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ORIGINAL ARTICLE

RELATIONSHIP BETWEEN EXHALED HYDROGEN AND HUMAN NEUTROPHIL FUNCTION IN THE JAPANESE GENERAL POPULATION

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Abstract We examined the relationship between the amount of exhaled hydrogen and neutrophil ROS production to investigate the effect of hydrogen on oxidative stress at normal state among the general population comprising subjects who had participated in "The Iwaki Health Promotion Project in 2007", which was held in the Iwaki area, Hirosaki-city in Aomori prefecture in northern Japan. Subjects with diabetes mellitus (diagnosed by a medical doctor), malignant tumors, immune disorders or those who were pregnant at the time of the study, taking antimicrobial drugs, anticancer medication or hormones were excluded from the study, and a total of 656 subjects (252 males and 404 females) were finally enrolled. Smoking habits, alcohol use, exercise habits and HbA1c were surveyed. A positive correlation was seen between exhaled hydrogen concentration and total reactive oxygen species production in stimulated neutrophils in subjects less than 60 y.o. ($p<0.05$), but such a trend was not seen in other age groups or in female subjects. In conclusion, levels of body hydrogen as an antioxidative substance were suggested to have increased as a response to increased production of ROS by neutrophils as mechanisms against oxidative stress.

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Key words: exhaled hydrogen; neutrophil; reactive oxygen species; oxidative stress; general population.

原　著

一般住民における呼気水素と好中球機能の関係

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抄録　一般住民の呼気中水素濃度と好中球産生活性酸素種量の関係をみることで，酸化ストレスにおける水素の体内での役割を検討した．対象者は，2007年の岩木健康増進プロジェクトの参加者で，糖尿病，悪性腫瘍，免疫疾患罹患者，抗生剤，抗がん剤，ホルモン剤など炎症，免疫に関係する薬物と便秘薬服用者，欠損値のあるものを対象から除外した656名（男性252名，女性404名）であった．呼気中水素濃度と異物刺激時と刺激前の好中球活性酸素種産生量を測定した．その結果，60歳未満の男性では，呼気水素濃度と異物投与時の活性酸素種産生量に正の相関関係がみられた（$p<0.05$）が，60歳以上男性と女性ではそのような関係は認めなかった．以上より，抗酸化物質である水素は，好中球の産生する活性酸素種産生量の増加に対して，酸化ストレスに対する防御機構として反応的に増加した可能性が推測された．

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Introduction

It has been reported that reactive oxygen species (ROS) causes oxidative stress in the human body and is a contributory factor for ageing and lifestyle diseases[1]. It is also associated with exacerbation mechanisms as well as occurrence of cancer and cerebrovascular diseases[2,3]. However, the human body has an endogenous antioxidative mechanism, whereby the oxidative function of ROS can be suppressed and/or removed[4]. The body is therefore said to be under oxidative stress when the antioxidative function is insufficient to remove ROS that can harm the body tissues. Despite its importance, the relationship between the production mechanism of ROS and innate antioxidative function has yet been thoroughly elucidated.

In recent years, the function of hydrogen has been gaining attention as an antioxidative substance[5,6]. In 2007, Ohsawa et al.[7] have reported that inhalation of gas containing hydrogen in rats with cerebral infarction prevented the outspread of the infarct and that this was suggested to be due to the removal of one of the ROS family, the hydroradicals, from the focal site. In 2009, the same result was observed in a Parkinson disease mouse model through the same mechanism, suggesting the neuroprotective action of water with high hydrogen content[8].

Considering the reports above, Kajiya et al pointed out the importance of hydrogen to physiological function in the body, not only the effect of endogenous H_2, but especially levels of H_2 produced endogenously[9]. These authors reported that the condition of mice with drug-induced hepatitis worsened after introducing an antibacterial agent to remove H_2-producing intestinal flora. Also, it has been suggested that an α glucosidase inhibitor, which is one of the disaccharidases, reduces oxidative stress through increased production of intestinal H_2, suppressing cardiovascular events[10].

As mentioned above, the roles of hydrogen in the antioxidative mechanism include not only to the direct removal of ROS, but also the suppression of ROS production mechanisms[11]. One of those mechanisms can be explained by the H_2-mediated increase of the antioxidative enzyme, superoxide dismutase (SOD) thereby accelerating the deletion of superoxide, which is a primary substance produced by ROS[12]. Also, TNF-α activates NADPH oxidase, which is involved in the ROS production mechanism, however, hydrogen lowers NADPH oxidase concentrations, leading to an anti-inflammatory reaction[13,14]. Unfortunately, the relationship between hydrogen occurring within the human body and neutrophils, which produce ROS that in excessive quantities cause oxidative stress, has not yet been investigated.

Neutrophils are immune cells that destroy and sterilize foreign substances by engulfing them and then by producing ROS. It is believed that hyperactivity of neutrophil ROS production contribute to oxidative stress[15]. Thus, increase of ROS production in the normal state (hyperactivity) and overreaction against foreign substances (hyperreactivity) can lead to excess ROS production and induce tissue damage through oxidative stress[16]. On the other hand, decreased neutrophil reaction against foreign substances has been reported to cause increased susceptibility to infection[17].

The present study has investigated the relationship between the amount of exhaled H_2 and neutrophil ROS production among normal healthy members of the general population. The concentration of H_2 detected in exhaled breath has been reported to reflect the amount of total H_2 produced by intestinal flora, and thus was used as an index for endogenous H_2[18].

Subjects and Methods

1. Subject

Subjects were selected from members of the general population who had participated in "The Iwaki Health Promotion Project in 2007", which was held in the Iwaki area, Hirosaki-city in Aomori prefecture in northern Japan, for ten days from 28th May until 6th June, 2007. The purpose of this project is to maintain and to promote the health of local community in order to prevent lifestyle-related diseases and to prolong their lifespan. Subjects with diabetes mellitus (diagnosed by a medical doctor), malignant tumors, immune disorders or those who were pregnant at the time of the study, taking antimicrobial drugs, anticancer medication or hormones were excluded from the study, and a total of 656 subjects (252 males and 404 females) were finally enrolled.

2. Lifestyle habits and physical measurements

Self-reported questionnaires were sent to subjects prior to the investigation day and were collected after reviewing the answers during personal interviews on the day of the study. In the questionnaire, subjects were asked about their age, sex, present illnesses, past illnesses, medication histories, smoking habits (daily number of cigarettes), alcohol use (daily alcohol volume) and exercise habits (days of weekly exercise). Body mass index [BMI, weight (kg) /height $(cm)^2$] was calculated as an index of obesity.

3. Blood parameters

Blood samples were collected from peripheral veins of subjects under fasting conditions in the morning. Neutrophil counts were measured using an automated blood cell analyzer (SE9000; Sysmex, Kobe, Japan). Measurements of blood glucose and HbA1c levels were consigned to Mitsubishi Chemical Medience after serum was separated from whole blood by centrifugation. Blood glucose was measured using the IATORO LQ GLU® chemical reagent kit and a biochemistry autoanalyzer (H7700; Hitachi High-Technologies Corporation, Tokyo, Japan). HbA1c was measured using the Cin Q HbA1c® chemical reagent kit (JCA-BM9030; JEOL Ltd., Tokyo, Japan), according to the established methods adopted by the Japan Diabetes Society (JDS) as well as the National Glycohemoglobin Standardization Program (NGSP)[19].

4. Measurement method of neutrophil-related functions

Reactive oxygen species (ROS) generation and the phagocyte activity (PA) of peripheral blood neutrophils were determined with a FACScan system (Becton Dickinson, San Jose, CA) using two-color flow cytometry. Hydroethidine (HE; 44.4 μmol/L, Polyscience Inc., Warrington, PA) was used as an indicator for the ROS production capability, and opsonized zymosan (OZ) particles labelled with fluorescein isothiocyanate (FITC; Sigma Chemical Co., St. Louis, MO, USA) for assessment of PA. Zymosan was purchased from Sigma Chemical Co. (St. Louis, MO, USA).

Briefly, 100 μL heparinized whole blood was mixed with 22 μL HE (final concentration, f.c. 8 μmol/L) and incubated at 37°C for 5 min. After the addition of 25 μL FITC-labeled OZ (FITC-OZ; f.c. 5 mg/mL), the samples were incubated at 37°C for 35 min. Neutrophils labelled with only HE served as the control to measure nonstimulated neutrophil function, basal ROS production. After incubation, Lyse and Fix (IMMUNOTECH, Marseille, France) was added to lyse the erythrocytes and to fix the samples. The samples were washed twice in phosphate-buffered saline with sodium azide, and the fluorescence intensity (FI) in activated neutrophils was measured with the FACScan system (stimulated ROS production). 30 μL

Trypan blue (0.25 mg/mL, pH 4.5) was added just before the assay to differentiate between attached and ingested FITC-OZ by fluorescence quenching [20, 21].

FI was measured as the value of neutrophils per 10,000 screened with forward and side scattering light for each sample. The accumulated FI (cumulative FI, CFI) was calculated by multiplying the intensity and the rate of fluorescence-positive cells. The FI was used as a quantitative index of the function per one activated neutrophil. The CFI was used as a quantitative index of neutrophil function.

5. Exhaled hydrogen

The exhalation gases were obtained in the morning while subjects were still in the fasting state. The subjects were asked to breathe in lightly and held their breath for approximately 15 seconds. They then exhaled and between 100 and 200 ml of their final exhalation (expiratory reserve volume) was directed into a bag through a mouth-piece attached to the top of the breath bag (manufactured by Otsuka Pharmaceutical Co., Ltd., Tokyo, Japan)[22]. The inside of the bags were thoroughly washed in advance at 70℃ and left overnight, then washed again 3 times by inflating with pure synthetic air (G1 grade: impurity less than 0.1 ppm) which is free from volatile organic compounds to exclude some contaminated gases generated from the inner surface of the bags. Just before the measurement, the breath bag was warmed in an incubator to 45℃ for approximately 15 minutes. 1 ml of breath was taken from the breath bag with a special syringe and injected into a Biogas CO AnalyzerTM, BAS-1000 (designed and developed by Mitleben R&D Associates, Osaka, Japan), to analyze H_2 and CH_4 concentrations simultaneously. This apparatus is based on gas chromatography. All the analyses were performed on the day in which the exhalation gases were collected. The analysis device used was a gas chromatograph equipped with a high-sensitivity semiconductor gas detector. The lower limit for detection of gases was 0.1 ppm, and reproducibility was ± 2%. Pure synthetic air was used as a carrier gas and calibration was performed every 100 samples using mixed gas of 5 ppm concentration for the two gases.

As human body cells do not produce hydrogen, all H_2 detected in the breath is produced by intestinal flora[23], so that the exhaled hydrogen concentration we detected could reflect each subject's endogenous H_2 concentration.

6. Statistical analysis

Subjects were classified by gender and age into 4 groups: 60 y.o. or over and under 60 y.o. for each gender. A non paired-t-test was used to test for differences in the values of age, lifestyle, leukocyte/ neutrophil count and neutrophil functions. A multiple linear regression analysis was conducted to examine the linear effect of the H_2 level in expiratory gas on the neutrophil ROS production and phagocytic activity. Data were corrected for age, BMI, habits of smoking, drinking, physical exercise, and the presence of the menopause as the confounding factors.

Data analysis was performed with SPSS version 17.0 J for Windows (SPSS Inc., Chicago, IL, USA). The statistical differences were considered to be significant at $p < 0.05$, and to be marginally significant at $p < 0.1$.

7. Ethical consideration

Prior to the investigation, following points were explained to all participants and written consents were obtained: 1) the use of the documents obtained during the research would only be used for study purposes; 2) participants had a right to decline or withdraw from the project at any time; 3) confidentiality and anonymity of subjects would be protected; and

Table 1 Characteristics of subjects

	Male		Females	
	under 60 years (N=139)	60 years over (N=113)	under 60 years (N=198)	60 years over (N=206)
Age, years	47.7 ± 8.4	69.4 ± 5.7**	48.1 ± 9.2	67.9 ± 5.1**
BMI, kg/m^2	23.9 ± 2.7	23.7 ± 2.9	22.4 ± 3.1	23.3 ± 2.9
Exercise, time/week	1.5 ± 1.1	1.85 ± 1.4*	1.6 ± 1.2	1.9 ± 1.3*
Smoking, cigarettes/day	16.2 ± 17.9	16.9 ± 21.9	1.9 ± 5.3	0.3 ± 3.3**
Alcohol intake, g/day	51.5 ± 55.4	41.7 ± 52.3	9.1 ± 24.3	1.8 ± 8.7**
Menopause, number (%)	-	-	86(43.4%)	206(100%)

Date are expressed as mean ± standard deviation, compared with age: non-paired t-test
BMI, body mass index
* p<0.05, **p<0.01: compared with under 60 year

Table 2 Characteristics of subjects

	Male		Females	
	under 60 years	60 years over	under 60 years	60 years over
Exhaled hydrogen (H_2) concentration (ppm)	11.2 ± 12.6	8.8 ± 9.9	10.4 ± 12.4	11.3 ± 14.2
white blood cell count (/uL)	5727.4 ± 1723.2	5228.3 ± 1407.7*	4809.6 ± 1323.5	4722.8 ± 1269.9
Nutrophils cell counts (/uL)	3162.8 ± 1237.1	2947.7 ± 1077.7	2705.7 ± 963.1	2628.2 ± 10003.9
Basal ROS production, CFI	9308.6 ± 9384.8	12078.1 ± 11712.3*	11901.2 ± 11270.2	14919.2 ± 14973.7*
Stimulated ROS production, CFI	99253 ± 110744.4	116740.8 ± 112215.8	79133.5 ± 76939.8	91669.3 ± 89580.4
Phagocytic activity, CFI	164258.5 ± 126081.8	191326.9 ± 158882.9	169681.4 ± 117047.4	208215 ± 236567.9*

Date are expressed as mean ± standard deviation, compared with age: non-paired t-test
ROS: reactive oxygen species, CFI: cumulative fluorescence intensity
* p<0.05: compared with under 60 year

4) the storage of data would be properly and securely managed. The Iwaki Health Promotion Project and the present study were approved by the Ethics Committee of Hirosaki University Graduate School of Medicine.

Results

1. Subjects background (Tables 1 and 2)

For males, exercise frequency per week was greater in the 60 y.o. or over group than in the under 60 y.o. group (p<0.05). For females, exercise frequency was greater in 60 y.o. or over than in the under 60 y.o. group (p<0.05). Smoking prevalence and alcohol consumption were greater in the under 60 y.o. group than in the 60 y.o. or over group (both p<0.01). For females in the under 60 y.o. group, 43.4% had reached menopause and 100% were menopausal in the 60 y.o. or over group.

2. Exhaled hydrogen concentration and neutrophil function according to two age groups

There were no significant differences in H_2 levels in exhaled gas between the two age group for both genders (Table 2). For males, the basal ROS production was significantly greater in the 60 y.o. or over than that in the under 60 y.o. group (p<0.05). For females, the basal and phagocytic activity were significantly

Table 3 Multiple regression analysis with Expiration hydrogen (males)

	under 60 years			60 years over		
dependent variables	β-coefficient	P-value	R^2	β-coefficient	P-value	R^2
Exhaled hydrogen						
Basal ROS production, CFI	0.112	0.202	0.045	0.005	0.961	0.059
Stimulated ROS production, CFI	0.237	0.006	0.088	−0.081	0.426	0.033
Phagocytic activity, CFI	0.026	0.770	0.032	−0.023	0.820	0.018

Values are adjusted for age, body mass index, cigarette smoking, alcohol intake, exercise
ROS, reactive oxygen species; CFI, cumulative fluorescence intensity

Table 4 Multiple regression analysis with Expiration hydrogen (females)

	under 60 years			60 years over		
dependent variables	β-coefficient	P-value	R^2	β-coefficient	P-value	R^2
Exhaled hydrogen						
Basal ROS production, CFI	0.033	0.651	0.044	−0.060	0.397	0.018
Stimulated ROS production, CFI	−0.011	0.880	0.026	0.063	0.374	0.036
Phagocytic activity, CFI	−0.043	0.559	0.035	−0.025	0.721	0.039

Values are adjusted for age, body mass index, cigarette smoking, alcohol intake, exercise
ROS, reactive oxygen species; CFI, cumulative fluorescence intensity

greater in the 60 y.o. or over than that in the under 60 y.o. group (both $p<0.05$).

3. The association between exhaled hydrogen and neutrophil function (Tables 3 and 4)

In less 60 y.o. males, a significant positive correlation was seen between exhaled H_2 concentrations and stimulated ROS production ($p<0.05$) (Table 3). On the other hand, there was no correlation between exhaled H_2 concentrations and neutrophil function (Table 4).

Discussion

To the best of our knowledge, the present study is the first to investigate the relationship between exhaled breath concentrations of H_2 and neutrophil functions (ROS production quantities at normal state and when reacting against foreign substances) in subjects from the general population. In recent years, antioxidative features of H_2 have been reported to include not only the elimination of ROS which is the cause of oxidative stress, but also the suppression of ROS production [7].

According to the results obtained in this study, a positive correlation was observed between the quantity of ROS production in stimulated neutrophils and exhaled breath concentration of H_2 in male subjects under 60 years of age. This finding suggested that those with a higher amount of neutrophil-produced ROS in the circulation tend to have higher exhaled H_2 breath concentrations, whereas those with lower amounts of circulating neutrophil-produced ROS tended to have lower H_2 concentrations in exhaled breath. It has already been pointed out that excess production of ROS by neutrophils induces oxidative stress in the body[24, 25]. However, the human body has an endogenous antioxidative mechanism that reacts against ROS[4]. Thus, the amount of exhaled H_2, which is an antioxidative substance, as an index of H_2 in the body, had probably increased as

a response to increased production of ROS by neutrophils.

As H_2 is not produced by cells of the human body, all H_2 detected in the breath are produced by intestinal flora[23]. In recent years, the association between intestinal flora H_2 production by and the human immune system has been reported, including the H_2-mediated suppression of the inflammatory reaction, which reduces nitrogen monoxide (NO) produced from macrophages[26]. H_2 has also been reported to reduce the amount of ROS produced by lymphocytes[27]. Moreover, hydrogen water has been found to suppress the reduction of leukocytes caused by radiation[28]. However, no previous studies have reported on the association between neutrophil function and endogenous levels of H_2.

Several studies have reported on the association between intestinal flora and neutrophil functions. According to one such study, reduced functions of circulating neutrophils were observed in a mouse model whose intestinal flora had been removed by antibiotics[29]. Also, intestinal flora was found to activate the neutrophil foreign body response against Streptococcus pneumoniae and Staphylococcus aureus[30]. However, the underlying mechanisms have remained unclear. Thus, H_2 produced by intestinal flora was suggested to play certain role in the association mechanism between intestinal flora and neutrophils.

The fact that such an association was observed only in the subjects 60 years old and under in the present study, was suggested to be the effect of increased inflammatory cytokines with age. From the past research, hydrogen was found to suppress ROS production mechanism by reducing the concentration of inflammatory cytokines[31]. However, some inflammatory cytokines including TNF-α are known to increase with age[32]. Thus, no association between H_2 and neutrophil functions was observed in subjects over 60 years of age, which can be explained by the decreasing effect of H_2 against inflammatory cytokine concentration concomitant with ageing.

In females, no significant associations were observed between exhaled breath concentration of H_2 and neutrophil functions. In previous studies a gender-related difference in immune function has been reported: females tended to have a higher immune function[33] and higher neutrophil function[34] than males. Also, female hormones are known to maintain various aspects of the immune function[35]. Thus, unlike the case in males, no significant association was observed in females as the effect of H_2 against neutrophil function may have been smaller than the effect of female hormones on neutrophil function.

References

1) Rains JL, Jain SK. Oxidative stress, insulin signaling, and diabetes. Free Radic Biol Med 2011;50:567-75.

2) Weinberg F, Chandel NS. Reactive oxygen species-dependent signaling regulates cancer. Cell Mol Life Sci 2009;66:3663-73.

3) Finkel T. Holbrook NJ. Oxidant oxidative stress and the biology of ageing. Nature 2000;408:239-47.

4) Yoshikawa T, Naito Y, Kondo M. Free radical involvement in the aging process. Neurosciences 1990;16:603-12.

5) Ohta S. Recent Progress Toward Hydrogen Medicine: Potential of Molecular Hydrogen for Preventive and Therapeutic Applications. Curr Pharm Des 2011;17:2241-52.

6) Dixon BJ, Tang J, Zhang JH. The evolution of molecular hydrogen: a noteworthy potential therapy with clinical significance. Med Gas Res 2013;3:10.

7) Ohsawa I, Ishikawa M, Takahashi K, Watanabe M, Nishimaki K, Yamagata K, Katsura K, et al. Hydrogen acts a therapeutic antioxidant by

selectively reducing cytotoxic oxygen radicals. Nat Med 2007;13:688-94.

8) Fujita K, Seike T, Yutsudo N, Ohno M, Yamada H, Yamaguchi H, Sakumi K, et al. Hydrogen in drinking water reduces dopaminergic neuronal loss in the 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine mouse model of Parkinson's disease. PLoS One 2009;4:e7247.

9) Kajiya M, Sato K, Silva MJ, Ouhara K, Do PM, Shanmugam KT, Kawai T. Hydrogen from intestinal bacteria is protective fur Concanavalin A-induced hepatitis. Biochem Biophys Res Commun 2009;386:316-21.

10) Suzuki Y, Sano M, Hayashida K, Ohsawa I, Ohta S, Fukuda K. Are the effects of alpha-glicosidase inhibitors on cardiovascular events related to elevated levels of hydrogen gas in the gastrointestinal tract? FEBS Lett 2009;583:2157-9.

11) Itoh T, Fujita Y, Ito M, Masuda A, Ohno K, Ichihara M, Kojima T. et al. Molecular hydrogen suppresses FcepsilonRI-mediated signal transduction and prevents degranulation of mast cells. Biochem Biophys Res Commun 2009;389:651-6.

12) Xie K, Yu Y, Pei Y, Hou L, Chen S, Xiong L, Wang G. Protective effects of hydrogen gas on murine polymicrobial sepsis via reducing oxidative stress and HMGB1 release. Shock 2010;34:90-7.

13) Gharib B, Hanna S, Abdallahi OM, Lepidi H, Gardette B, De Reggi M. Anti-inflammatory properties of molecular hydrogen: investigation on parasite-induced liver inflammation. C R Acad Sci III 2001;324:719-24.

14) Xie K, Yu Y, Zhang Z, Liu W, Pei Y, Xiong L, Hou L, Wang G. Hydrogen gas improves survival rate and organ damage in zymosan-induced generalized inflammation model. Shock 2010;34:495-501.

15) Smith JA. Neutrophils, host defense, and inflammation: a double-edged sword. J Leukoc Biol 1994;56:672-86.

16) Peake J, Suzuki K. Neutrophil activation, antioxidant supplements and exercise-induced oxidative stress. Exerc Immunol Rev 2004;10:129-41.

17) Weiss SJ. Tissue destruction by neutrophils. N Engl J Med 1989;320:365-76.

18) Levitt MD. Production and excretion of hydrogen gas in man. N Engl J Med 1969;281:122-7.

19) Kashiwagi A, Kasuga M, Araki E, Oka Y, Hanafusa T, Ito H, Tominaga M, et al. International clinical harmonization of glycated hemoglobin in Japan: From Japan Diabetes Society to National Glycohemoglobin Standardization Program values. J Diabetes Investig 2012;3:39-40.

20) Hed J. The extinction of fluorescence by crystal violet and its use to differentiate between attached and ingested micro-organisms in phagocytosis. FEBS Lett 1977;1:357-61.

21) Sahlin S, Hed J, Rundquist I. Differentiation between attached and ingested immune complexes by a fluorescence quenching cytofluorometric assay. J Immunol Methods 1983;60:115-24.

22) Nitta H, Kinoyama M, Watanabe A, Fujita Y, Ueda H, Shirao K. Measurement of carbon monoxide in exhaled breath as a possible marker of stress. J Health Sci 2007;53:132-6.

23) Levitt MD. Production and excretion of hydrogen gas in man. N Engl J Med. 1969;281:122-7

24) Pyne DB. Exercise-induced muscle damage and inflammation: a review. Aust J Sci Med Sport 1994;26:49-58.

25) Duarte JA, Appell HJ, Carvalho F, Bastos ML, Soares JM. Endothelium-derived oxidative stress may contribute to exercise-induced muscle damage. Int J Sports Med 1993;14:440-3.

26) Itoh T, Hamada N, Terazawa R, Ito M, Ohno K, Ichihara M, Nozawa Y, et al. Molecular hydrogen inhibits lipopolysaccharide/interferon γ-induced nitric oxide production through modulation of signal transduction in macrophages. Biochem Biophys Res Commun 2011;411:143-9.

27) Yang Y, Gao F, Zhang H, Hunag Y, Zhang P, Liu C, Li B, et al. Molecular hydrogen protects human lymphocyte AHH-1 cells against 12C6+ heavy ion radiation. Int J Radiat Biol. 2013. [Epub ahead of print]

28) Yang Y, Li B, Liu C, Chuai Y, Lei J, Gao F, Cui J, et al. Hydrogen-rich saline protects immunocytes

from radiation-induced apoptosis. Med Sci Monit 2012; 18: 144-8.

29) Karmarkar D, Rock KL. Microbiota signaling through MyD88 is necessary for a systemic neutrophilic inflammatory response. Immunology 2013;140:483-92.

30) Clarke TB, Davis KM, Lysenko ES, Zhou AY, Yu Y, Weiser JN. Recognition of peptidoglycan from the microbiota by Nod1 enhances systemic innate immunity. Nat Med 2010;16:228-31.

31) Stuck AE, Walthert JM, Nikolaus T, Büla CJ, Hohmann C, Beck JC. Risk factors for functional status decline in community-living elderly people: a systematic literature review. Soc Sci Med 1999;48:445-69.

32) Burger D, Dayer JM. Cytokines, acute-phase proteins, and hormons: IL-1 and TNF-alpha production in contact-mediated activation of monocytes by T lymphocytes. Ann N Y Acad Sci 2002;966:464-73.

33) Cannon JG, St Pierre BA. Gender differences in host defense mechanisms. J Psychiatr Res 1997; 31:99-113.

34) Skafar DF, Xu R, Morales J, Ram J, Sowers JR. Clinical review 91: Female sex hormones and cardiovascular disease in women. J Clin Endocrinol Metab 1997;82:3913-8.

35) Molloy EJ, O'Neill AJ, Grantham JJ, Sheridan-Pereira M, Fitzpatrick JM, Webb DW, Watson RW. Sex-specific alteration in neutrophil apoptosis: the role of estradiol and progesterone. Blood 2003;102:2653-9.