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Isolation of Saponin-free Fraction from Ginseng (*Panax ginseng* C.A. Meyer) and its Effects on the Function of Neutrophils

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Abstract—A method of isolation of bioactive water-soluble saponin-free subfraction (RG-ws-1-1-1) from Korean red ginseng using solvent extraction, ultrafiltration and chromatography on Diaion HP-20 resin was developed. To evaluate the potency of RG-ws-1-1-1 to effect on the heat stress (HS) response and anti-inflammatory response in neutrophils, the oxygen consumption (OC) and formation of superoxide radicals (FSR) were assayed in suspensions of neutrophils from mice. Supplementation of RG-ws-1-1-1 to mice *in vivo* (200 mg/kg, 14 days intraperitoneally) results in the increase OC by neutrophils assayed *ex vivo* at 37 °C. OC and FSR by neutrophils were suppressed when cells obtained from the control animals were incubated at 42 °C. Preincubation of neutrophils with RG-ws-1-1-1 reduces HS related depression of FSR. Supplementation of RG-ws-1-1-1 to mice *in vivo* decreases HS related suppression of OC. RG-ws-1-1-1 subfraction significantly improves an oxygen-dependent anti-microbial function of neutrophils, also studied under the HS conditions.

Key words: Chromatographic Method, Ultrafiltration, Ginseng, Neutrophils, Heat Stress

INTRODUCTION

There is a substantial body of evidence which suggests that *Panax ginseng* C.A. Meyer enhances the host resistance against infection [Soldati, 1988; Song et al., 1998; Tomoda et al., 1993]. On the other hand, *Panax ginseng* was found to possess strong anti-stress properties [Kang et al., 1995; Yoshimatsu et al., 1993]. Main active ingredients of *Panax ginseng* are known to be ginseng saponins - ginsenosides [Bridges et al., 1988; Kang et al., 1995; Yoshimatsu et al., 1993; Kim et al., 1998; Park et al., 1999]. However, the diverse effects of *Panax ginseng* do not originate solely from the actions of saponins. The investigations of the isolated ginsenosides and non-saponin individual compounds revealed that both provide some synergistic effects [Park et al., 1999]. A number of methods have been developed for the isolation of biologically active fractions and individual compounds from red ginseng, which possessed anti-stress properties. Most of them were concerned with ginsenosides or ginsenoside-containing fractions [Bridges et al., 1988; Kang et al., 1995]. But in experiments searching for protection against radiation injury supplementing a powder or different extracts from ginseng strongly suggested the significance of non-saponin compounds [Chen et al., 1998].

To evaluate the anti-HS activity of bioactive substances, a number modeling systems (monitoring of selected physiological parameters that are a reflection of the HS related degree of physiological strain such as heart rate, skin temperature, various biochemical parameters) were reported. Each of them has some advantages and imperfections [Emel'yanova et al., 1996]. We proposed to use the functional model system, namely murine neutrophils with regard to their physiological functions. This cell system is quite suitable to evaluate anti-inflammatory and anti-heat stress effects of Korean red ginseng (KRG) supplemented either *in vivo* or *in vitro*.

In fact neutrophils are responsible for defense against pathogens. Neutrophils are the primary cells that are involved in the killing of bacteria via an oxygen-dependent anti-microbial system, that is able to form strong oxidants - reactive oxygen species including superoxide ion (O_2^-), hydrogen peroxide (H_2O_2), hypochlorous acid (HOCl) and hydroxyl radical ($OH^\cdot$). When neutrophils are exposed to microorganisms, an increase in the rate of O_2 consumption (the respiratory burst) is induced. An increased formation of reactive oxygen species is the consequence and strong oxidants like hypochlorous acid and H_2O_2 are released into the intracellular space of neutrophils [Badwey and Karnovsky, 1980]. On the other hand, the exposure of neutrophils in suspensions as well as animals to elevated temperatures results in the heat stress response (HSR) in cells [Borman et al., 1998]. In particular, during the HS a depression in the physiological functions of phagocytes is observed, including a decrease in consumption of oxygen and in the rate of formation of superoxide radicals after physiologically relevant stimulations [Clerget et al., 1990; Polla, 1991; Polla and Cossarizza, 1996; Polla et al., 1998]. The level of the bactericidal activity of neutrophils can be controlled by quantifying the rate of formation of the predominantly selected agent (H_2O_2 or, O_2^- or $OH^\cdot$). The control of the bactericidal activity of neutrophils can be also done by measuring the integral parameter of the metabolism, the variation of O_2 consumption by neutrophils. In our study we have measured the kinetics of O_2 consumption and formation of superoxide radicals in suspensions of neutrophils under comfort conditions (37 °C) as well as under heat

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stress conditions.

The specific nature of our model system originates from fact that the bactericidal activity of neutrophils is controlled at least by the induction of elevated formation of oxidants and by the induction of heat stress proteins (HSP) that are vital for the mechanism of protection of host cells (neutrophils) against the elevated formation of oxidants released by neutrophils themselves during the respiratory burst [Polla et al., 1998]. To simplify the system under the study we can suppose that the bactericidal activity of neutrophils is a function of two mutually dependent factors.

The exposure of neutrophils to an HS breaks the optimal mode of metabolism in neutrophils inducing another relation between two mutually dependent factors (the induction of formation of strong oxidants and HSR). An oxygen-dependent anti-microbial system of neutrophils activates the defense mechanisms to find a new and optimal mode of operation in order to provide sufficient physiological activity under HS conditions. Since ginseng possesses both anti-stress, and anti-inflammatory properties, we hypothesized that a supplementation of fractions of ginseng to animals *in vivo* will affect both factors and may provide an increase in the bactericidal activity of neutrophils assayed *ex vivo* under HS conditions. We suggest that ginseng influences the mentioned processes via involving the different kinds of substances. At the same time the physiological processes we wanted to study are known to be closely interrelated. So both of them were the subjects of our study. Therefore, it was reasonable to test the biological effects of the multi-compound fractions rather than of the individual compounds obtained from ginseng. The main attention in this case was primary focused on polypeptides and polysaccharides.

The present study was aimed at elaborating a method of isolation of a water soluble non-saponin subfraction (RG-ws-1-1-1) from KRG and to test *in vivo* and *in vitro* the effects of this subfraction upon the activation of respiratory burst and formation of superoxide radicals by neutrophils *ex vivo* under comfortable conditions (37 °C) and under the HS conditions. We have elaborated the convenient methods for the isolation of a water-soluble non-saponin subfraction (RG-ws-1-1-1) from KRG. We have also shown that RG-ws-1-1-1 subfraction enhances an oxygen-dependent anti-microbial function of neutrophils under comfortable conditions as well as under heat stress conditions. This effect is important due to intensive searching for new, effective therapeutic interventions designed to improve the heat stress-induced decrease of the resistance of humans and animals to the infections.

MATERIALS AND METHODS

1. Chemicals

All chemicals were reagent grade and purchased from Sigma Chemical Co., USA unless otherwise noted.

2. Ginseng Sample

Six years old fresh *Panax ginseng* C.A. Meyer was steamed and sun-dried to process it into KRG. KRG roots were cut to mill. Ginseng powder from inside root part (xylemy+pith) was used for the extraction.

3. Extraction of Ginseng Powder and Extract Fractionation

Ginseng root powder was mixed with water followed by stirring during 24 hours at 4 °C. Extracted mixture was centrifuged at 22,000 g during 30 min at 4 °C, then supernatant was centrifuged at 37,000 g during 30 min at 4 °C. The procedure was twice repeated to achieve the optically transparent solution. The ultrafiltration procedure (Amicon type ultrafiltration cell, model L8400, USA) through filters of PM 10 type with a molecular weight cut-off >10,000 were used for the fractionation of the crude extract. Filtrate containing the compounds with molecular weight<10,000 was concentrated under vacuum.

For a crude saponin extraction we applied the method of adsorption on a Diaion HP-20 resin (Mitsubishi Kasei, Japan) as described elsewhere [Kim et al., 1998]. Resin was washed with 4 volumes of water, 4 volumes of methanol and finally with 4 volumes of water to eliminate excess of methanol. Concentrated filtrate was applied to a chromatographic column (4.5 cm×50 cm) preloaded with Dianion HP-20 resin. The column was washed consecutively with the following solvents: water (RG-ws-1-1-1 subfraction), 25% methanol (RG-ws-1-1-2 subfraction) and 95% methanol (RG-ws-1-1-3 subfraction). Finally, each subfraction eluted was concentrated under vacuum and lyophilized. The summary of all procedures used during extraction is depicted in Fig. 1.

4. Chemical Compositions

For determination of saponins in the subfractions an analytical thin layer chromatography (TLC) method was employed (TLC aluminium sheets 20 cm×20 cm, silica gel 60 F_{254}, «E.Merck," Germany). Developing solvent used was a chloroform-methanol-water

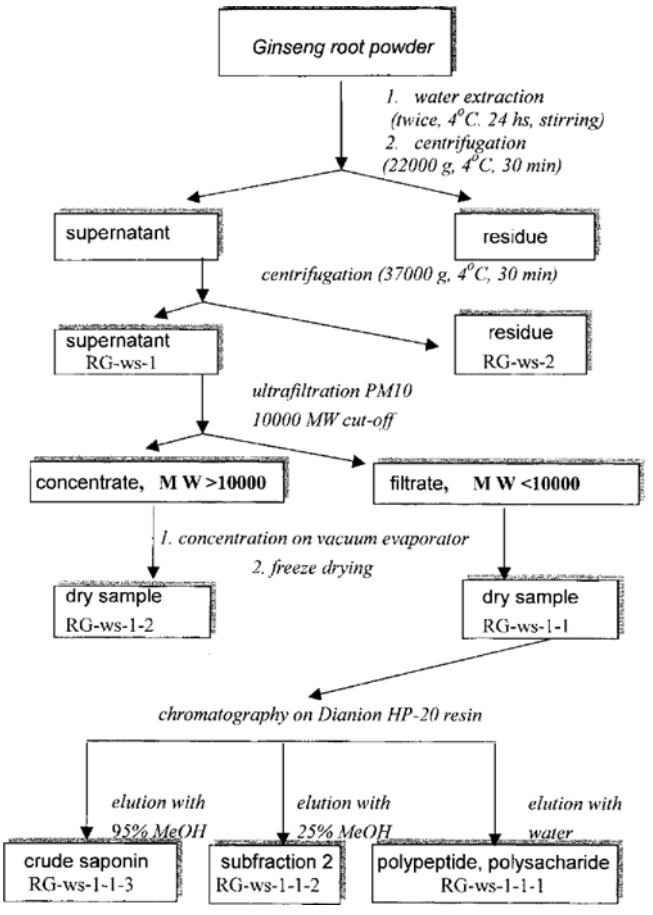

Fig. 1. Isolation of bioactive crude saponin and saponin-free subfractions from ginseng root powder.

mixture (65 : 35 : 10, v/v), the lower phase of a mixture. A spray reagent was a Liebermann-Buchard reagent. The sprayed plate was warmed up to 100 °C for 5-10 min to observe the TLC profiles of the fractions studied.

Phenol-sulfuric acid assay was used for total sugar content determination [Chaplin and Kennedy, 1986]. A protein assay based on the method of Bradford [Bradford, 1976] was used to determine the concentration of water-soluble proteins. The dye reagent concentrate, containing Coomassie Brilliant Blue G-250 dye, phosphoric acid and methanol was purchased in a kit with bovine serum albumin (kit II, catalog No. 500-002, Bio-Rad, USA).

5. Animals and Experimental Design

Mice of strain C57BL/6, female 7 weeks of age were used for experiments [Larina et al., 1985]. For each experiment mice were uniformed to be within 3 g of weight. The mice from control (C) group and RG-ws-1-1-1 subfraction-treated group (R) were injected intraperitoneally (i.p.) with a 0.9% sodium chloride solution (placebo) or with the substances studied, respectively. Subfraction RG-ws-1-1-1 was dissolved in a 0.9% sodium chloride solution at the concentration of 14.7 mg/ml. The doses applied during i.p. injection were 200 mg/kg body weight. All the mice groups were treated with RG-ws-1-1-1 or placebo (0.9% NaCl) and injected daily during two weeks. The final concentration of subfraction used *in vitro* experiments with suspensions of neutrophils was 1 mg/ml in all the experiments.

6. Intraperitoneal Elicitation

Mice were injected i.p. with 2.5 ml of 4% fluid thioglycollate medium. The medium used was autoclaved for 15 min under the pressure of 15 psi, 121 °C. Peritoneal exudates, containing neutrophils as a major fraction of cells, were collected 5 hours after the medium was injected. [Ivanova et al., 1988; Vishell and Shiigi, 1993].

7. Peritoneal Exudate Cell Collection

Mice were sacrificed by cervical dislocation. 5 ml of ice-cold HEPES-buffered medium No. 199 (17 mM HEPES-buffer, pH 7.4) supplemented with a 5% (v/v) heat-inactivated fetal calf serum and heparin (10 USP U/ml) was injected into the peritoneal cavity of a mouse. The cells suspension obtained was sedimented by centrifugation at 100 g for 5 min, cells were washed with this elicitation medium and resuspended in the same medium. The peritoneal exudate contained neutrophils and also lymphocytes and macrophages. All results were normalized to the content of neutrophils in the exudate used.

8. Zymosan Suspension

Opsonized zymosan was freshly prepared before every experiment. Zymosan particles (Sigma, Germany) were incubated with autologous serum in a concentration of 20 mg/ml at 37 °C for 60 min. It was sedimented by centrifugation (10,000 g for 10 min), washed once with phosphate-buffered saline by centrifugation and adjusted to 7×10^8 particles per ml in Hanks balanced salt solution.

9. Oxygen Consumption

To study the kinetics of oxygen consumption by cells, the polarography experimental technique with a Clark type oxygen electrode (model Gilson 5/6H, France) was used. The oxygen concentration in suspensions of cells was monitored at temperatures 37 °C, 40 °C and 42 °C. The calibration of oxygen concentration was provided in the assumption that 1 ml of distilled water contained 217 nmol of molecular oxygen at 37 °C. Neutrophils were injected and incubated in electrochemical cell. The final concentration of neutrophils was adjusted to be 6.72×10^6 per ml. The respiratory burst was induced by serum-treated zymosan particles as a stimulus.

10. Exposure of Neutrophils to Various Temperatures

To measure the influence of the heat stress upon the respiratory burst, neutrophils were incubated in electrochemical cell. Experiments were performed at various temperatures including 37 °C, 40 °C and 42 °C. Incubation of neutrophils at the fixed temperature was done for 13 min. Then the stimulation of neutrophils by zymosan particles was initiated.

11. Superoxide Anions Determination

The generation of O_2^- in the extra cellular space in the suspensions of neutrophils was determined by measuring the stoichiometric reduction of cytochrome *c* by superoxide radicals. Measurements were carried out 15 min after the stimulation of neutrophils with phorbol 12-myristate 13-acetate (PMA, 100 ng/ml) was done as described elsewhere [Rose, 1992]. For these experiments all reagents and cells were equilibrated at the proper temperature before mixing them up. The reaction took place in test tubes incubated in a water bath at 37 °C, 40 °C and 42 °C. The reaction was stopped after 15 minutes by placing on ice the test tubes containing neutrophils. The supernatants were removed, and the amount of reduced cytochrome *c* was determined by measuring the optical density of probes at the wavelength 550 nm. Formation of O_2^- was calculated by using the extinction coefficient of the reduced cytochrome c E (μM)= 0.0211 [Rose, 1992]. The rate of O_2^- formation was calculated by using the linear part of the calibration curve and is expressed as nmol O_2^-/min/10^6 cells.

12. Statistical Analysis

Data were obtained from three independent experiments (neutrophils were obtained from 5 mice for each group of cells) and expressed as mean±SD. Statistical analysis was carried out by using Student's t-test. Values of $p<0.05$ were considered as significant.

RESULTS AND DISCUSSION

1. Isolation of Bioactive Saponin-Free Subfraction

Several solvents and buffers are used for isolation bioactive compounds from red ginseng. In our study water extraction was applied due to its efficiency and food safety. Taking into account a significant difference in the bioactivity of a low and high molecular weight compounds obtained, the ultrafiltration through filters with >10,000 MW cut-off was used on first step of fractionation of crude extract. Preliminary tests of consumption of oxygen by neutrophils have shown a high activity of the low molecular weight fraction RG-ws-1-1 that was the object studied here. TLC profiles of RG-ws-1-1 have shown that this fraction contains saponins as one of main components. The biological activity of ginseng's saponins is well known [Bridges et al., 1988; Kang et al., 1995; Yoshimatsu et al., 1993; Kim et al., 1998; Park et al., 1999]. To study the bioactivity of other compounds it is important to remove saponins completely from the RG-ws-1-1 fraction. Usually, an extraction with butanol is used for this purpose, but this procedure is not highly efficient and sometimes it is difficult to remove the residual amount of butanol from the extract. To exclude *in vivo* the effect of butanol as a toxic substance that usually appears when the conventional solvent phase separation method is used for crude saponins extraction, we

Table 1. *In vivo* RG-ws-1-1-1 effects on number of neutrophils recovered in peritoneal exudates per mouse and on percentage of total peritoneal exudate cells

Test solutions	Number of neutrophils per mouse ($\times 10^6$)	Percentage of total peritoneal exudate cells		
		Neutrophils	Macrophages	Lymphocytes
0.9% NaCl	37.2±2.0	84±3	3±1	13±3
RG-ws-1-1-1 in 0.9% NaCl	39.4±3.2	85±2	4±1	11±2

had to apply adsorption on a Diaion HP-20 resin. Column chromatography using a Dianion HP-20 resin has a high efficiency and is a convenient method able to separate a water-soluble subfraction (RG-ws-1-1-1) from saponins that were eluted only using a 95% methanol as a solvent (subfraction RG-ws-1-1-3). TLC profiles have indicated that only last subfraction eluted contains saponins. So RG-ws-1-1-1 appeared to be a saponin-free subfraction. Meantime in RG-ws-1-1-1 subfraction the main components detected appeared to be polypeptides and polysaccharides.

2. Peritoneal Exudates

The number of neutrophils per mouse and the percentage of neutrophils, macrophages and lymphocytes present in the peritoneal exudates collected after the time provided to obtain a high yield of neutrophils (5 h) are depicted in Table 1.

Experimental results indicated that the number of neutrophils recovered from the C group of mice, 3.7×10^7 neutrophils/mouse, did not differ significantly from the number of neutrophils recovered from mice treated with an RG-ws-1-1-1 subfraction (3.9×10^7 neutrophils/mouse). Cells' viability, as assayed using a trypan blue exclusion test, was about 98%.

3. Zymosan Stimulated O_2 Consumption by Neutrophils

These experiments were designed to evaluate the physiological activity of neutrophils recovered from murine peritoneal exudate. It was shown that the O_2 consumption by the resting cells is not changed during 12 hours. Here we established under what conditions zymosan would activate optimal respiratory burst of neutrophils assayed *ex vivo* to study *in vivo* and *in vitro* the effects of fractions of ginseng and the effects induced by HS in neutrophils. Neutrophils were incubated for 13 min in the chamber of oxygen electrode (electrochemical cell), and were stimulated by opsonized zymosan particles. A strong effect was observed for zymosan particles to cells ratio to be around 25. The relative increase in consumption of oxygen (a metabolic burst, assayed by ratio V_{ph}/V_0) reached the value of around 9.2, which indicates that functional activity of neutrophils was extremely high. Therefore we reduced the level of the metabolic burst to the optimal level (V_{ph}/V_0~4.4) changing the zymosan particles cells ratio to 15. So the respiratory burst was activated by zymosan with particles to cells ratio 15 in all our subsequent experiments.

The experimental results indicate that the harvesting of neutrophils from peritoneal cavities of C 57BL/6 mice strain (induction time 5 hours, inductor - 4% fluid thioglycolate medium) results in reproducible and high yield of neutrophils, that have high functional activity.

4. *In vivo* Effect of RG-ws-1-1-1 Subfraction on the O_2 Consumption by Neutrophils

During phagocytosis of serum-opsonized zymosan the O_2 consumption by neutrophils isolated from mice injected with RG-ws-1-1-1 subfraction increased 6.1-fold (Table 3). The respiration of neutrophils from C group of mice increased only 4.4-fold (Table 2). The effect of RG-ws-1-1-1 subfraction resulted in the increase of O_2 consumption by neutrophils isolated from the R group of mice compared to the O_2 consumption by neutrophils obtained from the C group of mice. The O_2 consumption by resting (non-stimulated) cells from both group of mice did not differ significantly (Tables 2, 3). The maximal rates of O_2 consumption by neutrophils from mice of both C and R groups were reached within 4 min after the stimulation. The rates of O_2 uptake by neutrophils from C and R groups of mice were decreased to zero during 10 min and 13 min, respectively. These results led us to the conclusion that supplementation of RG-ws-1-1-1 subfraction to mice *in vivo* increases the bactericidal activity of neutrophils as assayed *ex vivo*.

Table 2. The effects of exposure of neutrophils from control group of mice to heat stress conditions upon the oxygen consumption by zymosan-stimulated neutrophils assayed *ex vivo*

Temperature of incubation of neutrophils	Parameters of oxygen consumption		
	V_0	V_{ph}	V_{ph}/V_0
37 °C	0.12±0.03	0.52±0.12	4.42
40 °C	0.16±0.05*	0.81±0.27*	5.06*
42 °C	0.14±0.04*	0.27± 0.03*	1.91*

V_0 - the rate of oxygen consumption by resting neutrophils, V_{ph} - the rate of oxygen consumption by zymosan-stimulated neutrophils, V_0 and V_{ph} are expressed in nmol/min of oxygen per 10^6 neutrophils.

Table 3. Effect of supplementation of RG-ws-1-1-1 subfraction to mice *in vivo* upon the oxygen consumption by neutrophils assayed *ex vivo* at different temperatures

Temperature of preincubation of neutrophils	Parameters of oxygen consumption		
	V_0	V_{ph}	V_{ph}/V_0
37 °C	0.17±0.05*	1.01±0.11*	6.13*
40 °C	0.17±0.06*	1.17±0.21*	6.91*
42 °C	0.21±0.07*	1.01±0.32*	5.21*

V_0 - the rate of oxygen consumption by resting neutrophils; V_{ph} - the rate of oxygen consumption by zymosan-stimulated neutrophils; V_0 and V_{ph} are expressed in nmol/min of oxygen per 10^6 neutrophils. *$p<0.05$ in comparison with control.

5. *In vivo* Effect of RG-ws-1-1-1 Subfraction on O_2 Consumption by Neutrophils Assayed Under Heat Stress Conditions

The neutrophils from C and R mice groups were incubated in a thermostabilized chamber of a Clark electrode for 13 min at 40°C and 42 °C. Exposure of neutrophils from both groups to the tem-

perature of 40 °C causes an increase in O_2 consumption by neutrophils from C group (a 20% increase) and practically the same increase in the rate of O_2 uptake by neutrophils obtained from R group (a 15% increase, Tables 2, 3). It is known that a low degree of hyperthermia could enhance the leukocyte function.

Exposure of neutrophils to a temperature of 42 °C causes a decrease in O_2 uptake by neutrophils obtained from the mice of C group (down to 48%) as compared to O_2 uptake by neutrophils from the same group of animals measured at 37 °C. Actually no influence of HS was observed on the rate of the O_2 uptake by resting cells obtained either from the control group of animals or RG-ws-1-1-1 treated animals when the neutrophils were incubated at 42 °C (Table 2). RG-ws-1-1-1 supplementation to mice *in vivo* causes a decrease in HSR, and the respiratory burst was only about 19% lower compared to the respiratory burst measured at 37 °C (Table 3). But the maximum rate of O_2 uptake by the cells was reached rather quickly (within 1.3 min after stimulation of neutrophils) and then rate of O_2 consumption decreased rapidly to a zero level just within 7 min.

6. *In vitro* Effect of the RG-ws-1-1-1 Subfraction on O_2^- Production by Neutrophils Assayed Under Heat Stress

In neutrophils obtained from the C group of animals and subjected to HS (42 °C) a decrease in O_2^- production during zymosan-induced phagocytosis was observed in neutrophils preincubated with RG-ws-1-1-1 subfraction as well as in neutrophils non treated *ex vivo* by RG-ws-1-1-1 subfraction (Table 4). In neutrophils non-treated with RG-ws-1-1-1 subfraction HS induced a 60% decrease in formation of superoxide radicals assayed after the stimulation of neutrophils by PMA. In neutrophils preincubated with RG-ws-1-1-1 subfraction the rate of formation of superoxide radicals by neutrophils during the phagocytosis under HS conditions was diminished only by 23% compared to the rate of formation of superoxide radicals assayed in neutrophils kept at the temperature of 37 °C (Table 4).

Our results confirm that HS in neutrophils is associated with inhibition of NADPH oxidase activity, an enzyme involved in the respiratory burst [Maridonneau-Parini et al., 1988]. HS depresses physiological functions of phagocytes, such as respiratory burst and O_2^- formation (Tables 2, 4). In fact, one of the primary events associated with development of the respiratory burst is an increased formation of superoxide anions due to the elevated activity of NADPH oxidase(s). Subsequent dismutation of superoxide anion lead to the elevated formation of H_2O_2, a substrate of the myeloperoxidase that forms HOCl, a strong oxidant able to form OH radicals in reaction with superoxide radicals in the intracellular space [Polla and Cossarizza, 1996]. The RG-ws-1-1-1 fraction is affecting the equilibrium responsible for the level of NADPH-oxidase activity that is the essential contributor to the overall formation of superoxide radicals in the extra cellular space in suspensions of neutrophils. This interpretation of our experimental results depicted in Tables 2, 3, 4 is in line with the data reported in [Polla and Cossarizza, 1996; Polla et al., 1998]. Our experimental data have shown that saponin-free RG-ws-1-1-1 subfraction of ginseng decreases HSR in neutrophils with regard to their physiological functions: O_2 consumption and formation of superoxide anions.

Table 4. *In vitro* effects of RG-ws-1-1-1 subfraction on the rate of formation of superoxide anions by PMA-stimulated neutrophils assayed under *ex vivo* conditions at the temperatures 37 °C and 42 °C. *p<0.05 in comparison with control

Solutions using for preincubation	O_2^- production (nmol/10^7 cells per 15 min)	
	Heat stress (42 °C)	Normal conditions (37 °C)
0.9% NaCl	33.1±3.7	83.3±9.7
RG-ws-1-1-1 in 0.9% NaCl	47.9±5.7*	62.3±7.5*

At the same time supplementation of RG-ws-1-1-1 subfraction to the animals *in vivo* significantly improved the bactericidal activity of neutrophils studied in *ex vivo* at 37 °C. In fact the zymosan-stimulated increase in O_2 consumption by neutrophils obtained from mice treated with RG-ws-1-1-1 subfraction increased 6.1-fold (Table 3). At the same time the zymosan-stimulated increase in respiration of neutrophils obtained from C group of mice increased only in 4.4-fold (Table 2), so the efficacy of the enzymatic system(s) involved in the formation of superoxide anions improved significantly. Although we do not know the details of the mechanisms involved in the influence of HSR upon the capacity of neutrophils to be activated, our data suggest that supplementation of RG-ws-1-1-1 subfraction causes significant protection of the enzymatic system of neutrophils that is partly inhibited by the HS. However, the interpretation of our experimental results is a bit complicated by the up and down regulation of the phagocyte function caused by heat stress itself [Kantengwa et al., 1995; Polla, 1991; Polla and Cossarizza, 1996]. During the infection the toxic oxidants released by activated neutrophils provide *in vivo* protection against pathogens [Badwey and Karnovsky, 1980]. On the other hand, the activation of neutrophils induces an oxidative stress that is able to damage the neutrophils themselves. It was found that toxic oxidants such as hydrogen peroxide (H_2O_2) and hydroxyl radicals ($OH^\cdot$) activate the heat stress response in neutrophils, inducing the HSP synthesis, in order to protect the cells against phagocytosis-related oxidative stress [Song et al., 1998; Polla, 1991; Polla and Cossarizza, 1996]. It seems that the same mechanisms are involved in protection against the partial inactivation of the bactericidal activity of neutrophils both by oxidative stress and heat stress. This interpretation is in line with the data reported elsewhere [Kantengwa et al., 1995; Polla, 1991; Polla et al., 1998].

CONCLUSIONS

1. A method of isolation of bioactive water soluble saponin-free subfraction (RG-ws-1-1-1) and crude saponin subfraction from Korean red ginseng was developed. It is based on solvent extraction, ultrafiltration and chromatography using Diaion HP-20 resin.

2. Supplementation of RG-ws-1-1-1 subfraction to mice *in vivo* results in the increase of O_2 uptake by stimulated neutrophils as assayed *ex vivo* at 37 °C (for 39%). So RG-ws-1-1-1 subfraction can be considered as a stimulator of the oxygen-dependent anti-microbial system in mammals.

3. *In vitro* the effect of RG-ws-1-1-1 subfraction supplemented to the suspension of neutrophils leads to the decrease of heat stress related suppression in the formation of superoxide anions by neutrophils during phagocytosis (a decrease of only 23% compared to a decrease of 60% observed without supplementation of RG-ws-1-

1-1 subfraction to the suspension of neutrophils). This is an important manifestation of an anti-heat stress activity of the saponin-free subfraction of ginseng.

4. *In vitro* heat stress (42 °C) decreases the level of activation of respiratory burst (oxygen consumption) of neutrophils down to 48% of the initial level. Supplementation of RG-ws-1-1-1 subfraction to mice *in vivo* decreases HSR down for 20%.

Finally, we can conclude that RG-ws-1-1-1 subfraction enhances an oxygen-dependent anti-microbial function of neutrophils under comfortable as well as under heat stress conditions. Heat stress protective features of KRG assayed as an improved bactericidal function of neutrophils under heat stress conditions are well observed after treatment of animals with a saponin-free subfraction of KRG.

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