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ORIGINAL ARTICLES

The *in vivo* and *in vitro* Effects of Caffeine on Rat Immune Cells Activities: B, T and NK Cells

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SUMMARY **The effect of caffeine (naturally occurring plant methylxanthine) on immunological cell activities in Sprague-Dawley rat both *in vivo* and *in vitro* was studied. A cytotoxic assay was done to study natural killer (NK) cells and a proliferation assay was performed for T and B cell activities. Three different doses of caffeine i.e., 2, 6 and 18 mg/kg/day were administered chronically to Sprague-Dawley rats to assess the effects *in vivo*. Both NK cell cytotoxicity and B cell proliferative response to pokeweed mitogen (PWM) showed significant decreases ($P < 0.05$) in rats treated with 6 mg/kg/day, whereas the T cell proliferative response to phytohemagglutinin-P (PHA-P) was significantly increased ($P < 0.05$) in the rats treated with 18 mg/kg/day. *In vitro*, caffeine significantly decreases ($P < 0.05$) B and T cell proliferative responses to PWM and PHA-P at added caffeine concentrations of 10, 20 and 40μg/ml. However, no effect was observed on NK cells activity. Furthermore, *in vitro*, a broader dose range of caffeine (1, 10, 100 and 1,000 μg/ml) exhibited dose-dependent inhibition of both B and T cell proliferative responses.**

Caffeine is a naturally occurring plant methylxanthine.[1] It has been used as a component of various types of food, beverages and medicines according to its stimulating, pain relieving, diuretic and other effects. Since 1980, caffeine has been considered to have interim food additive status instead of being generally recognized as safe (GRAS).[2] Apart from its pharmacological effects on the central nervous system (CNS), cardiovascular system (CVS), renal system, smooth and skeletal muscle[3-5] there are controversies concerning the carcinogenic effect of caffeine. Many epidemiological studies have investigated the relationship of caffeine (and/or coffee) consumption on cancer development of some organs, e.g., urinary bladder,[6] pancreas,[7,8] colon[9] and breast[10] although no definitive evidence has been forth coming. It is realized that many factors play roles in tumor development; these can be categorized into two main groups, the inherent carcinogenicity of the substances and the unbalance of immunological homeostasis.[11,12] The second is focused in this study in such a way that this work was designed to learn whether caffeine has any role on the activities of immunological cells i.e. B and T lymphocytes, and natural killer (NK) cells which may lead to disturbance of immune mechanisms. The effects of chronic caffeine consumption, or the *in vivo* and *in vitro* effects of caffeine at various concentrations on B, T and NK cell activities of adult male Sprague-Dawley rats were determined. Three different doses of caffeine (2, 6 and 18 mg/kg/day) were given to rats for 120 consecutive days (*in vivo* effect) and their lymphocytic cell activities were studied. The direct interfering *(in vitro)* effect of caffeine was studied separately on normal rat lymphocytic cells. The proliferative responses to mitogen stimulation i.e. pokeweed mitogen (PWM) for B cells and phytohemagglutinin (PHA) for T cells were measured to assess B and T cells activities, respectively. The conventional ^{51}Cr cytotoxic assay against the YAC-1 cell line was performed to study NK cell activities.

MATERIALS AND METHODS

Animals and diets.

Male weanling Sprague-Dawley rats, 2 months old, weighing 150-200

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gm obtained from The National Laboratory Animal Center, Mahidol University were used. They were fed with commercial laboratory rodent chow and clean drinking water, *ad libitum*. Five were housed in each stainless steel cage and maintained in a ventillated room.

Caffeine treatment.

Caffeine (Sigma, St. Louis, MO, USA, 50% caffeine and 50% benzoic acid) was used. Three different doses of caffeine, i.e., 2, 6 and 18 mg/kg/day were administered by tube feeding every day for at least 120 consecutive days. Each treated group and the control group consisted of 10 animals.

Splenic mononuclear cell suspension.

Rats were killed, spleens were collected aseptically immediately and kept in RPMI 1640 culture medium (RPMI 1640, Gibco, OH, USA : 2 mM L-glutamine, 2 gm/L $NaHCO_3$, 10 mM Hepes, 100 units penicillin/ml, 100 μg streptomycin/ml). Thereafter, the spleens were cut into small pieces and teased through a sterile stainless steel sieve in 5-7 ml RPMI 1640 culture medium. These spleen cell suspensions were washed once with RPMI 1640 medium by centrifugation at 400 × g, at 25° C for 10 minutes. Mononuclear cells from the spleen cell suspension were separated using Ficoll-Hypaque (final density 1.077 gm/ml). After centrifugation at 400 × g at 25° C for 10 minutes mononuclear cells at the interface of Ficoll-Hypaque and RPMI 1640 medium were harvested. These splenic mononuclear cells were washed 3 times with RPMI 1640 medium by centrifugation at 400 × g, at 25° C for 10 minutes and numbers were adjusted to the desired concentrations with serum supplemented RPMI 1640 medium.

Natural killer cell cytotoxic assay.

The YAC-1 lymphoma cell line (kindly provided by Professor George Klein, Karolinska Institute, Stockholm, Sweden) was maintained in RPMI 1640 medium with 10% fetal bovine serum and used as the target cells. To label YAC-1, these cells were washed 3 times with RPMI 1640 medium by centrifugation at 200 × g, at 25° C for 10 minutes, then adjusted to 2×10^6 cells/ml in phosphate-buffered saline; 100 μCi $Na_2{}^{51}CrO_4$ (Amersham International, England) were added, followed by incubation at 37° C, humidified 5% CO_2 for 2 hours. These labelled cells were washed once and placed at 4° C in a refrigerator for at least 1 hour or until used. Before using these labelled YAC-1 cells, they were again centrifuged at 200 ×g at 25° C for 10 minutes and suspended in RPMI 1640 medium with 5% fetal bovine serum, adjusted to 1×10^5 cells/ml then kept at 4° C in a refrigerator until use. To prepare effector cells, splenic mononuclear cells were incubated in sterile plastic petri dishes (Nunc, Kamstrup, Roskilde, Denmark) at 37° C, in a 5% CO_2, humidified incubator for 1 hour to obtain a non-adherent splenic mononuclear cell suspension which was adjusted to 1×10^7 cells/ml with RPMI 1640 medium containing 5% fetal bovine serum. The cytotoxic assay was performed by mixing ^{51}Cr-labelled YAC-1 1×10^4 cells/100 μl with splenic non-adherent mononuclear cells 5×10^5 cells/100 μl (effector : target = 50:1) into each well of tissue culture plates (NUNC 96 wells, U type) in triplicate, with some wells measuring spontaneous release (^{51}Cr labelled YAC-1 1×10^4 cells/100 μl with RPMI 1640 medium 100μl) and other measuring maximum release (^{51}Cr labelled YAC-1 1×10^4 cells/100 μl with 10% Triton X 100 μl). All steps were done aseptically. These plates were incubated at 37° C in a humidified 5% CO_2 atmosphere for 4 hours. The supernatant (120 μl) from each well was collected separately into appropriately labelled vials. The isotope was counted by using a gamma counter (Gamma 5500, Beckman, Irvin, USA). The results were expressed as percent cytotoxicity according to the following formula :

$$\% \text{ cytotoxicity} = \frac{\text{experimental release} - \text{spontaneous release}}{\text{maximum release} - \text{spontaneous release}} \times 100$$

To study *in vitro* caffeine (5, 10, 20 and 40 μg caffeine/ml) effects on NK cell activities, these various caffeine concentrations were added in triplicate to appropriate wells of effector-target cell mixtures and to wells containing only YAC-1 cells. Other procedures were as described above for the cytotoxic assay.

Proliferation assay.

Mitogens used in this study were pokeweed mitogen (PWM) (1 and 10 μg/ml) and phytohemagglutinin-P (PHA-P) (1, 50 and 100 μg/ml). Ten percent heat-inactivated normal Sprague-Dawley rat serum supplemented RPMI 1640 culture medium, with 1.0×10^{-4} M 2-mercaptoethanol (Sigma, St. Louis, MO, USA) was used. Splenic mononuclear cells of chronically caffeine treated Sprague-Dawley rats were tested for *in vivo* effects while those from normal Sprague Dawley rats were studied for *in vitro* effects of caffeine. Briefly, a splenic mononuclear cell suspension was distributed at 2×10^5 cells/100 μl into each well of a 96-well flat-bottom tissue culture plate (NUNC, Kamstrup, Roskilde, Denmark) then appropriate concentrations of PWM or PHA-P per 100 μl were added in triplicate. Wells containing only 2×10^5 cells/200 μl culture medium represented control cultures. All steps were done aseptically in a biohazard laminar flow hood. The plates were covered with lids, gently shaken and placed in a 5% CO_2 humidified atmosphere at 37° C for 3 days. Then 0.5 μCi/20 μl of ^3H-thymidine (2.0 Ci/m mol, New England Nuclear Boston, MA, USA) was added to each well and incubation

continued for another 18 hours. Cells in each well were harvested on glass fiber filters (Grade 934 AH, Reeve Angle Clifton, NJ, USA) using an automatic cell harvester (Skatronas, Lier, Norway). The labelling isotope was detected by liquid scintillation counter (LKB 1219 Rack Beta) and the stimulation index (SI) was calculated by following formula :

$$SI = \frac{\text{mean cpm stimulated culture}}{\text{mean cpm control culture}}$$

RESULTS

All four groups of caffeine treated rats (0, 2, 6 and 18 mg/kg/day) were tested for their *in vivo* NK cell cytotoxic activity against YAC-1 cells and by the proliferative response to PWM and PHA-P stimulation concurrently. Control Sprague-Dawley rats demonstrated a mean % cytotoxicity of splenic NK cell activity against YAC-1 cells at E:T (effector: target) 50:1 of 32.6% (Fig. 1), a mean stimulation index by PWM (10 μg/ml) and by PHA-P (50 and 100 μg/ml) of 36 (Fig. 2), 29.8 and 15.7 (Fig. 3) respectively. In 2 mg/kg/day caffeine (low dose) treated rats, no changes of NK cell cytotoxicity or proliferative stimulation index of PWM and PHA-P stimulation were observed. However, in 6 mg/kg/day caffeine (midle dose) treated rats, the % cytotoxicity of splenic NK cells and the stimulation index by PWM were significantly ($P < 0.05$) decreased (Fig. 1, 2). No such inhibitory effect of caffeine was observed for PHA-P stimulation at all concentration tested. Nevertheless, in the 18 mg/kg/day caffeine (high dose) treated rats, neither decrease in % cytotoxicity of NK cells activities nor PWM stimulation index were observed but the PHA-P stimulation index (50 and 100 μg/ml) was significantly ($P < 0.05$) increased (Fig. 3). The *in vitro* effect of caffeine on NK, B and T cell activities showed a different pattern. For 4 concentrations of caffeine (5, 10, 20 and 40 μg/ml) added directly into cultures no interfering effect

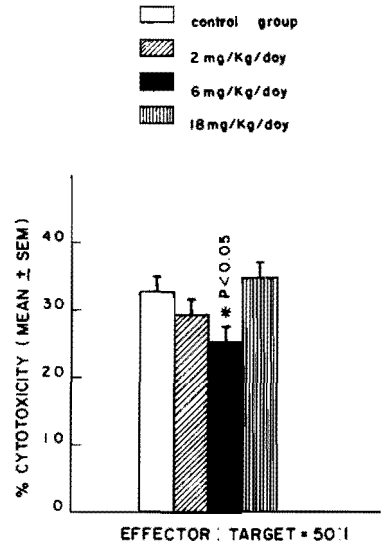

Fig. 1 The percent cytotoxicity of control and chronic caffeine treated rat NK cell activities against yac–1 in A 4–hour cytotoxicity assay.
*Statistical analysis against non–caffeine treated control group (Mann–Whitney U test).

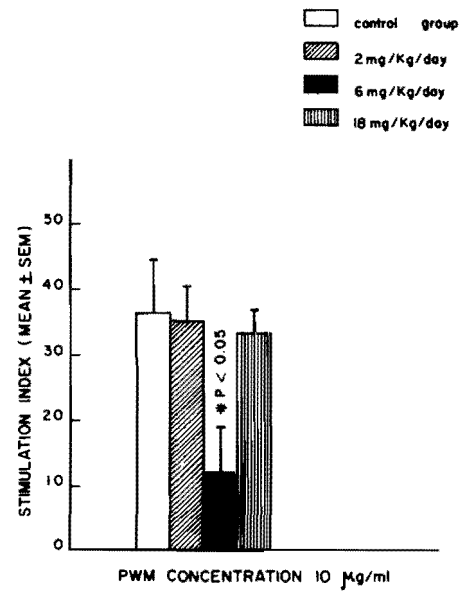

Fig. 2 The mean (± SEM) stimulation indices of control and chronic caffeine treated rat splenic mononuclear cells stimulated by PWM.
*Statistical analysis against non–caffeine treated control group (Mann–Whitney U test).

could be observed on NK cell activity (Table 1). On the other hand, caffeine exerted an inhibitory effect on both B and T proliferation (Table 2) due to stimulation with PWM (1 μg/ml) and PHA-P (50 μg/ml), respectively. This inhibitory effect showed a dose dependent pattern (Table 3).

DISCUSSION

Chronic caffeine treatment (the *in vivo* effect) in Sprague Dawley rats exhibited dose-dependent effects on splenic NK and mononuclear cell activities as shown by both enhancing and inhibiting effects. This pattern of effects of chronic caffeine consumption on NK, B and T cell activities concurs with the study of Mohr [13] who reported an inverted dose response relationship of chronic caffeine treatment and tumor incidence with respect to both frequency and multiplicity. Possible explanations for the inhibiting effect on NK and B cell activities may involve either the quantity or the distribution of surface membrane receptors, especially on NK cells involved in target cell recognition and receptors on B cells for PWM stimulation. This possibility could be clarified by the enumeration of NK and target cell binding activities. Alternatively, chronic caffeine consumption could interfere with the level of intracellular Ca^{2+} concentration, as it induced an increase in skeletal and muscle cells.[5,14] This may also occur inside NK and B cells, affecting the NK and B cell activities. Nevertheless, chronic caffeine consumption may result in a lower level of mononuclear cells, especially NK cells and B cells, than in normal spleen: quantitation of NK or B cells by using specific surface markers (e.g. CD 16 or CD 19) should be helpful. In contrast to this inhibition effect of chronic caffeine (6 mg/kg/day) consumption on NK and B cells activities, an enhancing effect could be observed on T cell proliferation in the group of rat treated with 18 mg caffeine/kg/day. Two PHA-P concentrations of 50 and 100 μg/ml were shown to be affected (Fig. 3). In general, the optimal PHA concentration stimulates all kinds of T lymphocytes while it is suggested that the high concentration stimulates preferentially some T cell subpopulations. Thus, changes of T cell subpopulations may possibly occur in Sprague Dawley rats treated with 18 mg/kg/day caffeine. However, hyperreactivity of T lymphocytes as a result of this caffeine high dose treatment for 120 consecutive day should be considered too.

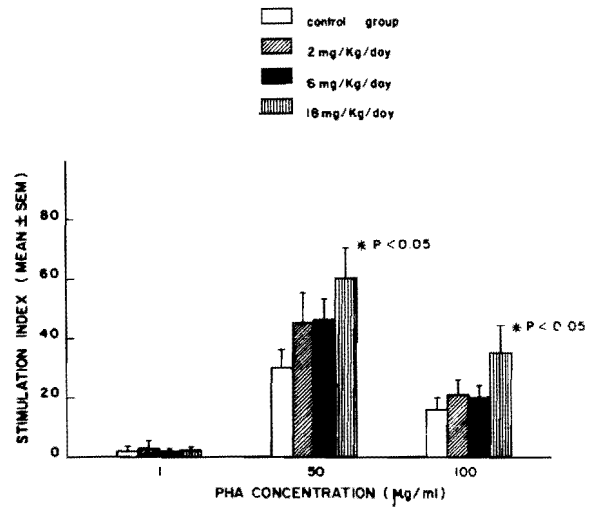

Fig. 3 Histogram shows stimulation indices of control and chronic caffeine treated rat splenic mononuclear cells by different doses of PHA–P
*Statistical analysis against non caffeine treated control group (Mann–Whitney U test).

Table 1. The effects of various concentrations of caffcine on rat NK cell activities in the *in vitro* assay system.

Caffcine cone (μg/ml)	Group size (n)	a % Cytotoxicity (Mean ± SEM)	b p–value
0	13	35.4 ± 2.2	> 0.05
5	13	30.5 ± 2.8	> 0.05
10	13	31.3 ± 2.8	> 0.05
20	12	28.2 ± 2.2	> 0.05
40	13	24.6 ± 2.6	> 0.05

[a]effector : target 50:1
[b]compared with value of caffeine conc. O μg/ml by Mann–Whitney U test.

Table 2. The effects of various concentrations of caffeine in the assay system on rat lymphocyte proliferative responses.

Caffcine conc. (μg/ml)	Without mitogen			With PWM	With PHA
	Group size (n)	a cpm	a SI	SI	SI
0	13	437.7 ± 44.9	1.00 ± 0.00	40.2 ± 4.4	31.6 ± 4.9
5	13	340.2 ± 46.8	0.84 ± 0.11	28.8 ± 3.8	20.8 ± 4.3
10	13	323.9 ± 53.5	0.78 ± 0.13	27.3 ± 3.4*	18.3 ± 3.7*
20	13	325.5 ± 42.9	0.80 ± 0.10	28.1 ± 2.6*	19.5 ± 2.6*
40	13	312.4 ± 46.0	0.75 ± 0.11	24.5 ± 3.1*	17.1 ± 3.2*

Final conc. PWM = 1 μg/ml; PHA–P 50 μ/ml
[a]Mean ± SEM
*Statistically significant by Mann–Whitney U test against the SI value at caffeine conc. O μg/ml ($p < 0.05$).

Table 3. The percent inhibition of stimulation indices of various concentrations of caffeine of rat lymphocyte proliferative response.

Caffeine conc. (μg/ml)	Group size (n)	% inhibition	
		PWM	PHA–P
1	10	13.0	8.8
10	10	36.0	28.8
100	10	82.3	75.6
1000	10	96.8	97.4

$$\%\text{inhibition} = 100 - \left(\frac{\text{SI of culture with caffeine}}{\text{SI of culture without caffeine}} \right) \times 100$$

The *in vitro* caffeine effect in this study suggested that caffeine itself (5-40 μg/ml, doses which were in the range of caffeine concentrations appearing in plasma according to caffeine using for feeding rats in this study) did not have any direct interfering effect on any step of NK cell cytolysis. In spite of that, caffeine at the same concentrations showed inhibitory effects on both B and T cell proliferation, which also demonstrated a dose-related inhibition (1-1,000 μg/ml). The possible mechanisms for these inhibitory effects include the following. Firstly, caffeine at certain concentration can interfere with biochemical events at the membrane, such as polyphosphoinositide hydrolysis or protein kinase C activation which were affected by changes of intracellular Ca^{2+} level induced by caffeine.[15] Secondly, caffeine may induce the accumulation of cAMP (caffeine acts as phosphodiesterase inhibitor), cell proliferation being prevented by the increased cAMP level.[16,17] Thirdly, it is possible that caffeine may interfere with biological activities of macrophages, such as the release of interleukin-1 (IL-1) or prostaglandin (PGE2) which would affect B and T cell proliferation. However, the exact mechanism(s) and the dose effect relationship need to be studied with possible application to the manipulation of B and T cells activities for the treatment of certain diseases.

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