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Chronic caffeine intake affects lysozyme activity and immune cells in mice

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Abstract

The aim of this study was to examine the effect of caffeine on the activity of lysozyme and some immune parameters of mice. The mice were divided into five groups. Group 1, the control group, was given water. The other four groups were administered various concentrations of caffeine by oral intubation (group 2, 2 mg kg^{-1}; group 3, 20 mg kg^{-1}; group 4, 40 mg kg^{-1}; group 5, 200 mg kg^{-1}). It was found that the activity of lysozyme in the serum depended on the caffeine dose. Compared with the control (group 1), lysozyme activity was 1.4-times higher in group 2, 1.6-times higher in the group 3, and 1.8-times higher in groups 4 and 5 ($P < 0.05$). In group 3 a significant increase in spleen weight was detected and the spleen index was 2.1-times ($P < 0.05$) higher compared with control. In group 3 the number of monocytes and neutrophils was 2.5-times higher ($P < 0.05$) compared with control. In group 5 the caffeine increased the number of neutrophils 2.7-times and increased the number of eosinophils 4.6-times ($P < 0.05$) compared with control. Our study revealed that caffeine played an important role in the development of protective immune response.

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Introduction

The immune system is strongly affected by a variety of physical and psychological stressors (Ottaviani & Franceschi 1996). Caffeine (1,3,7-trimethylxanthine) is a wide range stimulant consumed on a worldwide basis. It is the most common psychoactive substance consumed in the world. The consumption of caffeine occurs in a variety of forms i.e. drinking coffee, tea, maté, or soft drinks, chewing cola nuts, consuming cocoa products or taking over-the-counter pain or slimming medications. The mean daily caffeine consumption for all adult consumers and from all sources reaches 2.4–4.0 mg kg^{-1} for a 60–70 kg subject in the United States, UK and Canada as well as 7.0 mg kg^{-1} in Scandinavia (James 1991; Barone & Roberts 1996).

At submillimolar concentrations caffeine exerts a wide variety of physiological effects on different organisms from bacteria to man (Garattini 1993). It has a wide range of effects on cardiovascular activity, including vasoconstriction, total peripheral resistance, blood flow, and so forth. Caffeine produces acute elevations in systolic and diastolic blood pressure in most individuals (Nurminen et al 1999). It possesses analgesic properties (Sawynok & Yakahi 1993), enhances lipolysis and fat oxidation, reduces glycogen breakdown (Costill et al 1978; Pasman et al 1995), and inhibits carcinogenesis (Lu et al 2002); however, as a complex-forming agent it decreases the effective concentration of antitumour antibiotic actinomycin D (Veselkov et al 2002). Furthermore, caffeine influences many pathways involved in the cellular response to DNA damage, reducing the cell cycle delay caused by DNA damage and inhibiting repair of the damage (Murnane 1995). Caffeine is frequently used as a component of anti-rheumatic, anti-inflammatory and anti-pyretic drugs (Nogowska et al 1999). Caffeine is a potential stimulant of the central nervous system (CNS) (Gilbert 1984) and has a significant ergogenic effect (Davis et al 2003).

Lysozyme (1,4-β-*N*-acetylmuramidase), a small (14.3 kDa) cationic protein, is generally assumed to be a component of the first-line host defence against bacterial invaders. This protein is an ubiquitous host factor found in secretions and inflamed tissue at infection sites. Lysozyme belongs to the class of enzymes that lyses the cell walls of

bacteria as the bond between the C-1 of N-acetylmuramic acid and the C-4 of N-acetylglucosamine of the peptidoglycan is cleaved. The antimicrobial activity of lysozyme is directed against certain Gram-positive bacteria and to the lesser degree in-vitro against Gram-negative bacteria (Nakae & Nikaido 1975; Pellegrini et al 1992; Cottagnoud & Tomasz 1993). As well as its antimicrobial activity, lysozyme has many other functions including enhancing phagocytic activity of polymorphonuclear leucocytes and macrophages (Kokoshis et al 1978; Anderson 1990) and it stimulates proliferation and antitumour activity of monocytes (Dudley et al 1969). Neutrophilic granulocytes, monocytes and tissue macrophages produce lysozyme (Zeman & Kantorski 1990). Azurophilic and specific granules of neutrophilic granulocytes are the richest source of lysozyme (Prokopowicz et al 1985). Lysozyme is found intracellularly in various blood cells and their bone marrow precursors. Lysozyme is present in plasma/serum, tears, milk, spit, and intestinal mucus, whilst a particularly large level is determined in liver, spleen, kidney and lung. Lysozyme is present in many animals, but its concentrations vary over a wide range depending on the species (Gionfriddo et al 2000).

We have determined the potential influence of caffeine on the activity of lysozyme and some immune parameters (spleen index, number of lymphocytes, basophils, monocytes, neutrophils and eosinophils), which play an important role in the development of protective immune responses of the organism against infectious agents, in mice.

Materials and Methods

Reagents

Lysozyme from chicken egg white, *Micrococcus lysodeikticus*, edetic acid (EDTA) and caffeine were purchased from Sigma (St Louis, MO). Other chemicals were of analytical grade and used as received. All the solutions were prepared using deionized water purified with a Millipore S.A. water purification system (Molsheim, France).

Animals and housing

Approval of the Lithuanian Ethic Committee for Laboratory Animal Use was obtained before commencement of the experiments.

Twenty male BALBc mice (27–28 g) were obtained from the vivarium of the Institute of Immunology of Vilnius University (Vilnius, Lithuania). Mice were randomly divided into five groups of four animals, housed in groups in solid-bottomed cages containing bedding of wood shavings, with food and water freely available. Room temperature was maintained at 21–24 °C and a 12-h light/dark cycle was used.

The procedure for treatment with caffeine

The mice were given a 0.27–0.28 mL dose of caffeine, 2 mg kg^{-1} (group 2), 20 mg kg^{-1} (group 3), 40 mg kg^{-1} (group 4), or 200 mg kg^{-1} (group 5) solution in water by oral intubation. The doses were calculated individually for each mouse. The control group (group 1) was given water. The caffeine solutions were given over 30 days at 3-day intervals. On day 30 the mice were weighed and then killed by cervical dislocation.

Immune cells and spleen index

To analyse some immune parameters of mice after chronic caffeine exposure, blood samples were collected from the heart of all animals. To each 1-mL blood sample was added 50 μL Na-EDTA (6%). Blood count analysis of neutrophils, lymphocytes, monocytes and eosinophils was performed with a multiparameter automated haematology analyser (Mascot Hemavet, Intelimetric Ltd, Oxford, IN). Effective operation of the Hemavet required the use of Hema-Set^3 multispecies haematology reagents which have well-controlled properties. A diluent (Multi-Cell^3 Diluent) was required to disperse erythrocytes, leucocytes (lymphocytes, monocytes, neutrophils, eosinophils, basophils), and thrombocytes without fixing or distorting these cells during analysis. To count the leucocytes and measure haemoglobin, the lysing agents (Cellyse XI^3 and Cellyse XI^3) simultaneously lysed the erythrocytes and converted the haemoglobin to a stable pigment while allowing the leucocytes to remain intact. The Hemavet system generated a leucocyte differential by constructing a distribution cytogram based on the relative size and complexity of cells in a blood sample. Each cell passing through the instrument's sensing zone was analysed, compared with known criteria, and placed in a corresponding area in the cytogram, based on these criteria.

The spleen was excised from each mouse and weighed. The spleen index (SI) was calculated as (organ weight/body weight) $\times$ 1000.

Assay of enzymatic activity of lysozyme

The enzymatic activity of lysozyme against *M. lysodeikticus* cells was studied in 0.01 M sodium phosphate buffer, pH 6.2 at 37 °C. This suspension was freshly prepared by dispersing 0.7 mg mL^{-1} *M. lysodeikticus* cells in phosphate buffer. The serum from investigated animals was prepared by centrifugation with 5415 D Eppendorf centrifuge (Hamburg, Germany) at 3000 rev min^{-1} for 5 min. Each assay was initiated by adding and mixing 0.1 mL egg-white lysozyme solution or investigated serum to 1 mL bacterial cell suspension and 1 mL phosphate buffer. In the control sample we used 0.1 mL deionized water instead of lysozyme solution. The solution was mixed and incubated at 37 °C for 30 min. After that the test tubes were placed in ice. The decrease in turbidity at a wavelength of 540 nm due to digestion of *M. lysodeikticus* by lysozyme was measured with KFK-2 colorimeter Zomz (Zagorsk, Russia). No changes in turbidity occurred under these conditions in the absence of the enzyme.

The direct effect of caffeine on *M. lysodeikticus* cells was studied according to the above assay. This assay was

initiated by adding and mixing 0.1 mL caffeine solution (0.5, 1, 1.5, 2 mg mL^{-1}) instead of investigated serum.

The lysozyme activity in mouse serum sample was determined from the calibration curve (Ramanaviciene et al 2002) and expressed in U mL^{-1}.

Statistical analysis

The results were reported as the mean ± standard error of the mean (s.e.m.). Comparisons between groups for the specific measure were made using non-parametric multiple Kruskal–Wallis test. To identify differences between individual treatments Nemenyi's test was performed. Differences were considered statistically significant at $P < 0.05$.

Results

Body weight

The mean body weights of mice before and on day 30 of chronic caffeine treatment were assessed. The results of the Kruskal–Wallis test showed a significant caffeine treatment effect ($\chi^2 = 17.5$, df = 4, $P < 0.002$). The weight increment of mice treated with 2 mg kg^{-1} (group 2) and 20 mg kg^{-1} caffeine (group 3) was significantly less (Nemenyi's test, $P < 0.05$) compared with control mice (Table 1). However, mice treated with high caffeine doses (40 and 200 mg kg^{-1}, groups 4 and 5, respectively) weighed less ($P < 0.05$) than the control group, but similar to mice treated with 2 mg kg^{-1} caffeine.

Spleen index

The results of the Kruskal–Wallis test showed a significant caffeine-treatment effect on spleen index ($\chi^2 = 17.8$, df = 4, $P < 0.001$). The results illustrated that the mean weight of spleen was significantly higher (1.9-times, Nemenyi's test, $P < 0.05$) in group 3, where animals were given 20 mg kg^{-1} caffeine, however in groups 2 and 5 the mean weight of spleen was almost the same as in the control group. The similar tendency was observed during estimation of spleen indices in the same groups of animals. SI of group 3 was significantly higher (2.1-times, $P < 0.05$) compared with the control and other groups (Table 1). However, with the increase of the dose of caffeine SI had a tendency to decrease. In group 4 (40 mg kg^{-1} caffeine) the SI was 1.4-times ($P < 0.05$) higher compared with the control group.

Lysozyme activity

The different concentrations of caffeine increased lysozyme activity in the mice serum. The results of the Kruskal–Wallis test showed a significant caffeine treatment effect on lysozyme activity ($\chi^2 = 14.4$, df = 4, $P < 0.006$). The results showed that even a low concentration of caffeine (2 mg kg^{-1}) increased the activity of lysozyme in serum 1.4-times (Nemenyi's test, $P < 0.05$) if compared with the control group (Table 1). In the serum of mice treated with 20 mg kg^{-1} caffeine, lysozyme activity increased by 1.6-times, whilst for those treated with 40 or 200 mg kg^{-1} the activity had increased by 1.8-times ($P < 0.05$) compared with the control group.

We investigated the direct effect of caffeine using the lysozyme activity test. Caffeine concentration to 2 mg mL^{-1} in the test sample (0.095 mg mL^{-1} or 0.01% or 0.5 mM in the reaction solution) had no effect on the results of this test.

Immune cells

The results of the Kruskal–Wallis test showed a significant caffeine-treatment effect on immune cells: monocyte number ($\chi^2 = 11.6$, df = 4, $P < 0.02$), eosinophil number ($\chi^2 = 13.8$, df = 4, $P < 0.008$), neutrophil number ($\chi^2 = 14.6$, df = 4, $P < 0.006$). Measurements of immune-related haematological parameters in mouse blood showed that chronic caffeine exposure caused monocytosis, neutrophilia and eosinophilia, which depended on the dose of caffeine. In

Table 1 Effects of increasing doses of caffeine on the activity of lysozyme and some immune parameters of mice.

	Water (group 1, control)	Caffeine 2 mg kg^{-1} (group 2)	Caffeine 20 mg kg^{-1} (group 3)	Caffeine 40 mg kg^{-1} (group 4)	Caffeine 200 mg kg^{-1} (group 5)
Increment of weight (%)	7.66 ± 0.03	5.33 ± 0.11*	2.27 ± 0.04*	6.81 ± 0.14*	5.33 ± 0.09*
Lysozyme activity (U mL^{-1})	62.8 ± 5.2	87.8 ± 7.0*	100.5 ± 5.0*	113.4 ± 4.2*	113.3 ± 3.1*
Spleen index	11.5 ± 0.5	12.3 ± 0.3	24.5 ± 1.8*	16.0 ± 0.8*	8.7 ± 0.2
Monocyte number (10^3 μL^{-1})	0.257 ± 0.048	0.320 ± 0.073	0.652 ± 0.130*	0.398 ± 0.066	0.286 ± 0.023
Eosinophil number (10^3 μL^{-1})	0.078 ± 0.011	0.093 ± 0.034	0.270 ± 0.061	0.280 ± 0.045	0.365 ± 0.076*
Neutrophil number (10^3 μL^{-1})	1.16 ± 0.19	1.33 ± 0.27	2.99 ± 0.25*	2.28 ± 0.29	3.16 ± 0.32*

*$P < 0.05$ compared with control.

group 3 the number of monocytes was 2.5-times higher (Nemenyi's test, $P < 0.05$) compared with the control group (Table 1).

The mean number of lymphocytes and basophils proved to be similar in animals of all five groups. The mean number of erythrocytes and haemoglobin in all caffeine-treated mouse groups was approximately by 1.3-times higher than that in the control group, but no statistical significance was observed.

In the control group and group 2 (2 mg kg^{-1}) the number of eosinophils was within the normal range. However, increasing the dose of caffeine resulted in an increase in the deviation from the control group (Table 1). In groups 3 and 4 (20 and 40 mg kg^{-1}, respectively) departure from the control group was 3.5–3.6-times higher, while in group 5 (200 mg kg^{-1}) it was 4.6-times higher (Nemenyi's test, $P < 0.05$). The tendency of caffeine to influence the number of neutrophils proved to be similar. In groups 3 and 5 (20 and 200 mg kg^{-1}, respectively) the deviation of neutrophil number increased by 2.5–2.7-times (Nemenyi's test, $P < 0.05$) compared with the control group (Table 1).

Discussion

Caffeine has long been known to have numerous biological actions (Ribeiro-Alves et al 2003). The purpose of this study was to determine whether chronic caffeine exposure effected non-specific immunity and some immune parameters. It was important to take into account the influence of the CNS on this process, since caffeine is a potent adenosine antagonist, a CNS stimulant that easily crosses the blood–brain barrier due to its lipophilic properties (McCall et al 1982).

Neutrophils, monocytes and tissue macrophages produce a great number of cytokines and cation proteins, including lysozyme (Zeman & Kantorski 1990; Kendziorek & Tomaszewska 1994). Azurophilic and specific granules of neutrophils are the richest source of lysozyme (Pruzanski et al 1984). Lysozyme is associated with the monocyte–macrophage system (Prokopowicz et al 1985) and is believed to be a mechanism of non-specific immunity (Anderson 1990). It regulates molecular proliferative mechanisms, enhances the effectiveness of the properdin-complement system, stimulates the synthesis of immunoglobulins (Sugahara et al 2000), and affects differentiation and apoptosis of healthy and affected cells. In some diseases, for example cancer, neoplastic process plays an inhibitory effect on non-specific immunity (Mantur et al 1998).

Caffeine concentration up to 2 mg mL^{-1} in the test sample had no effect on the activity of lysozyme, i.e. had no effect on the bacteria used in the test. This result corresponded with other published results. Rocha et al (2001) observed insignificant effect of caffeine on mouse inner medullary collecting duct cells up to 1.0 mM (0.02%). We observed a statistically significant effect of caffeine concentration on *Escherichia coli* from 0.1% and on *Pseudomonas fluorescens* from 0.5% (Ramanaviciene et al 2003).

Comparison of the activity of lysozyme between the five groups of mice kept under identical conditions, however chronically treated with different doses of caffeine, showed that lysozyme activity in the serum depended on caffeine dose. A statistically confident influence of caffeine was observed in all groups of mice treated with caffeine in comparison with the control group. Our results showed that increased lysozyme activity was associated with an increased number of neutrophils, eosinophils and monocytes. All these cells, as well as their bone marrow precursors, could be a source of increased lysozyme activity (Jolles et al 1965; Kendziorek & Tomaszewska 1994). On the other hand, increased activity of lysozyme is a parameter of myelopoietic activity (Lollike et al 1995). Furthermore, those authors observed that plasma lysozyme activity rises before detectable increases in the number of circulating neutrophils. Intake of 2 mg caffeine kg^{-1} over 30 days is a small dose to observe an increment in cells, but an increment in lysozyme activity led us to propose that myelopoietic activity was affected. At 20 mg caffeine kg^{-1} an increment in lysozyme activity and immune cells was observed. Lysozyme activity at higher (40 and 200 mg kg^{-1}) caffeine intake was similar, while a decrease or similar number of immune cells was observed. These results showed that at high caffeine doses other processes predominated. Caffeine intake has an effect on increased urinary excretion of minerals (zinc, calcium, magnesium, sodium). The effect of caffeine intake on urinary zinc was reported by Massey & Berg (1985). Zinc is an essential trace element for the immune system, for the immune cells. In mammals, zinc deficiency is primarily observed in the immune system (Lothar & Philip 2001). Our findings led us to suppose that caffeine plays a particular role in regulation of the activity of lysozyme in the serum. This effect can be related to the stimulation of the CNS. Our findings are consistent with the observation of Novokrescenov (1964), who determined that the activity of lysozyme increased after the injection of caffeine into rabbit vein during medicinally-induced sleep.

The differences in increment of weight were the result of caffeine exposure, because weights were statistically similar between groups at the start of the experiment. It confirms previous observations that caffeine can have an anorexic effect in the rat (Gans 1984). We have observed a correlation between increased activity of movement and play fighting with a decrease in weight gain. A high caffeine dose (40–200 mg kg^{-1}) has less effect on increment of weight. It can be explained by intensive food intake, which occurs at times of increased anxiety, nervous or panic disorder. Also, it can be related to caffeine-induced ergogenic activity. This activity affects increment of weight differently, and significantly reduces it at 20 mg kg^{-1} caffeine. The differences in increment of weight are closely related to stimulatory and inhibitory activity of caffeine on the CNS and are consistent with the findings in previous studies (Barraco et al 1983; Garrett & Holtzman 1994).

In this study, spleen and spleen index enlargement were significantly higher in mice treated with 20 and 40 mg kg^{-1} caffeine compared with the control group. The spleen is a secondary lymphoid organ and its enlargement can be related to a more intensive production of lymphocytes and

monocytes. Also, spleen enlargement can be caused by an accumulation of cells such as macrophages and erythrocytes. In our experiment the significantly high number of monocytes correlated with the enlargement of the spleen index. These results confirmed a previous presumption. Monocytes circulate in the blood stream and then migrate into the tissues and differentiate into specific tissue macrophages, which are actively phagocytic cells capable of ingesting and digesting exogenous antigens like whole bacterial cells, virus particles, and injured or dead host cells (Kuby 1997). However, a high caffeine dose (200 mg kg^{-1}) induced a contrary effect on spleen index and had no effect on the number of monocytes.

An increased number of eosinophils was observed in response to high doses of caffeine. Eosinophilia occurs in a large number of diseases, and in some of them, eosinophils are the principal effector cells. The production of eosinophils involves the proliferation and differentiation of haematopoietic progenitor cells, while the accumulation of eosinophils involves interactions between eosinophils and endothelial cells, chemotaxis and cellular activation, and the balance between the survival and apoptosis of eosinophils (Rothenberg 1998). A marked accumulation of eosinophils occurs in several important disorders, such as allergic diseases, parasitic infections and cancer (Weller 1991). An increased number of eosinophils may be harmful, because of the pro-inflammatory effects of eosinophils (Gleich & Adolphson 1986), or it may be helpful, because of the antiparasitic effects of these cells (Capron 1991). Caffeine can increase migration of eosinophils and neutrophils from bone marrow to the blood stream. Eosinophils, like neutrophils, are motile, phagocytic cells that can migrate from the blood into the spaces of other tissues. Their phagocytic role is significantly less important than that of neutrophils, and it is thought that their major role consists in defence against parasitic organisms. On the other hand, neutrophils are the first cells that arrive at a site of inflammation during response to many types of infections, they form the primary defence against bacterial infection (Klebanoff 1975). We suggest that caffeine affected the bone marrow directly or through the CNS and it released more than the usual number of neutrophils and eosinophils. An increased number of these cells showed that the caffeine-treated mice were able to prepare a more effective defence against infection compared with mice treated with water. Our results and presumption that these cells were immunocompetent led us to believe that caffeine enhanced the protective immune responses of the organism against the infection. However, the increased number of cells could be due to a loss of attachment to endothelial walls. These results require further investigation.

Our study revealed that increasing caffeine doses increased non-specific immunity—activity of lysozyme, spleen index and number of some immune cells (monocytes, eosinophils and neutrophils)—while the number of lymphocytes and basophils were similar in all treatment groups. These results showed that caffeine played an important role in the development of the protective immune response. The effect of caffeine could be related to stimulation of the central nervous system or it could directly affect bone marrow. Thus, the results allow us to suppose that chronic consumption of caffeine reinforces the protective immune responses of the organism and can reinforce the efficiency of anti-microbial drugs.

Conclusions

The results showed that caffeine played a particular role in regulation of non-specific immunity—lysozyme activity in serum and the number of some immune cells (monocytes, eosinophils and neutrophils). It can be closely related to the high capability of caffeine to stimulate the activity of the central nervous system or to directly affect bone marrow.

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