0.001	148/57	144/43	0.275
Exercise habit (–/+)	169/187	79/114	90/73	0.430	86/105	83/82	0.320
Alcohol consumption (–/+)	170/222	69/152	101/70	<0.0001	93/112	77/110	0.334

Data are mean ± SEM or number of subjects. BMI, body mass index; WBC, white blood cell; ALT, aminotransferase; TG, triglyceride; LDL-c, low density lipoprotein cholesterol; HbA1c, hemoglobin A1c; FPG, fasting plasma glucose; MDA, malondialdehyde; SOD, superoxide dismutase; mRNA, messenger ribonucleic acid; GPX, glutathione peroxidase; CAT, catalase; mtDNA, mitchondrial deoxyribonucleic acid.

Table 3. Chracteristics of of several clinical parameters, oxidative stress-related markers, and mRNA of antioxidant enzymes

Variables	No smoking	Past smoking	Present smoking	*p*	Exercise habits (–)	Exercise habits (+)	*p*	Alcohol (–)	Alcohol (+)	*p*
	(*n* = 217)	(*n* = 75)	(*n* = 100)		(*n* = 169)	(*n* = 187)		(*n* = 170)	(*n* = 222)	
Age	46.52 ± 7.65	47.2 ± 7.06	46.37 ± 7.92	0.448	46.71 ± 7.21	46.27 ± 7.8	0.381	46.55 ± 7.65	46.83 ± 7.59	0.662
Sex (male/female)	74/143	60/15	87/13	<0.001	79/90	114/73	<0.01	69/101	152/70	<0.001
BMI	21.97 ± 4.44	24.30 ± 4.21***	24.90 ± 4.64***	<0.0001	23.94 ± 5.14	23.31 ± 4.47	0.385	22.36 ± 4.56	23.78 ± 4.62	0.001
Systolic blood pressure	111.7 ± 17.72	118.30 ± 16.26**	113.3 ± 15.97	0.006	112.9 ± 16.96	113 ± 17.18	0.839	110.4 ± 16.99	115.6 ± 16.98	0.002
Diastolic blood pressure	71.63 ± 12.09	74.75 ± 10.86	72.63 ± 11.57	0.06	72.50 ± 12.08	72.5 ± 11.75	0.778	70.16 ± 11.31	74.26 ± 11.82	0.001
WBC	4,927 ± 1,223	4,999 ± 1,236***	5,969 ± 1,960***	<0.001	5,374 ± 1,682	5,010 ± 1,291	0.049	5,182 ± 1,447	5,211 ± 1,554	0.861
ALT (U/L)	20.11 ± 5.25	24.97 ± 14.42*	27.28 ± 22.18***	<0.001	23.08 ± 18.91	21.57 ± 7.06	0.162	21.09 ± 12.72	24.23 ± 17.11	0.011
TG (mg/dl)	80.12 ± 49.86	97.83 ± 61.75*	131.2 ± 106.0***	<0.0001	101.3 ± 75.07	91.35 ± 70.59	0.906	83.93 ± 46.63	106.2 ± 87.58	0.016
LDL-c (mg/dl)	129.7 ± 28.60	133.5 ± 43.04	133.90 ± 33.24	0.608	132.7 ± 32.63	130.6 ± 33.68	0.382	137.2 ± 34.46	126.9 ± 31.10	0.002
Uric acid (mg/dl)	5.00 ± 1.33	5.97 ± 1.13***	5.97 ± 1.44***	<0.0001	5.35 ± 1.48	5.41 ± 1.36	0.537	5.03 ± 1.33	5.72 ± 1.40	<0.001
HbA1c (%)	5.63 ± 0.32	5.62 ± 0.24	5.72 ± 0.56	0.148	5.68 ± 0.49	5.62 ± 0.28	0.210	5.65 ± 0.29	5.65 ± 0.45	0.437
FPG (mg/dl)	97.88 ± 12.89	101.3 ± 9.45**	102.3 ± 12.83**	<0.001	100.4 ± 16.07	98.9 ± 8.81	0.855	96.94 ± 8.56	101.7 ± 14.37	<0.001
MDA (μM)	0.23 ± 0.05	0.24 ± 0.06	0.23 ± 0.06	0.287	0.23 ± 0.06	0.229 ± 0.06	0.305	0.225 ± 0.05	0.230 ± 0.06	0.009
SOD1	1.85 ± 4.91	1.62 ± 2.77	1.36 ± 1.64	0.376	1.45 ± 2.44	1.95 ± 5.13	0.268	1.70 ± 5.00	1.66 ± 2.87	0.07
SOD2	0.57 ± 0.61	0.60 ± 0.46	0.52 ± 0.48	0.152	0.50 ± 0.44	0.64 ± 0.66	<0.01	0.54 ± 0.65	0.58 ± 0.46	0.004
GPX1	0.47 ± 0.59	0.57 ± 0.60	0.45 ± 0.38	0.210	0.45 ± 0.53	0.53 ± 0.59	0.056	0.43 ± 0.50	0.53 ± 0.57	<0.001
CAT	0.44 ± 0.53	0.48 ± 0.43	0.39 ± 0.45	0.097	0.41 ± 0.52	0.46 ± 0.48	0.217	0.40 ± 0.42	0.46 ± 0.55	0.09
mtDNA	2.78 ± 1.00	3.04 ± 1.00*	3.20 ± 1.44**	0.002	2.80 ± 1.11	2.99 ± 1.07	0.033	2.02 ± 1.28	2.97 ± 1.02	0.06
Exercise habit (–/+)	90/112	31/40	48/35	0.170				78/78	91/109	0.40
Alcohol consumption (–/+)	119/98	16/59	35/65	<0.0001	78/91	77/110	0.334			

Data are mean ± SEM or number of subjects. *p<0.05, **p<0.01, ***p<0.001 vs nonsmoking group. BMI, body mass index; WBC, white blood cell; ALT, aminotransferase; TG, triglyceride; LDL-c, low density lipoprotein cholesterol; HbA1c, hemoglobin A1c; FPG, fasting plasma glucose; MDA, malondialdehyde; SOD, superoxide dismutase; mRNA, messenger ribonucleic acid; GPX, glutathione peroxidase; CAT, catalase; mtDNA, mitchondrial deoxyribonucleic acid.

doi: 10.3164/jcbn.21-24

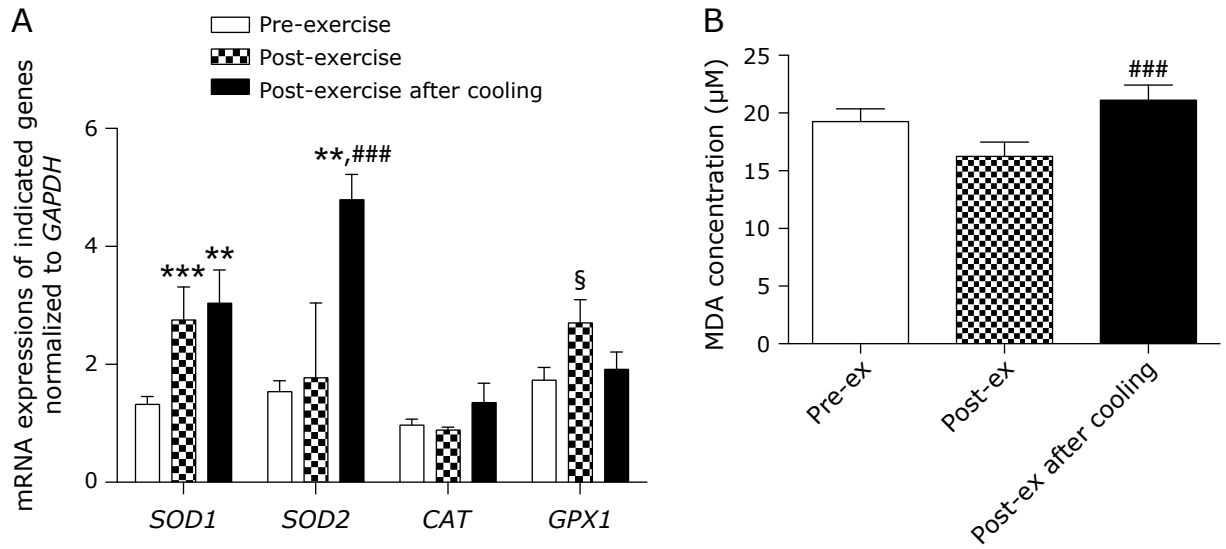

Fig. 2. Exercise-related changes in mRNA levels of antioxidant enzymes in PBMC and MDA levels in plasma. (A) Relative mRNA levels of *SOD1*, *SOD2*, *CAT*, and *GPX1* in PBMCs. Data are mean ± SEM of 14 individuals. $^{**}p<0.01$, $^{***}p<0.001$ vs pre-exercise. $^{\#\#\#}p<0.001$, vs post-exercise (multiple comparison test). $^{\S}p<0.05$, vs pre-exercise (*t* test). (B) Lipid peroxidation in plasma. MDA were measured using the fluorescence method. Data are mean ± SEM of 14 individuals. $^{***}p<0.001$, vs pre-exercise.

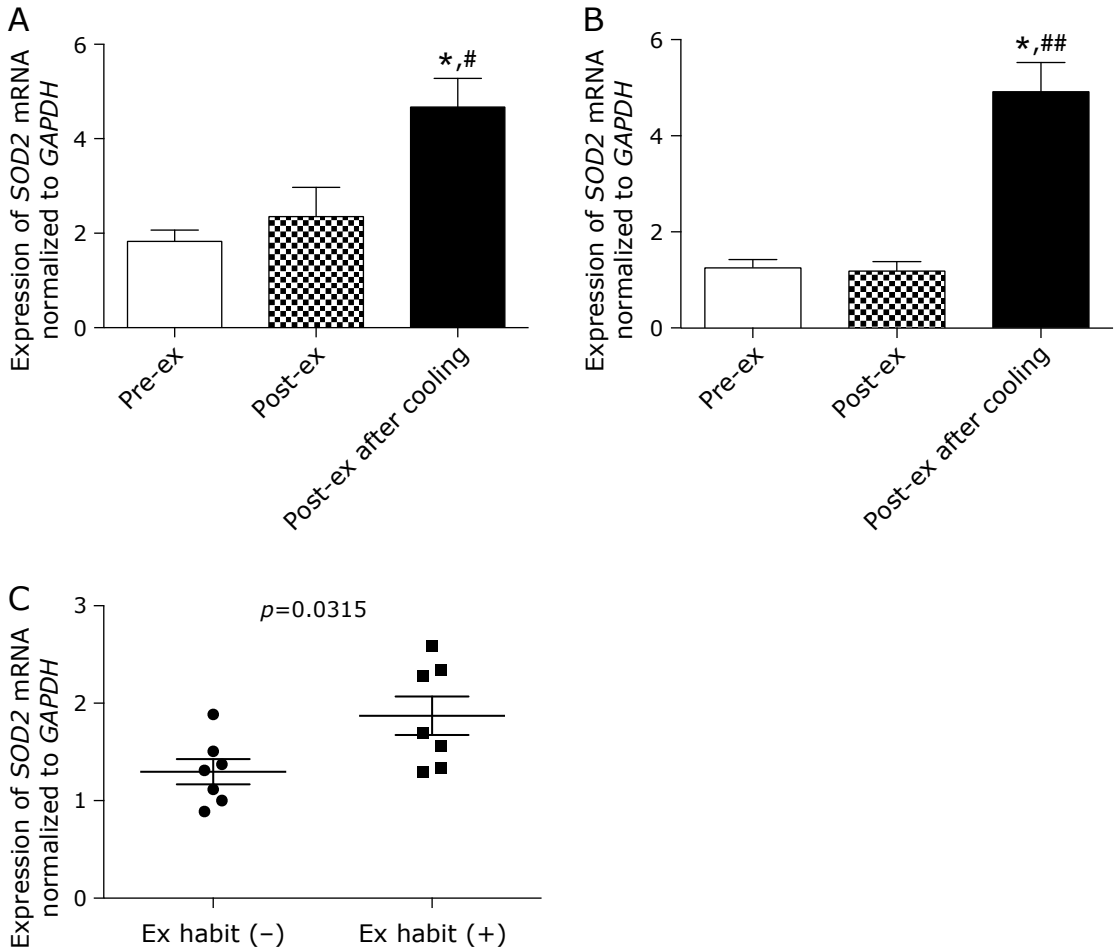

Fig. 3. Changes in *SOD2* mRNA in subjects of the exercise habit and no exercise habit groups. The changes of *SOD2* mRNA before and after exercise intervention in the exercise habit group (A) and no exercise habit group (B). $^{*}p<0.05$, vs pre-exercise, $^{\#}p<0.05$, $^{\#\#}p<0.01$, vs post-exercise. (C) The comparison of *SOD2* mRNA levels between the exercise habit group and no exercise habit group before exercise (at baseline). Data are mean ± SEM of 14 individuals.

Table 4. Correlation among mRNA of antioxidative enzymes, oxidative stress marker with several clinical parameters

	SOD1		*SOD2*		*GPX1*		*CAT*		MDA		mtDNA	
	r	*p*	*r*	*p*	*r*	*p*	*r*	*p*	*r*	*p*	*r*	*p*
Age	–0.050	0.323	0.050	0.325	0.068	0.187	0.008	0.871	0.054	0.285	0.025	0.625
Sex	–0.092	0.069	–0.231	<0.001	–0.206	<0.001	–0.092	0.069	–0.174	0.001	–0.242	<0.001
BMI	0.326	<0.001	0.152	0.003	0.186	<0.001	0.069	0.176	0.069	0.174	0.100	0.048
Systolic blood pressure	–0.024	0.632	0.133	0.008	0.117	0.021	0.016	0.747	0.165	0.001	0.045	0.379
Diastolic blood pressure	–0.013	0.802	0.051	0.313	0.128	0.011	–0.012	0.807	0.144	0.004	–0.005	0.917
WBC	–0.268	<0.001	–0.310	<0.001	–0.241	<0.001	–0.271	<0.001	–0.035	0.493	–0.114	0.026
ALT	–0.110	0.030	0.120	0.018	0.106	0.035	0.034	0.505	0.172	0.001	0.157	0.002
TG	–0.004	0.932	0.016	0.759	0.051	0.321	–0.027	0.591	0.148	0.003	0.125	0.013
LDL–c	0.004	0.937	0.002	0.975	–0.046	0.359	–0.015	0.773	0.122	0.015	0.017	0.738
Uric acid	–0.014	0.043	0.138	0.007	0.153	0.002	0.035	0.497	0.125	0.015	0.087	0.089
HbA1c	–0.109	0.031	0.076	0.133	<0.001	0.992	0.039	0.443	–0.010	0.884	0.035	0.487
FPG	–0.068	0.182	0.098	0.052	0.047	0.351	–0.053	0.299	0.141	0.005	0.157	0.002
MDA	0.064	0.209	–0.300	<0.001	–0.254	<0.001	–0.202	<0.001			0.126	0.012
SOD1			–0.721	<0.001	–0.688	<0.001	–0.736	<0.001	0.064	0.209	0.148	0.003
SOD2	–0.721	<0.001			0.720	<0.001	0.765	<0.001	–0.300	<0.001	0.130	0.010
GPX1	–0.688	<0.001	0.720	<0.001			0.671	<0.001	–0.254	<0.001	0.062	0.224
CAT	–0.736	<0.001	0.765	<0.001	0.671	<0.001			–0.202	<0.001	0.084	0.095
mtDNA	0.148	0.003	0.130	0.010	0.062	0.224	0.084	0.095	0.126	0.012		
Smoking habits	–0.046	0.359	–0.073	0.150	–0.023	0.644	–0.078	0.123	0.033	0.515	0.124	0.014
Alcohol consumption	0.092	0.068	0.147	0.004	0.209	<0.001	0.085	0.092	0.078	0.122	0.097	0.055
Exercise habits	0.068	0.198	0.147	0.006	0.102	0.055	0.066	0.217	0.006	0.864	0.114	0.003

BMI, body mass index; WBC, white blood cell; ALT, aminotransferase; TG, triglyceride; LDL-c, low density lipoprotein cholesterol; HbA1c, hemoglobin A1c; FPG, fasting plasma glucose; MDA, malondialdehyde; SOD, superoxide dismutase; mRNA, messenger ribonucleic acid; GPx, glutathione peroxidase; CAT, catalase; mtDNA, mitchondrial deoxyribonucleic acid.

Table 5. Correlation of *SOD2* mRNA with exercise habits

	No smoking		Past smoking		Present smoking	
	r	*p*	*r*	*p*	*r*	*p*
Total	0.171	0.015	0.078	0.527	0.094	0.399
Male	0.136	0.282	0.069	0.613	0.011	0.929
Female	0.092	0.282	0.003	1.000	0.300	0.545

Table 6. Odds for exercise habits according to *SOD2*mRNA

	Tertiles of SOD2			
	Q1 (≤0.34)	Q2 (0.35–0.54)	Q3 (≥0.55)	*p* for trend
Model 1	1	1.369 (0.822–2.278)	2.007 (1.200–3.356)	0.008
Model 2	1	1.259 (0.750–2.113)	1.781 (1.049–3.024)	0.032
Model 3	1	1.265 (0.742–2.155)	1.903 (1.090–3.323)	0.024
Model 4	1	1.126 (0.650–1.952)	1.592 (0.892–2.842)	0.116

Data are odds ratios and (95% confidence intervals). Model 1: no adjustment. Model 2: adjustment for age and sex. Model 3: adjustment for age, sex, BMI, ALT, TG, LDL-c, HbA1c, systolic blood pressure, MDA, mtDNA, and alcohol consumption. Model 4: smoking habit was added to model 3.

Logistic regression analysis. The logistic regression analysis for exercise habits according to *SOD2* mRNA levels are shown in Table 6. *SOD2* mRNA associated positively with exercise habit after adjustment for age, sex, BMI, ALT, TG, LDL-c, SBP, MDA, mtDNA, and alcohol consumption in model 3. When smoking habit was added to model 3, the association between *SOD2* mRNA and exercise habits disappeared in model 4.

Discussion

The main findings of the intervention study were significant increases in mRNA levels of *SOD1*, *GPX1*, and *SOD2* in PBMC after two weeks of exercise. The validation cross-sectional study demonstrated that *SOD2* mRNA in whole blood cells was a significant independent determinant of exercise habits in nonsmokers. This result also showed that increased *SOD2* was

doi: 10.3164/jcbn.21-24

accompanied by decreased lipid peroxidation and increased mitochondrial biogenesis. These indicated that *SOD2* mRNA level in the blood might be useful as a phenotypic biomarker of the mechanism of muscle-based health promotion by exercise.

In the intervention study, 30 min of daily jogging was implemented over a 2-week period, and blood samples were taken three times. In our previous intervention study of four weeks of bicycle exercise in a trained population, the mRNA levels of *CAT* and *GPX1*, but not *SOD1* or *SOD2*, were significantly elevated the day after the end of intervention. In another study of 30 min of treadmill exercise for 7 days, the mRNA levels of *GPX1*, *SOD1*, and *SOD2* were all significantly elevated in CD34(−) PBMC on the day after the end of intervention.[22] These differences might be due to the type of exercise (e.g., aerobic or anaerobic) and timing of sample collection.[23] It has been reported that lipid peroxidation rises immediately after acute exercise and returns to normal the following day.[24,25] Other studies reported that lipid peroxidation in plasma decreased after repeated exercise intervention for more than a week.[26,27] Although the slight decrease in MDA levels observed in our study the day after the last exercise could be consistent with these reports, it is difficult to explain a significant increase observed two weeks after the exercise intervention. This needs to be examined in future.

What is unique about our data is the elevated mRNA levels of *SOD2* in PBMC two weeks after exercise intervention, but not one day after. This finding may explain the results of our validation study, in which only *SOD2* but not *SOD1* or *GPX1*, *CAT* mRNAs were associated with exercise habits. Adaptation of the oxidative stress that first occurs in muscles might gradually spread to affect non-muscular tissues, including blood cells, for a certain period of time. Muscle cells is known to generate cytokines and other muscle fiber-derived peptides, such as myokines.[28] Myokines communicate with other organs such as adipose tissue, liver, bone, and brain to exert the beneficial effects of exercise at the whole-body level.

In the cross-sectional study, the genes candidates for exercise biomarkers in interventional studies were validated in the blood samples from 392 healthy adults without comorbidities. We also examined mtDNA levels, a marker of mitochondrial biogenesis, and analyzed lifestyle habits including exercise, alcohol consumption, and smoking. In this study, *SOD2* was the only significant factor found to be altered by exercise although the reports from other research have shown different types of antioxidant enzymes to be altered by exercise.[22,29,30] This discrepancy might be due to the fact that these studies did not take lifestyle into account. The lifestyles such as alcohol consumption, smoking and taking supplementation were reported to affect oxidative stress levels in the blood.[31–33] The results that exercise habit had the positive correlation with mtDNA and the negative correlation with MDA in our study reinforced the usefulness of *SOD2* as a biomarker for exercise. The similar mechanism have been reported regarding the impact of muscles on improving overall health.[13] Interestingly in our study, the level of mtDNA correlated significantly and positively with past and current smoking in addition to exercise, but not with alcohol consumption. This result differs from the previous another report, which showed that mtDNA level was negatively correlated with smoking, and not significantly correlated with exercise.[34] This may be related to differences in how smoking and non-smoking are separated from past smoking, or to the inclusion of disease groups such as diabetes or hypertension; therefore, the medications used by their subjects could have affected the blood levels of oxidative stress markers. In fact, in other studies of healthy subjects except for the patients, mtDNA was reported to increase after exercise.[35] However, another bench research has shown that nicotine increases MDA and decreases SOD activity, which could inhibit mitochondrial biogenesis.[36–39] Further verification is needed to elucidate the mechanism of *SOD2* and mitochondrial function after smoking.

The adaptive increase in antioxidant enzymes in skeletal muscles induced after exercise is involved in ROS production in the mitochondria due to high consumption of O_2. However, the mechanism responsible for the induction of antioxidant enzymes in PBMC is not clear yet. Our data suggest unknown inducers of ROS in the mitochondria of PBMC since induction of *SOD2* was not observed at the end of the intervention but 2 weeks after the end of the study.

Although our findings are significant, several study limitations should be discussed. First, the sample size was small. Second, the mRNA level was not measured in PBMC but in blood clots in the validation study. Third, causal relationships could not be determined because one of the two studies was a cross-sectional study. Fourth, the intervention study did not include a control study. Fifth, some reporting bias may have been introduced because the information on exercise training was obtained via self-reported questionnaire. Finally, for proper analysis of exercise training, information about clerical staff or physical labor is needed.

Our results suggest that *SOD2* mRNA in the blood is a potentially suitable biomarker of exercise training in non-smokers. Future investigation using cohort studies is needed to confirm the usefulness of *SOD2* mRNA in blood.

Author Contributions

Study concept and design, KO; acquisition of data, KT, NO; analysis and interpretation of data, KO; drafting of the manuscript, SO; critical revision of the manuscript for important intellectual content, YO and YT; statistical analysis, KO; obtained funding, SO and KO; administrative, technical, or material support, NO and ME; study supervision, NS and KO.

Acknowledgments

We thank the staffs of the Junpukai Health Maintenance Center for their help in collecting blood samples and questionnaires. This work was supported in part by JSPS KAKENHI grants #18K19681 and #19K21427.

Conflict of Interest

No potential conflicts of interest were disclosed.

References

1 Warburton DER, Nicol CW, Bredin SS. Health benefits of physical activity: the evidence. *CMAJ* 2006; **174**: 801–809.
2 Vina J, Borras C, Sanchis-Gomar F, *et al*. Pharmacological properties of physical exercise in the elderly. *Curr Pharm Des* 2014; **20**: 3019–3029.
3 Zhao M, Veeranki SP, Magnussen CG, Xi B. Recommended physical activity and all cause and cause specific mortality in US adults: prospective cohort study. *BMJ* 2020; **370**: m2031.
4 Ekelund U, Tarp J, Steene-Johannessen J, *et al*. Dose-response associations between accelerometry measured physical activity and sedentary time and all cause mortality: systematic review and harmonised meta-analysis. *BMJ* 2019; **366**: I4570.
5 Ferraro E, Giammarioli AM, Chiandotto S, Spoletini I, Rosano G. Exercise-induced skeletal muscle remodeling and metabolic adaptation: redox signaling and role of autophagy. *Antioxidants Redox Signal* 2014; **21**: 154–176.
6 Russell AP, Foletta VC, Snow RJ, Wadley GD. Skeletal muscle mitochon-

dria: a major player in exercise, health and disease. *Biochim Biophys Acta* 2014; **1840**: 1276–1284.
7 Memme JM, Erlich AT, Phukan G, Hood DA. Exercise and mitochondrial health. *J Physiol* 2021; **599**: 803–817.
8 Egan B, Zierath JR. Exercise metabolism and the molecular regulation of skeletal muscle adaptation. *Cell Metab* 2013; **17**: 162–184.
9 Chance B, Sies H, Boveris A. Hydroperoxide metabolism in mammalian organs. *Physiol Rev* 1979; **59**: 527–605.
10 Jenkins RR. Free radical chemistry. Relationship to exercise. *Sport Med* 1988; **5**: 156–170.
11 Liepinsh E, Makarova E, Plakane L, *et al*. Low-intensity exercise stimulates bioenergetics and increases fat oxidation in mitochondria of blood mononuclear cells from sedentary adults. *Physiol Rep* 2020; **8**: e14489.
12 Kasai S, Shimizu S, Tatara Y, Mimura J, Itoh K. Regulation of Nrf2 by mitochondrial reactive oxygen species in physiology and pathology. *Biomolecules* 2020; **10**: 320.
13 Kang C, Li Ji L. Role of PGC-1α signaling in skeletal muscle health and disease. *Ann N Y Acad Sci* 2012; **1271**: 110–117.
14 Gomez-Cabrera MC, Domenech E, Romagnoli M, *et al*. Oral administration of vitamin C decreases muscle mitochondrial biogenesis and hampers training-induced adaptations in endurance performance. *Am J Clin Nutr* 2008; **87**: 142–149.
15 Colley RC, Garriguet D, Janssen I, Craig CL, Clarke J, Tremblay MS. Physical activity of Canadian adults: accelerometer results from the 2007 to 2009 Canadian Health Measures Survey. *Heal Rep* 2011; **22**: 7–14.
16 Tsukiyama Y, Ito T, Nagaoka K, Eguchi E, Ogino K. Effects of exercise training on nitric oxide, blood pressure and antioxidant enzymes. *J Clin Biochem Nutr* 2017; **60**: 180–186.
17 Owen KB, Smith J, Lubans DR, Ng JY, Lonsdale C. Self-determined motivation and physical activity in children and adolescents: a systematic review and meta-analysis. *Prev Med* 2014; **67**: 270–279.
18 Busquets-Cortés C, Capó X, Martorell M, Tur JA, Sureda A, Pons A. Training and acute exercise modulates mitochondrial dynamics in football players' blood mononuclear cells. *Eur J Appl Physiol* 2017; **117**: 1977–1987.
19 Ohkawa H, Ohishi N, Yagi K. Assay for lipid peroxides in animal tissues by thiobarbituric acid reaction. *Anal Biochem* 1979; **95**: 351–358.
20 Truett GE, Heeger P, Mynatt RL, Truett AA, Walker JA, Warman ML. Preparation of PCR-quality mouse genomic DNA with hot sodium hydroxide and tris (HotSHOT). *Biotechniques* 2000; **29**: 52–54.
21 Kim MY, Lee JW, Kang HC, Kim E, Lee DC. Leukocyte mitochondrial DNA (mtDNA) content is associated with depression in old women. *Arch Gerontol Geriatr* 2011; **53**: e218–e221.
22 Jenkins NT, Landers RQ, Prior SJ, Soni N, Spangenburg EE, Hagberg JM. Effects of acute and chronic endurance exercise on intracellular nitric oxide and superoxide in circulating $CD34^+$ and $CD34^-$ cells. *J Appl Physiol (1985)* 2011; **111**: 929–937.
23 García-López D, Häkkinen K, Cuevas MJ, *et al*. Effects of strength and endurance training on antioxidant enzyme gene expression and activity in middle-aged men. *Scand J Med Sci Sport* 2007; **17**: 595–604.
24 Inglés M, Serra-Añó P, Gambini J, *et al*. Active paraplegics are protected against exercise-induced oxidative damage through the induction of antioxidant enzymes. *Spinal Cord* 2016; **54**: 830–837.
25 Turner JE, Bosch JA, Drayson MT, Aldred S. Assessment of oxidative stress in lymphocytes with exercise. *J Appl Physiol (1985)* 2011; **111**: 206–211.
26 Pasini AMF, Stranieri C, Rigoni AM, *et al*. Physical exercise reduces cytotoxicity and up-regulates Nrf2 and upr expression in circulating cells of peripheral artery disease patients: an hypoxic adaptation? *J Atheroscler Thromb* 2018; **25**: 808–820.
27 Soares JP, Silva AM, Oliveira MM, Peixoto F, Gaivão I, Mota MP. Effects of combined physical exercise training on DNA damage and repair capacity: role of oxidative stress changes. *Age (Dordr)* 2015; **37**: 9799.
28 Huh JY. The role of exercise-induced myokines in regulating metabolism. *Arch Pharm Res* 2018; **41**: 14–29.
29 Görlach A, Dimova EY, Petry A, *et al*. Reactive oxygen species, nutrition, hypoxia and diseases: problems solved? *Redox Biol* 2015; **6**: 372–385.
30 Busquets-Cortés C, Capó X, del Mar Bibiloni M, *et al*. Peripheral blood mononuclear cells antioxidant adaptations to regular physical activity in elderly people. *Nutrients* 2018; **10**: 1555.
31 Farinha JB, De Carvalho NR, Steckling FM, *et al*. An active lifestyle induces positive antioxidant enzyme modulation in peripheral blood mononuclear cells of overweight/obese postmenopausal women. *Life Sci* 2015; **121**: 152–157.
32 Amaral AL, Mendonça AM, Giolo JS, *et al*. The effects of isoflavone supplementation plus combined exercise on salivary markers of oxidative stress in postmenopausal women. *J Clin Biochem Nutr* 2020; **66**: 43–48.
33 Takami M, Aoi W, Terajima H, Tanimura Y, Wada S, Higashi A. Effect of dietary antioxidant-rich foods combined with aerobic training on energy metabolism in healthy young men. *J Clin Biochem Nutr* 2019; **64**: 79–85.
34 Vyas CM, Ogata S, Reynolds CF 3rd, *et al*. Lifestyle and behavioral factors and mitochondrial DNA copy number in a diverse cohort of mid-life and older adults. *PLoS One* 2020; **15**: e0237235.
35 Chang YK, Kim DE, Cho SH, Kim JH. Association between leukocyte mitochondrial DNA copy number and regular exercise in postmenopausal women. *Korean J Fam Med* 2016; **37**: 334–339.
36 Sudheer AR, Muthukumaran S, Kalpana C, Srinivasan M, Menon VP. Protective effect of ferulic acid on nicotine-induced DNA damage and cellular changes in cultured rat peripheral blood lymphocytes: a comparison with *N*-acetylcysteine. *Toxicol In Vitro* 2007; **21**: 576–585.
37 Das S, Neogy S, Gautam N, Roy S. *In vitro* nicotine induced superoxide mediated DNA fragmentation in lymphocytes: protective role of *Andrographis paniculata* Nees. *Toxicol In Vitro* 2009; **23**: 90–98.
38 Garrabou G, Hernàndez AS, Catalán García M, *et al*. Molecular basis of reduced birth weight in smoking pregnant women: mitochondrial dysfunction and apoptosis. *Addict Biol* 2016; **21**: 159–170.
39 Miró Ò, Alonso JR, Jarreta D, Casademont J, Urbano-Márquez Á, Cardellach F. Smoking disturbs mitochondrial respiratory chain function and enhances lipid peroxidation on human circulating lymphocytes. *Carcinogenesis* 1999; **20**: 1331–1336.

doi: 10.3164/jcbn.21-24