Impact of Caffeine on Tularemia Progression in a Mouse Model

Miroslav Pohanka[1] and Oto Pavlis[2]

[1] Faculty of Military Health Sciences, University of Defense,
Trebesska 1575, 50001 Hradec Kralove, Czech Republic.
[2] Centre of Biological Defense, 561 66 Techonin, Czech Republic.

Caffeine is an alkaloid acting as an antagonist on adenosine receptors; however, the other targets for caffeine are known as well. Not much is known about effect of caffeine on immunity. In this work, we have hypothesized that caffeine can cause alteration of infectious disease progression. Tularemia, a zoonotic disease caused by *Francisellatularensis*, was used as the model disease and BALB/c mice were used as an in vivo model. Interleukines (IL) 1b, 2, 4, 6 and interferon g (IFN-g) were assayed by enzyme linked immuno-sorbent assay (ELISA) from plasma and bacterial burden was tested in spleen. We proved that caffeine caused increase of bacterial burden in the spleen in dose response manner. Oppose to this, IL-6 and IFN-g were decreased in dose response manner. Caffeine seems to be able to modulate tularemia progression. Impact on neutrophils via adenosine receptors and on macrophages via cholinergic anti-inflammatory pathway is the most probable explanation.

Key words: Inflammation; Tularemia; Caffeine; Regulation; Adenosine receptor; Innate immunity; Acetylcholine; Cholinergic anti-inflammatory pathway.

Caffeine, a simple alkaloid with proper chemical name 1,3,7-Trimethylpurine-2,6-dione or trimethylxanthine, is a secondary metabolite from *Coffea* plants. In the current time, caffeine is well accessible as an effective compound in some drugs and beverages[1]. From a toxicological point of view, caffeine is a low toxic compound with minimal adverse effects[2,3]. Mechanism of caffeine action is based on antagonism with adenosine on adenosine receptors[4,5]. However, caffeine is implicated in the other pathways and neurotransmissions such as the one based on acetylcholine, epinephrine, norepinephrine, serotonin, dopamine and glutamate can be mentioned as the neurotransmitter being interfered[6-10].

Not much is known about interaction between caffeine and immune system. In a previous work, link between caffeine and production of antibodies was revealed[11]. In another work, association between caffeine and oxidative stress which is also in a proximity to inflammation was proved and discussed[12,13]. Implication of caffeine in ongoing infectious diseases can be inferred regarding to the quoted papers; however, detailed evidence of such process is missing. In the current manuscript, impact of caffeine on immunity during an infectious disease is hypothesized. In order to demonstrate relevance of the findings, tularemia, a zoonotic disease caused by bacterium *Francisellatularensis*, was chosen as a model. The disease is similar in the used mice like humans[14] hence the findings about caffeine can be easily extrapolated to the human population.

* To whom all correspondence should be addressed.
E-mail: miroslav.pohanka@gmail.com

MATERIALS AND METHODS

Microorganism

In this experiment, *Francisellatularensis* LVS (strain code ATCC 29684) was chosen as a model pathogenic microorganism. Sub-passage of the bacterium was inoculated on standardMcLeod agar containing bovine hemoglobin and IsoVitaleX enrichment (Becton-Dickinson, San Jose, CA, USA). After one day planting, grown bacteria were harvested and washed by centrifugation (2,000×g for 10 minutes) using phosphate buffered saline (pH 7.4). Finally, bacterial suspension $2.35×10^6$ CFU/ml was prepared and used for laboratory animals infection. Exact concentration of*F. tularensis* suspension was determined by re-cultivation one day later.

Experiment on BALB/c mice

BALB/c mice were chosen as an animal model. The female mice were two months old (with approximate weight 21±2 g) in the experiment beginning and they were purchased as specific pathogen free animals from BioTest(Konarovice, Czech Republic). Conditions for animals husbandry were following: no limitation in access to feed and water, temperature 22±2°C, humidity 50±10%, and light period from 7 a.m. to 7 p.m. The part experiment based on live animals was done in vivarium of the Centre of Biological Defense in Techonin (Czech Republic). The experiment was permitted by ethical commission of Ministry of Defence, Czech Republic.

The animals were divided to six groups each composed from nine animals. Suspension of *F. tularensis* and/or solution of caffeine (analytical purity, purchased from Sigma-Aldrich, St. Louis, MO, USA) in saline were applied into rear limbs. While *F. tularensis* suspension was applied subcutaneously to left rear limb, caffeine was applied intradermally to right rear limb. Division of the groups was following:

1. The animals received 100 µl of *F. tularensis* suspension only.
2. The animals received 100 µl of *F. tularensis* suspension and 100 µl of caffeine solution providing dose 5 mg/kg of body weight.
3. The animals received 100 µl of *F. tularensis* suspension and 100 µl of caffeine solution providing dose 15 mg/kg of body weight.
4. The animals received 100 µl of *F. tularensis* suspension and 100 µl of caffeine solution providing dose 45 mg/kg of body weight.
5. The animals received 100 µl of caffeine solution providing dose 45 mg/kg of body weight.
6. The animals received only saline.

Second day after experiment beginning, the animals received the same dose of caffeine like the first day or received saline (animals without application of caffeine i.e. groups 1 and 6). Three days after immunization, the animals underwent euthanasia in CO_2 anesthesia by cutting of carotid artery. The excurrent blood was collected into tube with lithium heparin. Plasma was separated from the blood by centrifugation at 1,000×g for 5 minutes.Spleen was sampled and homogenized by pressing through a nylon net (diameter of a hole 1 mm). Bacterial burden was determined using standard cultivation.

Cytokines assays

The immunochemical markers interferon gamma (IFN-g), interleukin 1beta, 2, 4 and 6 (IL-1b, IL-2, IL-4, IL-6) were determined using standard enzyme linked immuno-sorbent assay(ELISA) kits from Sigma-Aldrich. The assay was made in compliance with protocol provided by the manufacturer.

Statistical anis

SoftwareOrigin 9 (OriginLab Corporation, Northampton, MA, USA) was used for the experimental data processing. Data from the experimental groups were compared one to each other and significances of alterations between the groups were estimated using one-way analysis of variance with Fisher test for probabilities levels $P = 0.05$ and $P = 0.01$ (size of group n = 9).

RESULTS AND DISCUSSION

In the experiment, no laboratory animal perished prior to euthanasia. The infected animals had typical symptoms for tularemia such as slow motility, exhaustion, and stubby fur[15,16]. When compared the infected animals one to each other, no visible difference between the infected animals treated with caffeine and the infected one without caffeine was observed. Controls that received saline and the controls that received only caffeine had no uncommon manifestation and no difference between the two groups has been revealed since the experiment starting. It is necessary to rationalize dose of caffeine used here. The lowest dose of

caffeine used here roughly corresponds with a dose of caffeine received by drinking of coffee or caffeine containing drinks. In an example, a small cup of coffee contains approximately 100 – 200 mg of caffeine[17,18]. The upper dose of caffeine used

here is approximately three times under median lethal dose[19].

Experimental data regarding to bacterial burden in spleen are summarized in figure 1. Spleen has been chosen as an organ suitable for bacterial burden testing because *F. tularensis* is retained there within host cell hence analysis of the organ is a standard protocol[20,21]. In the control animals (i.e. groups 5 and 6), no bacteria were found. The finding was expected and it is a confirmation of the fact that the disease was not transmitted to the controls. In the infected animals, average bacterial burden per spleen was around 22,500 CFU for the animals not treated with caffeine. The lowest dose of caffeine (5 mg/kg) had no effect on the bacterial burden; however, the upper doses 15 and 45 mg/kg caused significant ($P = 0.01$) increase of bacterial burden up to average 33,745 for the caffeine dose 15 mg/kg and 38,425 CFU for the dose 45 mg/kg. When considered importance of the finding, it can be stated that caffeine deteriorates the disease. However, the negative effect followed administration of the upper doses of caffeine. When extrapolated the data to humans, worsening of the disease can expected after caffeine administration; however, doses exceeding common drinking of coffee or tea should be accepted in
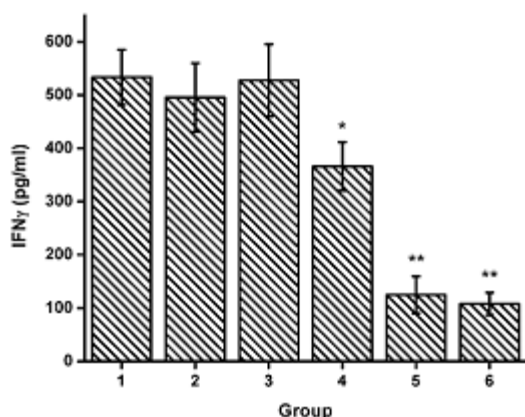

Fig. 1. Bacterial burden expressed as colony forming units (CFU)for *Francisellatularensis*in spleen of mice infected with tularemia. Error bars indicates standard error of mean. Symbol ** is an expression for statistical difference against the first group on probability level P=0.01. Meaning of groups: 1 – animals infected with tularemia; 2 – infected animals with caffeine dose 5 mg/kg; 3 – infected animals with caffeine dose 15 mg/kg; 4 – infected animals with caffeine dose 45 mg/kg
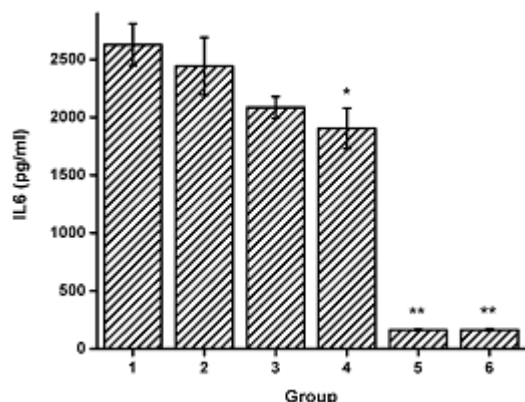

Fig. 2. Plasmatic level of interleukin 6 (IL-6) Error bars indicates standard error of mean. Symbol * respective ** is an expression for statistical difference against the first group on probability level P0.05 respective 0.01. Meaning of groups: 1 – animals infected with tularemia; 2 – infected animals with caffeine dose 5 mg/kg; 3 – infected animals with caffeine dose 15 mg/kg; 4 – infected animals with caffeine dose 45 mg/kg; 5 – animals with caffeine dose 45 mg/kg; 6 – controls receiving saline only

Fig. 3. Plasmatic level of interferon g (IFN-g). Error bars indicates standard error of mean. Symbol * respective ** is an expression for statistical difference against the first group on probability level P0.05 respective 0.01. Meaning of groups: 1 – animals infected with tularemia; 2 – infected animals with caffeine dose 5 mg/kg; 3 – infected animals with caffeine dose 15 mg/kg; 4 – infected animals with caffeine dose 45 mg/kg; 5 – animals with caffeine dose 45 mg/kg; 6 – controls receiving saline only
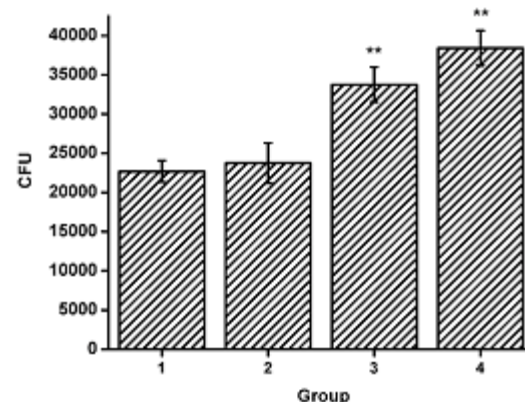
order to reach the described effect. On the other hand, such overdosing cannot be excluded since caffeine is used in food supplements and drugs as well and combination of the drugs, supplements and drinks can easily lead to overdosing[22,23].

ELISA for IL-1b, IL-2 and IL-4 did not prove any significant effect of caffeine. Comparing to the aforementioned, caffeine seems to be implicated in regulation of IL-6 and IFN-g production that can be perceived from figure 2 for IL-6 and figure 3 for IFN-g. Caffeine caused decrease of the IFN-gand IL-6 in a dose response manner. The effect of caffeine was significant (P = 0.05) for the upper dose of caffeine (45 mg/kg). The finding is not surprising in a true sense of the world because implication of caffeine in suppression of inflammatory was proposed by some investigators[24,25]. Caffeine can be involved in control over inflammation via adenosine A_3 receptors regulating neutrophils degranulation[26,27]. Neutrophils are necessary for tularemia resolving [28] and involvement of caffeine in neutrophils activation could explain alteration in the bacterial burden. However, it cannot explain the involvement in IFN-g and IL-6 levels. It is more probable that caffeine meets another pathway beside the regulation of neutrophils. There can be hypothesized impact of caffeine on cholinergic system when considered current literature. Caffeine is able to interact with cholinergic system by regulation of acetylcholine release[29] and cholinergic system is tightly linked to adenosine neurons so activation of adenosine receptors leads to inhibition of acetylcholine release and caffeine prevents from this[30-32]. Beside this, caffeine is an inhibitor of enzyme acetylcholinesterase[33-35]. The inhibition of acetylcholinesterase results in higher accessibility of acetylcholine as well. Increased level of acetylcholine can interact with acetylcholine receptors including á7 nicotinic acetylcholine receptor located on macrophages[36]. It is an compartment of so called cholinergic anti-inflammatory pathway[37-42]. The proved suppression of cytokines level can be attributed just to the cholinergic anti-inflammatory pathway. The whole effect caused by caffeine is probably a combination of impact on the neutrophils via adenosine A_3 receptors and on macrophages via the acetylcholine.

CONCLUSIONS

Caffeine is a simple organic compound with a broad impact on the body. In the current experiment, we proved that caffeine is involved in immunity regulation and can interfere with an infectious disease. The effect is probably a result of impact on the neutrophils via adenosine A_3 receptors and on macrophages via the acetylcholine. Considering the experimental data, caffeine should be considered as a drug with potentially serious role when taken during a disease. High doses of caffeine during the diseases could even have serious consequences.

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