EXPERIMENTAL STUDY

Galantamine has impact on immunity in mice exposed to keyhole limpet hemocyanin

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ABSTRACT

OBJECTIVES: In this work, we hypothesized whether galantamine could interact with the cholinergic anti-inflammatory pathway and modulate immunity this way.

BACKGROUND: Galantamine is a drug used for the therapy of Alzheimer disease. The drug inhibits enzyme acetylcholinesterase in the central nervous system, which causes better availability of neurotransmitter acetylcholine.

METHODS: In the experiment, we immunized BALB/c laboratory mice by keyhole limpet hemocyanin (KLH) in combination with galantamine in a dose 0.02–0.5 mg/kg. The animals were sacrificed from 1 to 7 days after the substances applications and plasma was collected in order to examine immunochemical markers by enzyme-linked immunosorbent assay.

RESULTS: We found significant drop in production of immunoglobulins and interleukin (IL) 4 level while IL2, IL4 and tumour necrosis factor α remained unaltered for the whole experiment. We infer that galantamine causes better availability of acetylcholine also in blood system, where the neurotransmitter interacts with nicotinic acetylcholine receptors on macrophages and initiates cholinergic anti-inflammatory pathway.

CONCLUSIONS: In a conclusion, galantamine can cause lower efficacy of vaccination or immunity response to an infectious disease and the phenomenon should be taken into consideration in the current therapy *(Tab. 1, Fig. 2, Ref. 24)*. Text in PDF *www.elis.sk.*

KEY WORDS: acetylcholinesterase, alzheimer disease, cholinergic anti-inflammatory pathway, galantamine, inflammation, macrophage, inhibition.

Introduction

Currently, galantamine is used as a drug for the therapy of Alzheimer disease and other memory impairment close to the Alzheimer disease (1). The drug acts as a competitive inhibitor of enzyme acetylcholinesterase (AChE), which causes better availability of neurotransmitter acetylcholine on the nicotinic (nAChR) and muscarinic (mAChR) acetylcholine receptors (2, 3). Beside the inhibition of AChE, galantamine is able to modulate nAChR as an allosteric potentiating ligand and thus enhance the inhibitory mechanism on the AChE (4, 5).

Not much is known about the interaction of galantamine with immune system. Like other inhibitors of AChE, galantamine causes better availability of the neurotransmitter acetylcholine not only in

central nervous system, but also in the other parts of cholinergic nerves. Cholinergic anti-inflammatory pathway is one of the crucial parts of the nerves having acetylcholine as a neurotransmitter and is involved in neuronal control over immunity (6–9). Though the most common way how to initiate cholinergic anti-inflammatory pathway is by application of an agonist on nAChR, inhibitors of AChE appears also as potent stimulants of the pathway. It is supposed that AChE on erythrocytes became inhibited and acetylcholine then stimulates α7 nAChR on macrophages (10, 11).

Regarding to galantamine, the supposed impact on immunity can be mediated by both interactions with AChE and nAChR. The proposed experiment hypothesized that galantamine would be a modulator of immunity just by the interaction with cholinergic system. Chemically pure keyhole limpet hemocyanin (KLH) was chosen for the experiment purposes as a suitable model antigen (12–15).

Methods

The animals immunization

In the experiment, six weeks old female mice BALB/c were purchased from Velaz, Unetice, Czech Republic. The mice weighted 20 g in average and they were kept in stable temperature 22 ± 2 °C, humidity 50 ± 10 % and light period lasting 12 hours per a day. The whole experiment and manipulation with the animals was

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Tab. 1. Composition of the experimental groups and the time of euthanasia after the experiment start – number of animals per group.

Euthanasia after:		1 day	4 days	7 days
saline solution		15	15	15
KLH 2 mg/kg		15	15	15
galantamine 0.5 mg/kg		15	15	15
galantamine 0.02 mg/kg	KLH 2 mg/kg	15	15	15
galantamine 0.1 mg/kg		15	15	15
galantamine 0.5 mg/kg		15	15	

the both permitted and supervised by ethical committee of Faculty of Military Health Sciences, University of Defense (Hradec Kralove, Czech Republic).

The animals received either saline solution, when used as control group or KLH or galantamine and they were sacrificed 1, 4 and 7 days after the exposition. Composition of the experimental groups is given in Table 1. The tested compounds were solved in saline solution and 100 µl of the solution was injected to rear limb. Composition of the experimental groups and the time of euthanasia can be found in the Table 1. After the elected intervals, the mice underwent euthanasia by cutting of jugular vein. Finally, fresh blood was taken to lithium heparin tube (Dialab, Prague, Czech Republic) and centrifuged at 1,000 ×g for 5 minutes. The isolated plasma was separated from the tubes and then stored at -80 °C until immunochemical markers examination.

Antibodies assay

Total level of immunoglobulins was assayed by the Enzyme-Linked Immunosorbent Assay (ELISA) on standard 96 well microplates Maxisorp (Nunc; Thermo Fisher Scientific; Waltham, Massachusetts, USA). The assay was performed as follows: 100 µl of ten times diluted blood plasma in phosphate buffered saline was injected per one well and incubated at laboratory temperature overnight. After washing by phosphate buffered saline, the wells were blocked with 100 µl of 0.1 % (w/v) gelatin for one hour and then rinsed again. Finally, 100 µl of secondary antibody against mouse immunoglobulins (Sigma-Aldrich, St. Louis, USA) labelled with horse radish peroxidase (HRP) was applied for another 60 minutes at 37 °C and wells were rinsed by phosphate buffered saline with Tween 0.05 % w/w. Enzyme reaction was initiated by a fresh solution of 0.5 mg/ml o-phenylenediamine and 5 mM H_2O_2 – 100 µl was added for 15 min while the whole plate was kept in dark. The reaction was stopped with 100 µl/well of 2 mol/l H_2SO_4. Wells with captured albumin (20 µl; 5 mg/ml) were used as negative controls. Standard immunoglobulin (Sigma-Aldrich) was chosen for the calibration purposes. Optical density was measured by the ELISA reader such as Sunrise (Salzburg, Austria) at 450 nm.

Interleukins assay

Interleukins (IL) 2, 4, 6 and tumour necrosis factor alpha (TNFα) were measured by ELISA kit from Sigma-Aldrich. The kit contained 96-well microplates with an already immobilized recognition antibody and a secondary antibody labelled with horseradish peroxidase. Standard cytokines were also provided and they

were used for calibration purposes. The kits were processed in compliance with manufacturer's instructions. Optical density was measured on the aforementioned instrument Sunrise.

Statistics

In this work, software Origin 8 Pro (OriginLab Corporation, Northampton, MA, USA) was chosen for statistical analysis. Analysis of variance with Tukey test was performed in order to judge significance of differences between the groups of animals on the probability level 0.05.

Results and discussion

In the experiment, animals were exposed to the tested compounds, as described in the method part. None of the animals perished during the experiment and no pathological manifestation in the mice was observed. We found that KLH was able to initiate immunity response in mice since the fourth day after immunization. In the seventh day, animals receiving KLH had total level of immunoglobulins 15 mg/ml in plasma comparing to the controls receiving saline only where average 7 mg/ml level was found (Fig. 1). Galantamine caused significant decrease of the immunoglobulin level in a dose dependent manner. The upper dose of galantamine caused a drop of antibodies up to average 8.8 mg/ml, which was significant on p = 0.01. The findings correlated with levels of IL-4 (Fig. 2), which dropped with growing dose of galantamine and the drop was in dose – response relationship. While the highest level of IL-4 (20.5 pg/ml) was seen in the animals challenged by KLH for 7 days, animals with the highest dose of galantamine dropped IL-4 level to 14.7 pg/ml, which was insignificant comparing to controls (10.3 pg/ml). Comparing to IL-4 levels, there was no significant difference in IL-2 and IL-6 levels.
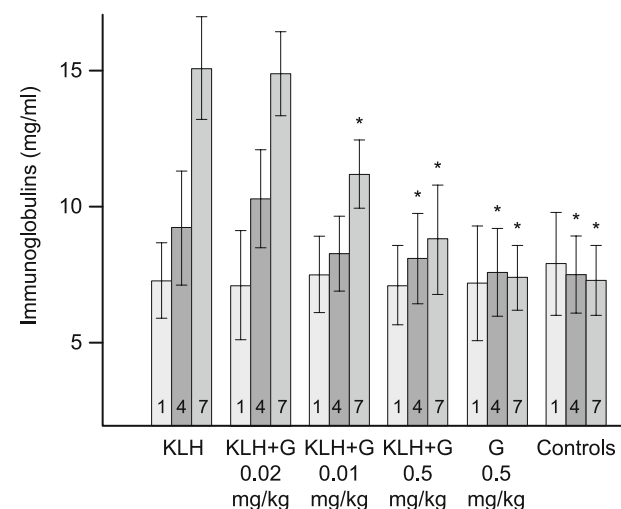
Fig. 1. Total level of immunoglobulins. Error bars indicates standard error of the mean and the number inside bars indicates the number of days (1, 4 and 7) following stimulation by galantamine and/or KLH. Symbol * is an expression for statistical difference (p = 0.05) against the equivalent group exposed to KLH only.
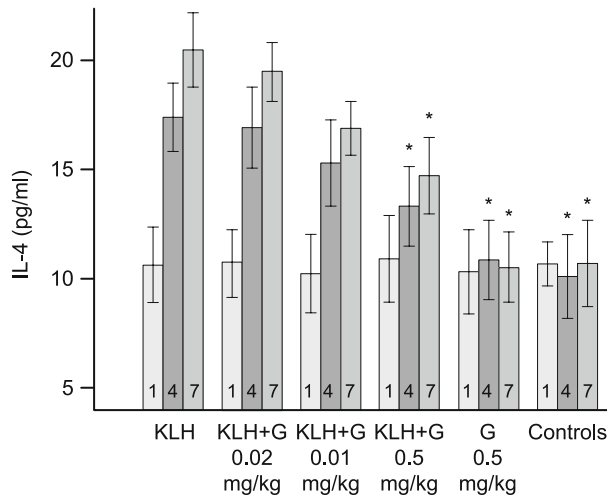
Fig. 2. Total level of IL-4 in plasma of laboratory mice. The description is same like in the Figure 1.

No inflammation occurred during the experiment and KLH alone had no initiation role in release of assayed inflammatory cytokines IL-6 and TNFα. This finding is not surprising because KLH is a protein antigen with low potency to initiate inflammation when no pro-inflammatory agent administered. The finding is also in a good agreement with the quoted papers (16–18) though KLH is able to cause local inflammatory reaction under some conditions and in some sites like a model of skin inflammation (19). Production of antibodies against KLH is not a surprising idea. On the contrary, the production was expected in the experiment (20, 21). Though the inflammation did not occur in the experiment, we expected that galantamine acted just via the macrophages. It is in compliance with cited studies where the effect was described. As an example, Acosta-Ramirez and co-workers described that macrophages were regulating CD4 T lymphocytes under vaccination with recombinant adenovirus (22). From this conclusion, we can consider the macrophages as significant actuators in specific immunity initiation because of the antigens presentation and evolving of specific immunity including antibodies production. Considering our older results, we hypothesize that modulation of cholinergic anti-inflammatory pathway can have a broad impact on specific immunity arising (23). The finding presented is also in a good correlation with experiment with caffeine, where the cholinergic anti-inflammatory pathway was expected as regulatory pathway initiated by the caffeine (24). Summarizing the achieved data, we interpreted the results as an impact of galantamine on the cholinergic anti-inflammatory pathway. The mechanism is based on inhibition of AChE, which causes better availability of the neurotransmitter acetylcholine. In blood system, AChE is also inhibited and acetylcholine can easily interact with the α7 nAChR on macrophages in higher scale when compared to the untreated organisms. In this experiment, we did not test an inflammatory mechanism which can also be modulated by galantamine and rather tested specific immunity evoked by KLH. We see that galantamine is involved in specific immunity regulation

and in drop of antibodies production. This fact should be taken into consideration when galantamine is administered to a patient suffering from Alzheimer disease, who suffers from an infectious disease. On the other hand, the phenomenon would be interesting in the next development of new drugs and therapy processes focused on immunosuppression.

Conclusions

We proved that galantamine can cause drop of antibodies production when laboratory animals are exposed to a protein antigen. Though the most evident responses to galantamine were discovered in the mice receiving the amount of galantamine much higher to that common in human therapy when therapeutic dose calculated on body weight is considered, the finding would be relevant for patients treated for Alzheimer disease and suffering from autoimmune or infectious diseases. Under these conditions, galantamine would have either additive effect to the other drugs or cause a mild alteration of the autoimmune or infectious disease progression. The link between cholinergic anti-inflammatory pathway and inhibition of acetylcholinesterase is inferred to be responsible for the revealed phenomenon.

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