ORIGINAL ARTICLE

Influence of *Ganoderma lucidum* (Curt.: Fr.) P. Karst. on T-cell-mediated immunity in normal and immunosuppressed mice line CBA/Ca

Influence of *Ganoderma lucidum* (Curt.: Fr.) P. Karst. on T-cell-mediated immunity in normal and immunosuppressed mice line CBA/Ca

Iryna V. Nizhenkovska • Vitalii T. Pidchenko • Nina G. Bychkova • Nina A. Bisko • Angela Y. Rodnichenko • Natalya O. Kozyko

Received 31 August 2015 / Accepted 8 September 2015

Summary

The article presents the results of the investigation of the effect of biomass powder of the fungus *Ganoderma lucidum* on T-cell-mediated immunity in normal and immunosuppressed mice CBA/Ca. Delayed-type hypersensitivity assay was used. Experimental immunodeficiency was established with intraperitoneal injection of the immunosuppressant cyclophosphamide at a single dose of 150 mg/kg on the first day of the experiment. Results of the study show that the administration of biomass powder of *Ganoderma lucidum* in a dose of 0.5 mg/kg orally for 10 days increases the delayed-type hypersensitivity response in normal mice CBA/Ca. Administration of 0.5 mg/kg of biomass powder of the fungus *Ganoderma lucidum* for 10 days blocked the development of the T-cell-mediated immunosuppression, induced by administration of cyclophosphamide and restored the delayed-type hypersensitivity response in immunosuppressed mice.

Key words: fungus *Ganoderma lucidum* • cyclophosphamide • immunodeficiency • T-cell-mediated immunity • delayed-type hypersensitivity

Souhrn

Práce předkládá výsledky výzkumu vlivu prášku houby *Ganoderma lucidum* na imunitu zprostředkovanou T-buňkami u zdravých myší a myší s potlačenou imunitou linie CBA/Ca. Použita byla zkouška opožděného typu hypersenzitivity. Experimentální imunodeficience byla navozena první den pokusu intraperitoneální injekcí imunosupresivní látky cyklofosfamidu v dávce 150 mg/kg. Výsledky studie ukazují, že podání prášku *Ganoderma lucidum* v dávce 0,5 mg/kg perorálně po 10 dní zvyšuje odpověď opožděného typu hypersenzitivity u zdravých myší CBA/Ca. Podání 0,5 mg/kg prášku houby *Ganoderma lucidum* po 10 dní blokovalo rozvoj imunosuprese zprostředkované T-buňkami vyvolané podáním cyklofosfamidu a obnovilo reakci opožděného typu hypersenzitivity u myší s potlačenou imunitou.

Klíčová slova: houba *Ganoderma lucidum* • cyklofosfamid • imunodeficience • imunita zprostředkovaná T-buňkám • hypersenzitivita opožděného typu

Nizhenkovska I. V., doctor of medicine, professor (✉)
Head at Department of Biological, Bioorganic and Pharmaceutical Chemistry of Bogomolets National Medical University, Kyiv, Ukraine
Kyiv, 22 Pushkinska str, 01601
e-mail: dekan-farm@ukr.net

Pidchenko V. T. • Bychkova N. G. • Kozyko N.O.
Bogomolets National Medical University, Kyiv, Ukraine

Bisko N. A.
M.G. Kholodny Institute of Botany NASU, Kyiv, Ukraine

Rodnichenko A.Y.
The State Institute of Genetic and Regenerative Medicine NAMS Ukraine, Kyiv, Ukraine

Introduction

Ganoderma lucidum is a wood-degrading basidiomycete, fruiting bodies of which have been used in traditional Asian medicine for more than 2000 years to prevent and/or treat various human diseases. It has been widely used for centuries as a famous and precious traditional Chinese herb in China, Japan, Korea, and other Oriental countries as a longevity-promoting and tonic herb of the non-toxic class. *Ganoderma lucidum* is capable of strengthening body resistance and improving

constitutive homeostasis in patients[1–3]. Modern uses of *Ganoderma* therefore include the treatment of coronary heart diseases, arteriosclerosis, hepatitis, arthritis, nephritis, bronchitis, asthma, hypertension, cancer, and gastric ulcer[1, 2].

As *Ganoderma lucidum* is very scarce in nature, artificial cultivation has become essential to meet the demands of international markets[1]. The main traditional *Ganoderma lucidum* fruiting body cultivation methods remain sawdust cultivation in bags or bottles and cultivation on natural logs. The cultivation in this way takes 3 to 5 months. Today submerged cultivation on culture media attracts the attention of researchers and has such advantages as reducing the production cycle to 2–3 weeks, a higher and more stable yield, cultivation is possible throughout the year by creating the optimal conditions required for biomass, the possibility of mechanization and automation of technological processes[4]. Authors use different substrate compositions for the submerged cultivation of *Ganoderma lucidum* mycelia. The chemical components of *Ganoderma lucidum* are complicated: they involve polysaccharides, flavonoids and alkaloids, amino acids, steroids, oligosaccharides, proteins, mannitol, etc.[5]. Submerged fermentation of *Ganoderma lucidum* is viewed as a fast and cost-effective alternative for efficient production of polysaccharides and ganoderic acids from *Ganoderma lucidum*[1, 4].

The majority of scientific works are devoted to the study of fruiting bodies of the fungus *Ganoderma lucidum*. Wherein the vegetative mycelium is poorly studied. Mostly the works on the study of mycelium represent the investigation of polysaccharide or triterpene extracts[6–10]. Wherein the influence of biomass of fungus *Ganoderma lucidum*, grown by submerged cultivation, on various links of immunity in immunodeficient conditions is not understood.

Therefore, the aim of our study was to investigate the influence of biomass powder of the fungus *Ganoderma lucidum* on T-cell-mediated immunity in normal and immunosuppressed mice.

Experimental Part

The biomass of the fungus *Ganoderma lucidum* was grown by submerged cultivation at the M.G. Kholodny Institute of Botany (Kyiv) under the supervision of N. A. Bisko, Doctor of Biological Sciences at the Department of Mycology. Submerged cultivation of *Ganoderma lucidum* (strain1900) was performed at the glucose-peptone-yeast medium (glucose – 20.0, peptone – 3.0, yeast extract – 2.0, KH_2PO_4 – 1.0, K_2HPO_4 – 1.0, $MGSO_4$ – 0.5, water – 1 l) for 14 days at the temperature of 26–28 °C. The biomass was dried and powdered.

Animal studies were conducted in compliance with the basic provisions of the European Convention for the Protection of Vertebrate Animals used for Experimental and Other Scientific Purposes (Strasbourg, 18. 3. 1986), Council Directive 86/609/EEC of 24 November 1986 on the approximation of laws, regulations and administrative provisions of the Member States regarding the protection of animals used for experimental and other scientific purposes, Order of the Ministry of Health of Ukraine from February 13, 2006 No. 66, Law of Ukraine "On protection of animals from cruelty" (2006).

The study was performed in 2 stages. During stage I, the influence of biomass powder of the fungus *Ganoderma lucidum* on T-cell-mediated immunity in normal mice was studied. The study was performed on sexually mature (aged 3–5 months) male CBA/Ca mice. All mice received a water suspension of the biomass powder, which was placed onto pieces of bread before their main food. The animals were divided into 5 groups: 1st – control group (n = 9) of mice, which received the pieces of bread with water, groups 2nd, 3rd, 4th and 5th orally received 0.5 mg/kg (n = 9), 5 mg/kg (n = 9), 50 mg/kg (n = 6) and 500 mg/kg (n = 6) of biomass of the fungus *Ganoderma lucidum*, respectively.

A feature of Delayed Hypersensitivity Reactions is almost a complete absence of visible manifestations at the stage of formation of the immune response after the administration of a sensitizing dose of antigen (sheep red blood cells). All visible manifestations are displayed only after re-introduction of the antigen (final dose). After the introduction of the biomass powder to mice for 10 days (in experimental groups) or water (in control group), all mice were immunized with a single intraperitoneal injection of sheep red blood cells in a dose of 2×10^5 cells in a volume 0.5 ml of saline at 20 g of body weight (sensitizing dose). After 5 days, experimental animals were administered 10^8 sheep red blood cells in a volume of 0.02 ml (final dose) in the sole of the left rear paw (experimental group) and were injected isotonic saline in the same volume in the sole of the right rear paw (control group). Assessment of reaction was carried out after 24 hours measuring the weight difference between the experimental (E) and control (C) paws. To do this, both legs were cut off higher heel joint immediately after slaughter[11].

During stage II, the influence of biomass powder of the fungus *Ganoderma lucidum* on T-cell-mediated immunity in immunosuppressed mice was studied. The study was performed on sexually mature (aged 3–5 months) male CBA/Ca mice. Experimental immunodeficiency was established with intraperitoneal (i.p.) injection of the immunosuppressant cyclophosphamide (Endoxan® – "Baxter Oncology GmbH", cyclophosphamide powder for injection, Germany), at a single dose of 150 mg/kg at the first day of the experiment.

Cyclophosphamide belongs to antineoplastic drugs and has cytotoxic, antitumor and immunosuppressive activity. After the injection of cyclophosphamide mice were administered the biomass powder of the fungus *Ganoderma lucidum* orally at a dose rate of 0.01 mg per 20 g of body weight (0.5 mg/kg). As a reference product we used *Echinacea* (Echinacea-Astrapharm – "Astrapharm", tablets, Ukraine). The dose of the reference product was calculated using the coefficient which determines the ratio between the doses of remedies for humans and experimental animals of different species. For mice it is 387.9.

To investigate the influence of biomass powder of the fungus *Ganoderma lucidum* on T-cell-mediated immunity in immunosupressed mice, the animals were divided into

4 groups: 1st – control group of mice (no immunosuppression, no treatment) (n = 6) – mice received pieces of bread with water before their main food, 2nd (CY) – immunosuppressed mice without treatment (n = 12) – mice were administered an i.p. injection of cyclophospamide 150 mg/kg, then they received pieces of bread with water for 10 days, 3rd (CY+G.l) – immunosuppressed mice which were treated with *Ganoderma lucidum* (n = 6) – mice were administered an i.p. injection of cyclophospamide 150 mg/kg, then they received a water suspension of the biomass powder for 10 days, which was placed onto the pieces of bread before their main food, 4th (CY+E) – immunosuppressed mice which were treated with *Echinacea* – mice were administered an i.p. injection of cyclophospamide 150 mg/kg, then they received a water suspension of the *Echinacea* tablets for 10 days, which was placed onto the pieces of bread before their main food.

After the introduction of the biomass powder (3rd group) or *Echinacea* (4th group) or water (1st and 2nd group), all mice were immunized with a single intraperitoneal injection of sheep red blood cells in a dose of 2×10^5 cells in a volume of 0.5 ml of saline at 20 g of body weight (sensitizing dose). After 5 days experimental animals were administered 10^8 sheep red blood cells in a volume of 0.02 ml (final injection) in the sole of the left rear paw (experimental group) and were injected isotonic saline in the same volume in the sole of the right rear paw (control group). Assessment of reaction was carried out after 24 hours measuring the weight difference between the experimental (E) and control (C) paws. To do this, both legs were cut off higher heel joint immediately after slaughter[11].

The delayed hypersensitivity reaction index (RI) was calculated for each animal using the formula:

$$RI = \frac{E-C}{C} \times 100$$

The data were analyzed using "Statistica 6.1" for Windows. Student's t-test was used for statistical analysis. Results were considered to be statistically significant at $p < 0.05$[12, 13]

Results

Effect of Ganoderma lucidum on T-cell-mediated immunity in normal mice

During this study it was observed that the administration of 0.5 mg/kg of biomass powder of the fungus *Ganoderma lucidum* to normal mice for 10 days caused a statistically proven increase in the weight of paws 2.7 times. Other doses of *Ganoderma lucidum* also increased the weight of paws but it was not statistically proven (Figure 1). The reaction index was increased 2.4 times after administration of 0.5 mg/kg of *Ganoderma lucidum*. Other doses also caused an increase in the reaction index but it was not statistically proven (Figure 2).

This assay showed that *Ganoderma lucidum* in a dose of 0.5 mg/kg caused a more significant stimulatory effect

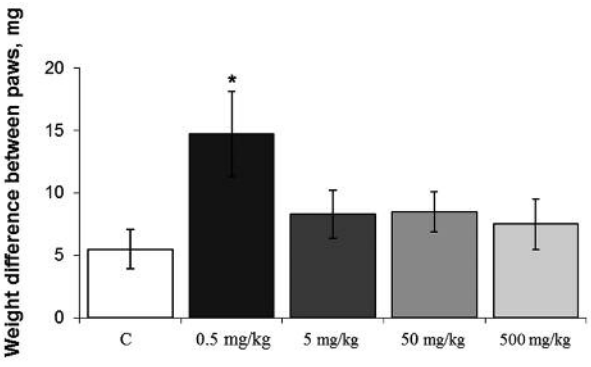

Fig. 1. Effect of different doses of Ganoderma lucidum on weight difference between the experimental and control paws in normal mice: C – control mice
** p < 0.05 concerning the control group*

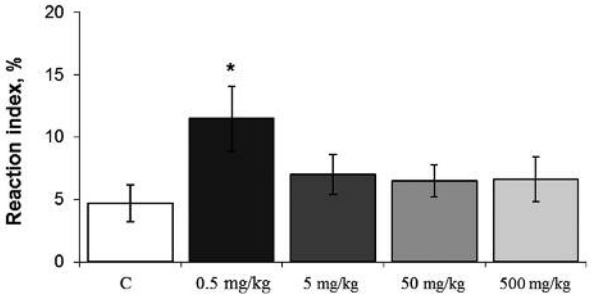

Fig. 2. Effect of different doses of Ganoderma lucidum on reaction index in normal mice: C – control mice
** p < 0.05 concerning the control group*

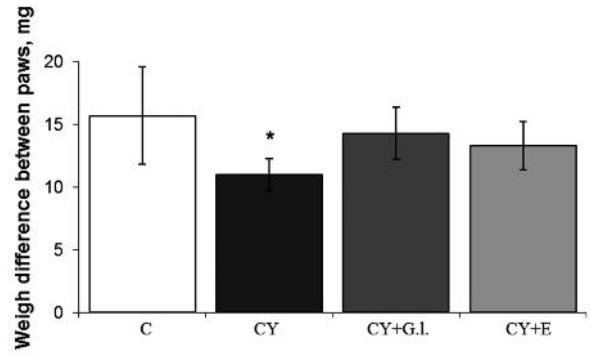

Fig. 3. Effect of cyclophosphamide (CY), Ganoderma lucidum (CY+G.l.) and Echinacea (E) on weight difference between the experimental and control paws in immunosuppressed mice: C – control mice
** p < 0.05 concerning the control group*

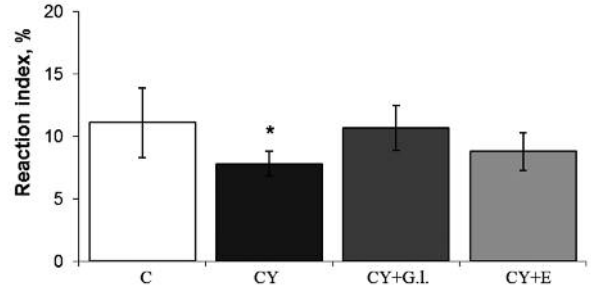

Fig. 4. Effect of cyclophosphamide (CY), Ganoderma lucidum (CY+G.l.) and Echinacea (E) on reaction index in immunosuppresserd mice: C – control mice
** p < 0.05 concerning the control group*

on T-cell-mediated immunity in normal mice. In the previous series of our research, the application of this dose of biomass powder of *Ganoderma lucidum* also experienced the most pronounced stimulatory effect on the humoral immune response in normal mice[14] and also in immunosuppressed mice[15]. Therefore we used this dose to investigate the influence of *Ganoderma lucidum* on T-cell-mediated immunity in immunosuppressed mice.

Effect of Ganoderma lucidum on T-cell-mediated immunity in immunosuppressed mice

The injection of cyclophosphamide caused a significant reduction of delayed-type hypersensitivity response by 30% compared to control mice.

Treatment with *Ganoderma lucidum* in a dose of 0.5 mg/kg during 10 days blocked the development of T-cell-mediated immunosuppression and restored the delayed-type hypersensitivity response. The delayed-type hypersensitivity response in the groups of mice that were treated with *Ganoderma lucidum* and *Echinacea* was at the level of the control group and it was higher than in the group without treatment but it was not statistically proven (Figures 3 and 4).

Discussion

T-cells belong to a group of white blood cells known as lymphocytes, and play multiple roles in cell-mediated immunity. These specialized cells respond to stimulation by DCs plus a peptide antigen[16]. Cell-mediated immunity is a T-cell-mediated defense mechanism against microbes that survive within phagocytes or infect nonphagocytic cells. Cell-mediated immunity functions to enhance antimicrobial actions of phagocytes to eliminate microbes. Cell-mediated immunity manifests as delayed type cellular immune responses as typically seen in Mantoux test. This T-cell-mediated activation of phagocytes depends on interferon gamma (IFN-γ), a major cytokine produced by type 1 T-helper (Th1) cells. Phagocytic cell activation and inflammation induced by cell-mediated immunity can cause tissue injury, typically called delayed-type hypersensitivity. In experimental animal models, delayed-type hypersensitivity responses are characterized by a granulomatous response consisting of macrophages, monocytes, and T-lymphocytes[17].

So in order to study the effect of biomass powder of the fungus *Ganoderma lucidum* on T-cell-mediated immunity in normal and immunosuppressed mice, we used delayed-type hypersensitivity assay. In the present study, we determined, for the first time, the effect of biomass powder of *Ganoderma lucidum* on T-cell-mediated immunity in normal and immunosuppressed mice. The major new findings are that the administration of biomass powder of *Ganoderma lucidum* in a dose of 0.5 mg/kg orally for 10 days increases the delayed-type hypersensitivity response in normal mice.

Cyclophosphamide is one of the most commonly used anticancer agents and immunodepressant drugs for preventing graft rejection, treating some chronic autoimmune diseases and inducing experimental immunosuppression. Cyclophosphamide is a cytotoxic agent that can inhibit both humoral and cellular immunity[18]. Although cyclophosphamide is the mainstay cancer chemotherapy agent, its immunosuppressing activity represents a major clinical challenge and is a main limiting factor for sustained clinical use[10]. In our studies the injection of cyclophosphamide caused a significant reduction of delayed-type hypersensitivity response by 30% compared to control mice. Treatment with *Ganoderma lucidum* in a dose of 0.5 mg/kg for 10 days blocked the development of the T-cell-mediated immunosuppression, induced by administration of cyclophosphamide, and restored the delayed-type hypersensitivity response. None of the mice treated with *Ganoderma lucidum* died, nor did their body weights change significantly during the experiment period.

Cao et al. isolated a kind of polysaccharide peptide (GLPS) with a molecular weight of 584.900 from a boiling water extract of wood-cultured *G. lucidum*, followed by ethanol precipitation, dialysis, and protein depletion. The GLPS can increase mRNA expression of interferon-γ (IFN-γ) and protein expression of granzyme B. It can also promote the cytotoxicity of specific cytotoxic T-lymphocytes induced by DCs, which were pulsed with P815 tumor antigen during the stage of antigen presentation. The mechanism of cytotoxicity is assumed to be mediated through the IFN-γ and granzyme B pathways[7]. Long-term (for 14 days) treatment of mice with an antler-shaped fruiting body of *G. lucidum* (Rokkaku-Reishi), which contains more than 40% β-D--glucan, can activate both T cells and splenic macrophages. β-D-glucan is the major ingredient of GLPS.

In a study performed by Wang et al. (1997), it was shown that polysaccharides extracted from the fresh fruiting bodies of *G. lucidum* can stimulate the production of IL-1β, IL-6, TNF-α, and IFN-γ in human monocyte-macrophages and T-lymphocytes[19, 20]. Fraction-3, a fucose-containing glycoprotein extracted from *Ganoderma lucidum*, stimulated the proliferation of murine spleen cells and expression of various cytokines, including IL-1, IL-2, and IFN-γ[21]. These results showed that polysaccharides obtained from *Ganoderma lucidum* could effectively increase the cellular immune activity *in vitro* as well as *in vivo*, by modulating cytokine production[22].

In addition to polysaccharides, a fungal immunomodulatory protein, Ling Zhi-8 (LZ-8), had been isolated from the mycelia of *G. lucidum* and was regarded as one of the major bioactive substances of *Ganoderma lucidum*. Chen-Hao Yeh et al. (2010) demonstrated that LZ-8 could activate murine macrophages and T lymphocytes but polysaccharides were merely the activators for macrophages, suggesting their diverse roles in activating the innate and adaptive immunity[23].

Probably the immunomodulating effect of biomass powder of *Ganoderma lucidum* on T-cell-mediated immunity can be explained by the synergistic effect of all these biologically-active substances, that could be also situated in biomass, since not always the immunomodulatory action of a pure form or the polysaccharide complex or another substance is confirmed[6, 9].

References

1. **Boh B., Berovic M., Zhang J., Zhi-Bin L.** Ganoderma lucidum and its pharmaceutically active compounds. Biotechnol Annu Rev. 2007; 13, 265–301.
2. **Lin Z. B., Zhang H. N.** Anti-tumor and immunoregulatory activities of Ganoderma lucidum and its possible mechanisms. Acta Pharmacol. Sin. 2004; 25, 1387–1391.
3. **Wicks S. M., Tong R., Wang C. Z., O'Connor M., Karrison T., Li S., Moss J., Yuan C. S.** Safety and tolerability of Ganoderma lucidum in healthy subjects: a double-blind randomized placebo-controlled trial. Am. J. Chin. Med. 2007; 35, 407–414.
4. **Wagner R., Mitchell D. A., Sassaki G. L., Amazonas M., Berovic M.** Current techniques for the cultivation of Ganoderma lucidum for the product of biomass, ganoderic acid and polysaccharides. Food Technology and Biotechnology 2003; 41(4), 371–382.
5. **Zjawiony J. K.** Biologically active compounds from Aphyllophorales (polypore) fungi. J. Nat. Prod. 2004; 67, 300–310.
6. **Cao Q. Z., Lin Z. B.** Antitumor and anti-angiogenic activity of Ganoderma lucidum polysaccharides peptide. Acta. Pharmacol. Sin. 2004; 25, 833–838.
7. **Cao L. Z., Lin Z. B.** Regulatory effect of Ganoderma lucidum polysaccharides on cytotoxic T- lymphocytes induced by dendritic cells in vitro. Acta. Pharmacol. Sin. 2003; 24, 321–326.
8. **Chang Y. H., Yang J. S., Yang J. L., Wu C. L., Chang S. J., Lu K. W., Kuo C. L., Hsia T. C., Chung J. G.** Ganoderma lucidum extract promotes immune responses in normal BALB/c mice in vivo. In Vivo 2009; 23(5), 755–759.
9. **Zhang J., Tang Q., Zimmerman-Kordmann M., Reutter W., Fan H.** Activation of B lymphocytes by GLIS, a bioactive proteoglycan from Ganoderma lucidum. Life Sci. 2002; 71, 623–638.
10. **Zhu X. L., Chen A. F., Lin Z. B.** Ganoderma lucidum polysaccharides enhance the function of immunological effector cells in immunosuppressed mice. J. Ethnopharmacol. 2007; 111, 219–226.
11. **Stefanov O. V. (ed.)** Doklinichni doslidzhennya likars'kih zasobiv [Preclinical studies of medicines]. Kyiv: Avicena 2001; 528.
12. **Gubler E. V., Genkin A. A.** Primenenie neparametricheskih kriteriev statistiki v mediko-biologicheskih issledovaniyah [Application of nonparametric statistics in biomedical research]. L: Medicina 1973;
13. **Mincer O. P., Ugarov B. N., Vlasov V. V.** Metody obrabotki medicinskoj informacii [Methods of processing of medical information]. iev: Vishha shkola 1991.
14. **Pidchenko V. T.** Vplyv riznyh doz poroshku bomasy Ganoderma lucidum (Curt.: Fr.) P. Karst. na gumoraľnu imunnu vidpoviď u myshej linii CBA/Ca [The effect of different doses of biomass powder of Ganoderma lucidum (Curt.: Fr.) P. Karst. on humoral immune response in mice line CBA/Ca]. Proceedings of the 3rd European Conference on Biology and Medical Sciences (Vienna, Austria, 28 october 2014), Vienna. »East West« Association for Advanced Studies and Higher Education GmbH, 2014; 211–219.
15. **Pidchenko V. T., Nizhenkovska I. V., Bychkova N. G., Bisko N. A., Rodnichenko A. Y.** Vplyv gryba Ganoderma lucidum (Curt.: Fr.) P. Karst. na gumoraľnu imunnu vidpoviď u myshej linii CBA/Ca z vtorynnym imunodefcytom [Influence of mushroom Ganoderma lucidum (Curt.: Fr.) P. Karst. on the humoral immune response in mice line CBA/Ca with secondary immunodeficiency]. Pharmaceutical review 2015; 94–100.
16. **Xu Z., Chen X., Zhong Z., Chen L., Wang Y.** Ganoderma lucidum polysaccharides: immunomodulation and potential anti-tumor activities. The American Journal of Chinese Medicine 2011; 39(1), 15–27.
17. **Jyonouchi H.** Delayed-type hypersensitivity: background, pathophysiology, epidemiology. http://emedicine.medscape.com/article/886393-overview#showall (13. 06. 2013).
18. **Huyan X.H., Lin Y.P., Gao T., Chen R.Y., Fan Y.M.** Immunosupressive effect of cyclophosphamide on white blood cells and lymphocyte subpopulations from peripheral blood of Balb/c mice. International immunopharmacology 2011; 11, 1293–1297.
19. **Wang S. Y., Hsu M. L., Hsu H. C., Tzeng C. H., Lee S. S., Shiao M. S., Ho C. K.** The antitumor effect of Ganoderma lucidum is mediated by cytokines released from activated macrophages and T lymphocyes. Int. J. Cancer 1997; 70, 699–705.
20. **Zhang Q. H., Lin Z. B.** Effect of Ganoderma lucidum polysaccharides B on TNF-α and INF-β 1. production and their mRNA expression. J. Beijing Med. Univ. 1999; 31, 179–183.
21. **Wang Y. Y., Khoo K. H., Chen S. T., Lin C. C., Wong C. H., Lin C. H.** Studies on the immuno-modulating and antitumor activities of Ganoderma lucidum (Reishi) polysaccharides: Functional and proteomic analyses of a fucose-containing glycoprotein fraction responsible for the activities. Bioorganic & Medicinal Chemistry 2002; 10(4), 1057–1062.
22. **Chi H. J. Kao, Amalini C. Jesuthasan, Karen S. Bishop, Marcus P. Glucina, Lynnette R. Ferguson.** Anti-cancer activities of Ganoderma lucidum: active ingredients and pathways. Functional Foods in Health and Disease 2013; 3(2), 48–65.
23. **Chen-Hao Yeh, Hsiao-Chin Chen, Jeng-Je Yang, Wen-I Chuang, Fuu Sheu.** Polysaccharides PS-G and protein LZ-8 from Reishi (Ganoderma lucidum) exhibit diverse functions in regulating murine macrophages and T-lymphocytes. J. Agric. Food Chem. 2010; 58, 8535–8544.