STUDY ON THE *IN VIVO* AND *IN VITRO* EFFECT OF CAFFEINE ON RAT IMMUNE CELL FUNCTIONS : B CELLS, T CELLS AND NK CELLS

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ชื่อวิทยานิพนธ์ การศึกษาผลของสารคาเฟอีนต่อหน้าที่การทำงานของภูมิคุ้มกันโดยเซลล์ในสัตว์ทดลองหนูและในห้องปฏิบัติการ

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บทคัดย่อ

คาเฟอีนเป็นสารประกอบที่พบได้ในกาแฟ ชา โกโก้ โคล่า และในรูปของส่วนประกอบของยาชนิดต่างๆมีรายงานการวิจัยถึงความสัมพันธ์ของการได้รับคาเฟอีนและ/หรือ กาแฟกับการเกิดมะเร็งของอวัยวะต่างๆในร่างกายของคนและสัตว์ทดลอง อย่างไรก็ตามมีรายงานอื่น ๆ คัดค้านเกี่ยวกับเรื่องนี้ การทดลองนี้ได้ศึกษาถึงผลของคาเฟอีนต่อความสามารถของเซลล์ในระบบภูมิคุ้มกัน (natural killer, B และ T เซลล์) โดยได้ทำการศึกษาในหนูพันธุ์ Sprague-Dawley ในภาวะของการได้รับคาเฟอีนติดต่อกันอย่างเรื้อรัง (120 วัน) และผลโดยตรงของคาเฟอีนต่อ natural killer (NK), B และ T เซลล์

ในภาวะของการได้รับคาเฟอีนติดต่อกันอย่างเรื้อรังด้วยความเข้มข้นต่างๆ กันคือ 2, 6 และ 18 มิลลิกรัม/กิโลกรัม/วัน ซึ่งเทียบได้กับผู้ที่ดื่มกาแฟ 1-2, 3-4 และ 9-10 ถ้วย/วัน พบว่า ในหนูกลุ่มที่ได้รับคาเฟอีน 6 มิลลิกรัม/กิโลกรัม/วัน นั้น ความสามารถของ NK เซลล์ และการตอบสนองของ B เซลล์ ต่อ pokeweed mitogen (PWM) มีค่าลดลงอย่างมีนัยสำคัญทางสถิติ ($p < 0.05$) เมื่อเทียบกับกลุ่มควบคุม ในขณะที่การตอบสนองของ T เซลล์ ต่อ phytohemagglutimin-P

(PHA-P) ในหนูกลุ่มที่ได้รับคาเฟอีนความเข้มข้นสูงสุดคือ 18 มิลลิกรัม/กิโลกรัม/วัน มีค่าสูงกว่าที่พบในกลุ่มควบคุมอย่างมีนัยสำคัญทางสถิติ ($p < 0.05$) อย่างไรก็ตาม จำนวนเม็ดเลือดขาวและเปอร์เซนต์ของเม็ดเลือดขาวชนิดต่าง ๆ ในหนูทดลองทั้ง 3 กลุ่ม มีค่าไม่แตกต่างจากกลุ่มควบคุม ($p > 0.05$)

การศึกษาผลโดยตรงของคาเฟอีนต่อ NK, B และ T เซลล์ ด้วยความเข้มข้นต่าง ๆ กันคือ 5, 10, 20 และ 40 ไมโครกรัม/มิลลิลิตร พบว่า การตอบสนองของ B และ T เซลล์ต่อ PWM และ PHA-P มีค่าลดลงอย่างมีนัยสำคัญทางสถิติ ($p < 0.05$) ที่ความเข้มข้นของคาเฟอีน 10, 20 และ 40 ไมโครกรัม/มิลลิลิตร เมื่อเทียบกับภาวะที่ไม่มีสารคาเฟอีน อย่างไรก็ตามการเปลี่ยนแปลงนี้ไม่พบในการตรวจหาความสามารถของ NK เซลล์ นอกจากนี้ยังพบว่าเมื่อเพิ่มความเข้มข้นของคาเฟอีนเป็น 1, 10, 100 และ 1,000 ไมโครกรัม/มิลลิลิตร การยับยั้งการตอบสนองของ B และ T เซลล์ ต่อ PWM และ PHA-P มีความสัมพันธ์กับการเพิ่มความเข้มข้นของคาเฟอีน

Thesis Title	Study on the *in vivo* and *in vitro* effect of caffeine on rat immune cell functions: B cells, T cells and NK cells
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ABSTRACT

Caffeine is a composition of coffee, tea, cocoa, cola drink and some medicines. A number of studies reported an association between caffeine and/or coffee consumption and cancer development, but controversy still exists for this effect of caffeine. This study tested for the effect of caffeine both *in vivo* and *in vitro* on Sprague-Dawlay rat immunological cell activities. Natural killer (NK) cell was studied by ^{51}chromium release cytotoxicity assay, and B and T cells were studied by lymphocyte proliferation assay.

In vivo effect was studied in chronic caffeine treated condition. Caffeine was given to three groups of rats at three different doses, *i.e.*, 2, 6 and 18 mg/kg/day (equivalent to caffeine in coffee consumption 1-2, 3-4 and

9-10 cups/day, respectively). Both NK cell cytotoxicity activity and B cell proliferative response to pokeweed mitogen (PWM) showed significant decrease ($p < 0.05$) in the group of rat treated with middle dose of caffeine (6 mg/kg/day). Whereas, the T cell proliferative response to phytohemagglutimin-P (PHA-P) was significant increased ($p < 0.05$) in the group of rat treated with high dose of caffeine (18 mg/kg/day). In addition, there was no difference ($p > 0.05$) in total and differential leukocyte count between all three groups of caffeine treated rats and control rats.

The direct (in vitro) effect of caffeine was studied by adding caffeine at the final concentrations of 5, 10, 20 and 40 ug/ml into the assay system. The results showed the significant decrease ($p < 0.05$) of B and T cell proliferative responses to PWM and PHA-P at caffeine concentrations of 10, 20 and 40 ug/ml. However, this effect was not observed in NK cell cytotoxicity activity. Furthermore, when the broader range of caffeine was tested (1, 10, 100 and 1,000 ug/ml), caffeine also exerted the dose-dependent inhibition on B and T cell proliferative responses.

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CHAPTER I

INTRODUCTION

Caffeine is a naturally occurring plant methylxanthine. It is a component present in many types of foods, beverages and medicines. Caffeine-containing beverages such as coffee, cocoa, chocolate and cola drink (Pepsi, Coke) are commonly consumed and quite popular in nearly all countries. One reason for their popularity is the stimulating effect of caffeine. In medicines, it is present in many combination drugs used as stimulants, pain relievers, diuretics, cold remedies and weight control products. The Food and Drug Administration (FDA) lists caffeine as Generally Recognized As Safe (GRAS). Since then, caffeine has been reviewed by the Select Committee on GRAS substances of the Federation of American Societies for Experimental Biology and The Flavor and Extractor Manufacturer Association's Expert Panel. After the 1980 inspection of all data available by the FDA, caffeine is no longer considered as GRAS but it is placed at an interim food additive status (1). Apart from the above effects of caffeine, controversy still exists in term of the correlation of caffeine and/or coffee consumption and cancer development in some organs, for example, urinary bladder (2), pancreas (3, 4), colon (5) and breast (6).

It is evident that many factors play the roles in tumour development. These can be categorised into 2 main groups. The first is the inherent carcinogenicity of the substance and the second is the unbalance of the immunological homeostasis (7, 8). To learn whether caffeine has any role on the activities of immunological cells which may lead to the disturbance of immune mechanism, the effect of chronic caffeine consumption (in vivo) and the direct effect of caffeine (in vitro), at various concentrations, on B, T and NK cell activities were determined in this study. An animal model using adult male Sprague-Dawley rats was employed for this study.

The interfering effects of three different doses of caffeine consumption on immune cell functions were studied in chronic caffeine treated (120 days) rats (in vivo effect). The direct interfering effect of caffeine (in vitro effect) was studied separately on normal rat immune cells. Proliferative responses to mitogen stimulation were measured to assay for B and T lymphocyte activities and the conventional ^{51}Cr cytotoxic assay against YAC-1 was performed to study NK cell activities. The morphological differentiation was studied to enumerate leukocytes. It was found that in chronic caffeine consumption, the activities of both NK cells and B lymphocytes showed maximal activity decreases at the same caffiene doses. In contrast, caffeine exerted an enhanced activity on T lymphocyte at the high dose. In in vitro however, caffeine exerted a dose-dependent inhibition of B and T lymphocyte activities, which was not observed in NK cell function.

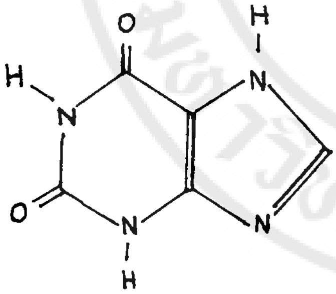
CHAPTER II

BACKGROUND

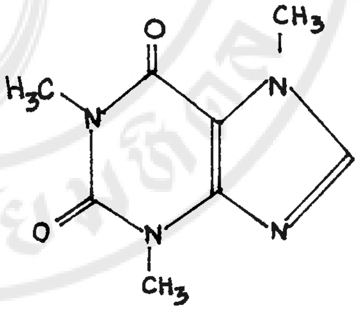
1. Caffeine

1.1 Chemistry and sources

Caffeine is consumed world-wide. It is a methylated xanthine (1, 3, 7-trimethylxanthine) with chemical formula $C_8H_{10}N_4O_2$ and molecular weight 194.19. It is composed of C 49.48%, H 5.19%, N 28.85%, O 16.48% (9). The structural formulas of xanthine and caffeine are as follow:



xanthine



caffeine

The solubility of caffeine is low and enhanced by complex formation, such as caffeine and sodium benzoate (1:1). In biological fluids, this salt can be dissolved and dissociated to yield caffeine. It can be found in many beverages and some kinds of drugs. The average caffeine content per drink is 12.5-169.0 mg for instant coffee, 39.8-110.4 mg for brewed coffee, 9.1-51.1 mg for tea (10), 5-10 mg for cocoa, 6 mg/ounce for solid milk chocolate, and 32-65 mg/12 ounce for cola drink (11). In drugs, the average per unit is 15-64 mg for cold tablets, allergy or analgesic preparations, 50-200 mg for appetite suppressants, and 100-200 mg for appetite stimulants (3).

1.2 Pharmacological effects

1.2.1 Absorption and excretion

At physiological pH, caffeine is un-ionized, stable and can be absorbed through permeating biological membrane. More than 99% of an oral administration is absorbed and peak plasma level is achieved within 1 hour. In humans, after a 250 mg dose intake, the peak plasma concentration is between 5 and 25 μg/ml (12). Similarly, after a 10 mg/kg dose intake in 40-day old rat the peak plasma concentration is 9.22 ± 0.41 μg/ml (13). Caffeine is poorly bound to plasma protein. The half-life of caffeine in plasma is variable among persons, the range is 3.0-7.5 hours (14, 15). Caffeine is eliminated primarily via metabolism in liver. Approximately 0.5-3.5% of administered dose is excreted unchanged in urine (16). In man, the primary metabolic pathway is N-3 demethylation and yields 1, 7-dimethylxanthine. The main excreted metabolites in urine are 1-methyluric acid, 1-methylxanthine, small amount of 1, 7- dimethyluric acid, 7-methylxanthine and 1, 7-dimethylxanthine (14). The rates of caffeine biotransformation and clearance are different within species and even also different within individual, for instance it is very slow in newborns and in severe liver diseases.

1.2.2 Central nervous system (CNS)

Caffeine is a potent stimulant of the CNS. It can induce less drownsiness, less fatigue, and alertness. At high dose, it provokes nervousness, restlessness, insomnia, tremors, hyperesthesia and other signs of CNS stimulation. It also has effects on mood, for example, in normal volunteers the feeling of alertness and anxiety can be observed (17). The effect on mood seem to be strongly influenced by individual variations of sensitivity as well as acquired tolerance to the drug.

1.2.3 Cardiovascular system (CVS)

Caffeine has prominent action on CVS. Robertson et al.(18) reported that after acute dose (250 mg) of caffeine ingestion, heart rate decreases during the first hour, then increases above the baseline during the next two hours. Five to ten percent increase in blood pressure is observed for 1-3 hours. Plasma epinephrine, norepinephrine, and plasma renin activity are also significantly increased. However, the chronic ingestion of caffeine has little or no effect on blood pressure, heart rate, plasma catecholamine level, and plasma renin activity in normal subjects (19).

1.2.4 Renal system

The acute ingestion of caffeine produces mild increase in urine volume and urinary sodium excretion in humans. It is suggested that the diuretic action of caffeine results form a decrease in renal tubular reabsorption of sodium and water (20).

1.2.5 Smooth muscle and skeletal muscle

Caffeine has an ability to relax smooth muscle of the bronchi, especially if the bronchi have been constricted by histamine or asthma. For skeletal muscle, it has been shown that at an approximate dose of 3.5 mg/kg, it increases twitch tension of the stimulated quadriceps muscle. The possible explanation for its mechanism is translocation of Ca^{2+} in striated muscle (21).

1.2.6 Metabolism

The dose of 3-9 mg/kg of caffeine can produce a slight increase (10%) in the basal metabolic rate of humans. Many studies support this observation. It seems quite certain that acute caffeine ingestion stimulates metabolism. However, there is no clear evidence indicating that chronic caffeine consumption has an influence on metabolism. Moreover, it seems to have a significant effect on plasma level of free fatty acids. Many studies have reported that acute caffeine ingestion increases serum free fatty acid 50-100% from normal values (22-23), even in habitual coffee drinking (24).

1.3 The carcinogenicity of caffeine

Many epidemiological studies have investigated the relationship of caffeine and/or coffee consumption on cancer development. In 1968, Dunham et al. (25) reported the first evidence of the correlation between urinary bladder cancer and coffee consumption. Subsequently, many studies showed risk of bladder cancer development in people who consume caffeine containing beverages. However, the studies have failed to show dose relationship (26-27). Some studies reported that this association, if any, is low (28-29) or negative (30). In 1981, MacMahon et al. (3) reported the strong association between coffee consumption and pancreatic cancer. Only a slight positive correlation between coffee intake and pancreatic cancer has been found by other (31). However, the effect of solvent using in decaffeination on cancer development is possible. Thus, caffeine obtained from coffee consumption may or may not be a carcinogen by itself and it may or may not interfere with some biological functions of cells, e.g., cells of the immunological system.

The development of breast cancer has been shown to be associated with caffeine and other methylxanthine intake. Minton et al. (6, 32) reported that methylxanthine consumption is associated with the development of fibrocystic breast disease in which the signs and symptoms often disappeared after elimination of methylxanthine from the diet. However, some variable in the group study, such as age of first pregnancy, number of children, method of contraception, and breast feeding practices were not considered. Lawson et al. (33) proposed the positive association between breast disease and coffee/tea consumption. Subsequent studies failed to demonstrate this association (5, 34, 35). In case-control studies, an increased risk of ovarian cancer among women who drink coffee was demonstrated (36, 37), but the results were not dose related (36), and other dietary habits were not examined. Lingeman (38) has reported the highest incidence of ovarian cancer in Swedes which have the highest coffee consumption per capita.

2. Biology of immunological cells

2.1 Lymphocytes

Lymphocytes are the subpopulation of leukocytes responsible for humoral and cell-mediated immune response. They are generally small (about 8-10 µm in diameter) with dense nuclei (39). The total numbers in human are approximately 2×10^{12}. Lymphocytes develop from pluripotent hemopoietic stem cells which are also the precursors of all blood cell types (40). In the fetus, the hemopoietic stem cells are located in the liver (41) and shift to the bone marrow in adult. Subsequently, they migrate via the blood circulation to central lymphoid tissue (thymus, bursa of Fabricius) that provide special microenvironments for the proliferation and differentiation into various types of lymphocytes (41-43). After development some lymphocytes die and others migrate from central- to peripheral lymphoid tissue (lymph node, spleen, and gut-associated lymphoid tissue), and are recognized as thymus-derived (T) lymphocytes and (in bird) bursa-derived (B) lymphocytes. T and B lymphocytes in the resting stage are very similar. However, they have different phenotypic markers that can be distinguished and separated, such as CD1, CD2 and CD3 on all T lymphocytes, CD4 on helper T lymphocytes, CD8 on cytotoxic/suppressor T lymphocytes, CD19, 20, 21 and 22 on B lymphocytes (44), etc. Lymphocytes in the circulation are committed and upon interaction with antigen, T or B lymphocytes will be activated and proliferate. These responses of T and B lymphocytes are oligo- and mostly polyclonal response (45).

In addition to antigens, mitogens can activate lymphocytes and induce them to mitosis. An agent that induces only early activation events, even DNA synthesis but not mitosis would not qualify as a mitogen (46). The common mitogens that are used for T lymphocyte activation are phytohemagglutinin (PHA) and concanvalin A (Con A), the lectins of *Phaseolus vulgaris* and *Canavalia ensifumis*, respectively. PHA binds N-acetyl-galactosamine-containing oligosaccharides and Con A binds saccharides containing a terminal D-mannose on T lymphocytes (46). For B lymphocyte activation, the common mitogens are lipopolysaccharide (LPS), pokeweed mitogen (PWM) and dextran sulphate (DxS). They are polyclonal B lymphocyte activators. LPS is a T lymphocyte independent B lymphocyte activator (47) while PWM is T lymphocyte dependent B lymphocyte activator (48). Both can activate B lymphocytes to proliferate and secrete immunoglobulin (49). The polyanion dextran sulphate (DxS) has been found to stimulate B lymphocytes (in a group overlapping with a group that responds to LPS) to proliferate rather than secrete immunoglobulin (50).

2.1.1 B lymphocytes

The expression of membrane-bound immunoglobulin on the surface is a common characteristic of B lymphocytes. They are mainly responsible for humoral immune response. The diversity of immunoglobulins produced by several million B lymphocyte clones react with numerous antigens (51). The precursor of B lymphocyte is the pre-B lymphocyte which produces cytoplasmic mu heavy chain inside the cell (52). Later, the light chain and delta heavy chain are synthesized (53). Both mu and delta heavy chains combine with light chain to from complete IgM and IgD molecules and cells expressing them on the surface are called mature B lymphocytes. Other expressed membrane-bound glycoproteins include receptor for complement component, Fc receptors, receptor for mitogen, <u>etc</u>. Some B lymphocytes switch from the expression of IgM and IgD to IgG, IgA and IgE (53). One of the most important surface components is Ia antigen (I-region-associated antigen), Class II MHC antigen. These molecules are polymorphic glycoproteins which function as recognition elements in the interaction with antigen-activated T lymphocytes (54). Some activated B lymphocytes do not undergo terminal differentiation but remain as memory cells (55). They are easily triggered by the previously sensitized antigen.

2.1.2 T lymphocytes

T lymphocytes play an important role in cell-mediated immune responses. There are at least three different subpopulations of T lymphocytes in regard to their functions: cytotoxic T lymphocyte (T_C); helper (or inducer) T lymphocytes (T_H); suppressor T lymphocyte (T_S).

There are two types of T_C: antigen-specific, MHC-restricted T_C that recognize Class I MHC glycoprotein associated with antigen on the target cells via T3/Ti antigen receptors (56, 57) and non-MHC restricted T_C that also recognize antigen on target cells via T3/Ti antigen receptors (57). For virus-infected cells, the viral antigens associated with Class I MHC glycoproteins and are recognized by the first type of T_C. In transplantation reactions, because the MHC is not compatible, the reactions are mediated by non-MHC restricted T_C, the second type T_C. Helper T lymphocytes are genetically programmed to induce (help) other cell types to divide and differentiate. After processing of antigens by antigen presenting cells (APC), the fragments of antigens are associated with Class II MHC glycoproteins (58). T_H recognize these antigen-MHC complexes through T3/Ti antigen receptors and are activated (59). Activated T_H are able to induce immune responses through the secretion of nonspecific peptides, e.g., IL-2, B cell growth factor (BCGF), B cell differentiation factor (BCDF) and T cell replacing factor (TRF). These can activate B lymphocytes to divide and secrete antibodies (60, 61). Suppressor T lymphocytes have negative effects on immune response. They can function only after being activated by T_H. Activated T_S secrete suppressor factor which can interact with T_H and inhibit the response of T_H to antigens (62), a phenomenon known as feedback inhibition. This feedback circuit is useful in self-regulation. The suppressor factor is a 70,000-dalton protein that has two subunits, the 45,000-dalton subunit and the 24,000-dalton subunit. Picogram amounts of suppressor factor are able to specifically turn off the ongoing immune response (63).

2.2 Natural killer (NK) cells

2.2.1 Characteristics and distribution of NK cells

A subpopulation of mononuclear cells with capacity to lyse certain types of tumour cells and normal cells was termed "natural killer cells" by Kiessling et al. (64). Lysis occurs without prior exposure to target cells. NK cells have been found in many vertebrates, e.g., man, mouse, hamster, rat, chicken, guinea pig and miniature swine. By using Giemsa stain, they appear as medium sized (12-15 μm), mononuclear cells with high ratio of cytoplasm to nucleus and eccentric slightly indented nuclei. The most distinctive characteristic is the azurophilic granules (large granular lymphocyte, LGL) in their cytoplasm that are thought to be involved in cytotoxic activity. They have surface receptors for the Fc portion of IgG leading to a capacity for antibody-dependent cytotoxicity (ADCC). These cells share some cell surface antigens with T lymphocytes, e.g., Thy 1 antigen in mice, and CD7, CD8, CR_3 and Leu9 in humans (65). However, T3/Ti antigen receptor genes do not rearrange or express functional transcription products (66, 67). The NK cell lineage is still controversial; some researchers have proposed a third lineage of lymphoid cells (65) while others suggest the relationship between NK cells and T lymphocytes (68, 69). In mouse, the distribution of NK cells is high in peripheral blood and spleen, but low in lymph node, peritoneum and bone marrow. There are no NK cells in thymus (64). In rat, the pattern of distribution is similar to mouse but there is no detectable activity in bone marrow cells (70).

2.2.2 Functional characteristics

NK cells play a direct role in resistance to viral infections and an indirect role in bacterial infections. In virus-infected mice their activities appear early, peaking before the peak of virus-specific cytotoxic T cells (71) and antiviral ADCC (72). They also play an important role in natural resistance to infection by mouse cytomegalovirus (73). For parasitic infections, they have natural cytotoxic effect on Trypanosome cruzi and malarial parasites (74, 75).

In tumour surveillance, they have potential roles in the rejection primary tumours. Several lines of evidence showed that NK cell activities decline in the advanced stages of malignant disease (76, 77). Patients with Chediak-Higashi syndrome show a marked deficiency in NK activity, and have a high incidence of lymphoproliferative disease (78); similarly for beige (bg) mutation mice (79). Considerable evidence suggests the role of NK cells in transplantation, for instance, haemopoietic graft (80), kidney graft (81) and in graft-versus-host (GVH) disease (82).

NK cells themselves are able to function as immunoregulatory cells. Many studies demonstrated the regulatory effect on hemopoiesis of myeloid and erythroid cells (83, 84). They suggested that there is a common determinant between human hemopoietic progenitor cells and K562 cell line (85). Another regulatory role is postulated to be involved in antibody homeostasis (86). There is evidence indicating that this regulatory role is mediated by the production of cytokines such as interferon (87) and interleukin-2 (88) which have effects on other cells and on NK cells themselves.

2.2.3 Mechanism of NK cell lysis

The killing process by NK cell can be divided into three stages.

i) Binding of the NK cell to the target cell

Primary recognition and conjugation between NK cell and target cell demonstrates the involvement of a lectin-type receptor on the NK cell and specific, differentiation-associated, carbohydrate moieties on the target cell (89). It occurs rapidly (< 1 min) at 4 °C as well as at 37 °C. This is a Mg^{2+}-dependent, Ca^{2+}-independent stage (90) and does not require energy.

ii) Programming for lysis

This stage occurs within 10-30 minutes and is Ca^{2+}- (91) and temperature-dependent, which is efficient at 37 °C and requires energy (92, 93). At this stage, the NK cell is activated and natural killer cytotoxic factor (NKCF) is released (94). This NKCF will bind to NKCF receptors on the target cell.

iii) Killer cell-independent cytolysis (KCIL)

This stage can be completed in 20-30 minutes after dissociation of effector from target cell. Target cell will be lysed by cytotoxic activity of NKCF. Recently, Tschopp and Conzelmann (95) proposed that the lytic mechanism may due to perforins, which are complement-like components. They polymerize on target cell and form transmembrane pores.

2.2.4 Modulation of NK cell activity

All types of species-specific interferon (IFN; α, β, γ) can augment NK cell function. After brief exposure to IFN, NK cells can kill a broader range of target cells with high efficiency (71, 96). Several studies have shown that there are three possible mechanisms for IFN enhancement: (i) increased recruitment of non-lytic pre-NK cells to become cytotoxic cells; (ii) increased kinetics of lysis by individual NK cells; and (iii) increased recycling ability (97, 98). IFN and IFN inducers such as poly(inosinic)-poly(cytidylic), BCG, C. parvum and retinoids are used as NK immunopotentiators. Interleukin-2 (IL-2) can also enhance NK cell function (99). In addition IL-2 induces the ability of NK cells to kill tumour target cells which are resistant to freshly-isolated NK cells.

IFN may suppress NK cell activity via the production of prostaglandins by many cell types (100). Several groups have indicated that prostaglandins depressed NK cell activity (98, 101). Another source of prostaglandins is tumour cells (102). Many tumours in animals and man cause elevation of prostaglandins and thereby decrease the NK cell activity. Furthermore, the activity of NK cells can be inhibited by other agents such as histamine and theophylline which elevate cyclic adenosine monophosphate (cAMP) which in turn promote the release of prostaglandins and other mediators (103).

2.3 Monocytes/Macrophages

Monocytes and macrophages develope from hemopoietic stem cells via the monoblast lineage in bone marrow (104). Monocytes in bone marrow enter the blood circulation and migrate to various tissues for further development into tissue macrophages. Macrophages can be found in many tissues, for instance, Kupffer cells in liver, alveolar macrophages, tissue histiocytes and microglia in central nervous system. The monocytes are less efficient in microbial killing than macrophages (105). There are a number of cell surface antigens on monocytes such as CD9, CD11b, CD11c and CD35. Some macrophage surface markers are CD39 and CD45 (44). However, these are not specific markers because they also are expressed on other leukocytes.

Macrophages participate in different functions of the immune response: (a) as scavengers to remove damaged or dying cells and nonmetabolizable inorganic materials; (b) killing microorganisms, particularly obligate intracellular microorganisms; (c) as accessory cells for lymphocyte activation; (d) as secretory cells which release some bioactive products that regulate other cellular functions; and (e) as cytocidal agents in the control of neoplasia (106). Macrophages act as accessory cells in the induction of immune response by antigen processing and presentation to T lymphocytes. The binding of antigens to accessory cells occurs by either specific receptors such as IgGFc and C_3 receptor or via non-specific, non-covalent interactions of antigens to undefined structures on the accessory cell membrane. The model for antigen processing prior to presentation involves antigen binding to receptors and activating the catalytic steps (107). These in turn involve the uptake and internalization of antigens within phagosomes followed by fusion of antigen-containing phagosomes with lysosomes. Antigen is degraded to antigenic fragments which are transfered to the cell membrane in the form of antigen-Class II MHC glycoprotein complexes. T3/Ti antigen receptors on T lymphoctye interact with these complexes and induce T lymphocyte activation (59). The activated T lymphocytes then can activate macrophages via either macrophage-T lymphocyte interaction or lymphokines secretion. Activated macrophages can eliminate antigen efficiently and can synthesize and secrete a number of bioactive products such as neutral protease, collagenase, elastase and plasminogen activator (105).

2.4 Granulocytes

Granulocytes develop from pluripotential hemopoietic stem cells in bone marrow, which subsequently divide and differentiate to myeloblasts, promyelocytes and myelocytes. The mature cells in the myeloid series that circulate in the blood are granulocytes. There are three types of granulocytes: neutrophils, eosinophils and basophils, according to the affinity of Romanovsky stains to their cytoplasmic granules.

a) Neutrophils

Neutrophils contain two types of cytoplasmic granules: the azurophilic granules (lysosomal granules) and specific granules which contain lysozyme, collagenase, iron-binding protein, lectoferrin, a vitamin B_{12}-binding protein and neutral proteases. Within a few hours of bacterial infection and the extensive necrosis of tissue, neutrophils migrate to this site, phagocytose and digest particles. The phagocytic process consists of the attachment of the particles to the cell membrane and ingestion of particles. The attachment involves membrane recognition of the particles by specific- (C_{3b} and immunoglobulins attach to C_{3b} or Fc receptor on neutrophils) or non-specific binding sites. The cell then engulfs the particle and a phagosome is formed. Granules fuse with the phagosome and release their contents: this is important for killing and digestion of bacteria (108). For non-phagocytosable particles, the granule contents are secreted to outside by fusion of the granules with the cell membrane.

b) Eosinophils

These cells have segmented lobular nuclei with large eosinophilic cytoplasmic granules. They contain more than one type of granule: large eosinophilic specific granules (which contain a bar of crystalloid and cationic proteins) and small granule (which are rich in enzymes). They possess both Fc and C_{3b} receptors (105). Eosinophils are able to phagocytose but their ingestive capacities are not as good as neutrophils. Blood eosinophilia is found in IgE-mediated hypersensitivity and helminthic infection, e.g., schistosomiasis (109).

c) Basophils

These cells have lobular nuclei with large crystalline granules. They possess receptors on the cell membrane specific for the Fc portion of IgE (110). The circulating basophils represent only a small proportion of granulocytes, however, a high number of basophils are found in tissue as mast cells. When basophils or mast cells become activated by the bridging of at least two IgE surface receptors (110), the activated cells release a variety of mediators such as histamine, eosinophil chemotactic factor of anaphylaxis, neutrophil chemotactic factor and slow reacting substance that can induce clinical state of anaphylaxis, profound shock associated with increased vascular permeability, contraction of smooth muscle, influx of inflammatory cells, etc.

CHAPTER III

MATERIALS AND METHODS

1. Animals and diets

Male, weanling Sprague-Dawley rats (The National laboratory Animal Centre, Mahidol University) at 2 months of age, weighing 150-200 g were used in the study. The animals were housed five per cage in stainless-steel cages. They were fed commercial laboratory rodent chow and clean drinking water *ad libitum*. All of them were maintained in ventilated room throughout this study.

2. Caffeine treatment

Caffeine (Sigma, St. Louis, MO, U.S.A.) composed of 50% caffeine and 50% benzoic acid was used. The animals were divided into four groups.

a) Control group, no caffeine treatment,

b) 2 mg/kg/day caffeine treatment,

c) 6 mg/kg/day caffeine treatment, and

d) 18 mg/kg/day caffeine treatment (for calculation of caffeine doses see appendix)

The chronic caffeine consumption was controlled by feeding rats with various doses of caffeine every day for at least 120 consecutive days.

3. Preparation of splenic mononuclear cell suspension

On the day of experiment, rats were anesthetized with diethyl ether (MERCK, Darmstadt, F.R. Germany) and then sacrificed by cardiac puncture. Spleens were removed aseptically immediately, kept in sterile petri dishes with cold culture medium and cut into small pieces with scissors. Then by using the closed end of sterile U shaped test tube as a plunger the small pieces of rat spleen were teased through a sterile stainless steel sieve into approximately 5-7 ml RPMI 1640 medium (Gibco, OH, U.S.A.). All steps for spleen cell preperation were done aseptically in the laminar flow hood (Biohazard, Gelaire). The working culture medium used is RPMI 1640 supplemented with 100 units penicillin/ml (MERCK, Rathway, NJ, U.S.A.), 100 µg streptomycin/ml (Glaxo, Samut Prakarn, Thailand), 2 mM L-glutamine, 2 g/1 $NaHCO_3$ (Sigma) and 10 mM HEPES buffer (Sigma). Splenic mononuclear cell suspension was washed once with RPMI 1640 medium by centrifugation at 400 g at 25 °C for 10 minutes. The pellet was resuspended with RPMI 1640 medium and mononuclear cells were separated using Ficoll-Hypaque mixture as described by Boyum (111). Ficoll-Hypaque contained 24 parts sterile 9% Ficoll (Ficoll 400, Pharmacia Fine Chemicals AB, Uppsala, Sweden) and 10 parts sterile Hypaque Sodium 50% (Wintrop Laboratories, New York, NY, U.S.A.), final density 1.007 g/cm^3. After centrifugation at 400 g for 30 minutes at 25 °C, mononuclear cells at the interface were gently removed into a 15 ml centrifuge tube and washed 3 times with RPMI 1640 medium by centrifugation at 400 *g*, 25 °C, 10 minutes. These splenic mononuclear cells were adjusted to the desired concentration with RPMI 1640 medium supplemented with 5% fetal bovine serum for natural killer cells or with 10% pooled rat serum for lymphocyte proliferation.

4. ^{51}Chromium release natural killer cell cytotoxicity assay

4.1 Target cells preparation

YAC-1 lymphoma was kindly provided by Professor George Klein, Kalorinska Institute, Stockholm, Sweden. It is a tissue culture cell line derived from a Moloney virus-induced lymphoma in A/Sn mice (112). The cells were maintained continuously in *in vitro* suspension culture of RPMI 1640 culture medium supplemented with 10% heat-inactivated fetal bovine serum (Gibco) and cultured in a humidified atmosphere of 5% CO_2 at 37 °C in an incubator. To assay for NK cell activity, radiolabelling of target cells was performed on the day of experiment as described by Kiessling *et al.* (113). Briefly, target cells were washed 3 times with complete RPMI 1640 culture medium (RPMI 1640 medium supplemented with 5% fetal bovine serum) by centrifugation at 200 *g*, 25 °C, for 10 minutes. The pellet was resuspended in phosphate- buffered saline (PBS, see appendix). Viability of the cell was checked by using the Trypan blue dye exclusion (114). Target cell viablility for the assay was at least 95%. The washed YAC-1 target cells were adjusted to a concentration of $2x10^6$/ml. Then 100 µCi

$Na_2{}^{51}CrO$ (specific activity = 250-500 mCi/mg, Amersham International, Amersham, England) was added and incubated in a 5% CO_2, humidified incubator at 37°C for 2 hours with occasional gentle mixing. Labelled target cells were washed twice with complete RPMI 1640 culture medium by centrifugation at 200 *g*, 25°C, 10 minutes. Subsequently, the labelled target cell suspension was placed at 4°C in a refrigerator for 1 hour to minimize spontaneous release counts. The labelled target cells were washed once more with complete RPMI 1640 culture medium by centrifugation at 200 *g*, 25°C, 10 minutes. The viability was again checked. The cells were adjusted to a final concentration of $1x10^5$ /ml. These labelled target cells were kept at 4°C in the refrigerator until used.

4.2 Effector cells preparation

4.2.1 To study the effect of various concentrations of chronic caffeine consumption in rats.

Splenic mononuclear cell suspensions obtained from both control and caffeine treated rats (as describe in section 3.) were diminished of adherent cells by placing 5 ml of the splenic mononuclear cell suspension in 35x10 mm plastic petri dishes (Nunc, Kamstrup, Roskilde, Denmark) (115). The cells were incubated in a 5% CO_2 , humidified incubator at 37° for 1 hour. (To allow adherent cells to attach to at the plastic petri dish). The supernatant, containing non-adherent cells, was gently aspirated and transferred to a 15 ml sterile centrifuge tube aseptically. These effector cells were washed with complete RPMI 1640 medium and then checked for viability ($\geqslant$ 95%). The effector cells were adjusted to 10^7 /ml with complete medium.

4.2.2 To study the direct effect of various concentrations of caffeine on the assay system.

The procedure for preparation of the effector cells was as described in 4.2.1 for control group (non-caffeine treated rats).

4.3 Cytotoxicity assay

4.3.1 The effect of various concentrations of chronic caffeine consumption in rats.

The method was modified from the technique of Kiessling *et al*. (113). Briefly, the ^{51}Cr release cytotoxic assays were performed in triplicate in 96-well round-bottom (U type) tissue culture plates (NUNC) with final volumes of 0.2 ml. In general, 1×10^4 labelled target cells (100 µl) was placed in each well. Then 5×10^5 effector cells (100 µl) were mixed with labelled target cells to obtain effector: target (E/T) ratio of 50:1 and then the plate was centrifuged at 200 g, 25 ^oC, 5 minutes. After centrifugation the plate was incubated at 37 ^oC for 4 hours in a humidified atmosphere of 5% CO_2 in air. After centrifugation at 200 g, 4 ^oC for 5 minutes, 120 µl of supernatant from each well was removed for ^{51}Cr radioactivity count in a gamma counter (Gamma 5500, Beckman, Irvine, CA, U.S.A.). Supernatants from the wells containing only labelled target cells served as the controls for baseline release of ^{51}Cr (spontaneous release). Maximum release of ^{51}Cr was determined using labelled target cells incubated with 10% Triton X-100 (Sigma). The results were expressed as percent cytotoxicity by using the formula as follows:

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

when: experimental release = mean counts per minute (cpm) of supernatants from triplicate wells containing a mixture of effector cells and labelled target cells

maximum release = mean cpm of supernatants from labelled target cells incubated with 10% Triton X-100

spontaneous release = mean cpm of supernatants from labelled target cells incubated in complete RPMI 1640 culture medium

4.3.2 The direct effect of various concentrations of caffeine on the assay system

To study the direct effect of various concentrations of caffeine on rat NK cell cytotoxicity, all procedures were as described in 4.2.1, except that caffeine was added into triplicate wells of the effector-target cell mixture at the various final concentrations of 5, 10, 20 and 40 µg/ml culture medium. The possible effect of caffeine on target cell viability was also studied; labelled target cells alone were incubated at 37 °C, 5% CO_2, in a humidified incubator for 4 hours with various concentrations of caffeine and released radioactivity was detected in the supernatants.

5. Lymphocyte proliferation assay

5.1 Normal rat serum

Blood was collected aseptically by cardiac puncture from normal Sprague-Dawley rats with 21G x 1½ needle (Nippon Medical, Japan). Then it was allowed to clot at room temperature and subsequently centrifuged at 400 g, 25 °C for 15 minutes. Serum was collected aseptically, pooled and the serum complement was inactivated by incubation at 56 °C for 30 minutes. Inactivated rat serum was kept in small aliquotes (3-5 ml) at -20 °C. This small aliquote of serum was thawed for use on the experimental day.

5.2 Preparation of mitogens

There were two kinds of mitogens used in the study. Pokeweed mitogen (PWM) was used as B cell mitogen and phytohemagglutinin-P (PHA-P) was used as T cell mitogen.

5.2.1 Pokeweed mitogen (PWM) preparation

One milligram of pokeweed mitogen (Sigma, cat. no.

L-9379) was dissolved in 1 ml of RPMI 1640 medium to prepare a stock solution. This stock solution (1 mg/ml) was kept at 4 °C in the refrigerator. The mitogen was diluted with complete RPMI 1640 culture medium to final concentrations of 1 µg/ml and 10 µg/ml for use in the assay.

5.2.2 Phytohemagglutinin-P (PHA-P) preparation

Five milligrams of phytohemagglutinin-P (Difco Laboratories, Detroit, MI, U.S.A., cat. no. 3110-56) was dissolved in 5 ml of RPMI 1640 culture medium, to prepare a stock solution (1 mg/ml). This stock solution was kept frozen at -20 °C in small aliquots until used. The stock solution was thawed and then diluted with RPMI 1640 culture medium to the concentrations of 1, 50 and 100 µg/ml for use in the assay.

5.3 Splenic mononuclear cells preparation

5.3.1 The effect of chronic caffeine consumption on rat mononuclear cells proliferative response.

Spleens from control rats and rats treated with various doses of caffeine treated rats were processed as described in Section 3. The viability of splenic mononuclear cells was checked by using Trypan blue dye exclusion. A suitable viability was at least 95%. The splenic mononuclear cell concentration was then adjusted to 2×10^{6}/ml with RPMI 1640 culture medium supplemented with 10% pooled, heat-inactivated normal Sprague-Dawley rat serum and 1.0×10^{-4}

M 2-mercaptoethanol (2-ME, Sigma).

5.3.2 The direct effect of various concentrations of caffeine on the rat mononuclear cell proliferation assay.

Spleens from normal Sprague-Dawley rats were processed as described above in 5.3.1 for control group.

5.4 Proliferation assay

5.4.1 The effect of chronic caffeine consumption

The assay was performed aseptically in triplicate in a larminar flow hood (Bioharzard, Gelaire). Splenic mononuclear cell suspension (2×10^6/ml) was distributed into each well of a 96-well flat-bottom tissue culture plate (NUNC) at 100 µl/well (2×10^5/well) by using a micropipette (Oxford, Lancer Division of Sherwood Medical, St. Louis, U.S.A.). Then 100 µl of freshly diluted of PWM or PHA-P at various concentration were added into each well. The final concentrations were: PWM, 10 µg/ml; PHA-P, 1, 50 and 100 µg/ml, each in triplicate. The cell control culture for each animal was prepared parallelly. It consisted of triplicate culture wells on the same plate containing only splenic mononuclear cell with RPMI 1640 culture medium. The final volume in each well was 200 µl and the concentration of 2-ME and pooled heat-inactivated normal rat serum were 5×10^{-5} M and 5%, respectively. The plate was carefully covered with the lid aseptically, gently shaken, and placed in a 5% CO_2, humidified incubator at 37°C for 3 days before 0.5 µCi of [^{3}H]-Thymidine (MW. 242.2, specific activity 2.0 Ci/mmol, New England Nuclear, Boston, MA) was added. The culture was further incubated for 18 hours in the same condition. The culture was stopped by harvesting the cells on glass fiber filters (Grade 934 AH, Reeve Angle, Clifton, NJ, U.S.A.), using an automatic cell harvester (Skatron semi automatic cell harvester, Skatronas, Lier, Norway) (116). The glass fiber filters were dried, placed in scintillation counting vials and 5 ml liquid scintillation fluid (see appendix) was added. [^{3}H]-thymidine incorporated into the proliferative cells was measured in a liquid scintillation counter (LKB 1219 Rack Beta). Mean cpm from triplicate cultures were calculated and the stimulation index (SI) was calculated by the following formula:

$$SI = \frac{\text{mean of cpm from stimulated culture}}{\text{mean of cpm from unstimulated culture (control culture)}}$$

5.4.2 The direct effect of various concentrations of caffeine on the rat mononuclear cell proliferation assay

Spleens from non-caffeine treated rats were processed for splenic mononuclear cell suspensions as described in Section 3, above. The splenic mononuclear cell proliferative responses to either B or T cell mitogens (PWM, PHA-P) were performed as described in Section 5.4.1, except that various concentrations of caffeine solution were added directly into each culture well of the assay system. The final concentrations of caffeine were 5, 10, 20 and 40 µg/ml. Each assay was performed in triplicate. The direct effect of various concentrations of caffeine on proliferation of splenic mononuclear cells (without mitogen) was studied in each experiment using a culture containing only splenic mononuclear cells and various concertrations of caffeine.

6. Total and differential leukocyte count

Blood used in this study was collected in EDTA by cardiac puncture. The blood was diluted with 3% acetic acid and total leukocytes were counted in a hemacytometer (117). For morphological differentiation, blood was smeared on a clean microscope slide. The preparation was air-dried and then the smear was fixed in absolute methanol and stained for 30 min with Giemsa stain (118). Differential leukocyte count was examined under oil immersion microscopy; 300 leukocytes were analysed from each slide.

7. Evaluation of the dose response relationship of caffeine on rat splenic mononuclear cell proliferative response

All procedures were as described in Section 5.4.2, except that the final concentrations of added caffeine in the culture wells were 1, 10, 100 and 1000 µg/ml. Control for the direct effect of caffeine on normal proliferation of rat splenic mononuclear cells were also performed in each experiment. These cultures contained only splenic mononuclear cells and caffeine.

CHAPTER IV

RESULTS

1. Effect of chronic caffeine consumption on rat splenic NK cell activity, lymphocyte proliferative response, total and differential leukocyte count

Splenic mononuclear cells from three groups of chronic caffeine treated rats, *i.e.*, 2, 6 and 18 mg/kg/day were studied for NK cell activity and proliferative response. The results of each group were analysed against the group of control rats as shown below.

1.1 Splenic NK cell activity

Four groups of chronic caffeine treated rats (more than 5 rats for each group) were studied for NK cell function by a 4-hr ^{51}Cr release cytotoxicity assay on YAC-1 cells. The results are expressed as percent cytotoxicity as shown in Table 1 and Figure 1. Percent cytotoxicity of the 6 mg/kg/day (middle dose) caffeine treated rats was significant lower than that of control rats (25.2±1.5 vs 32.6±1.8, $p < 0.05$). However, in the other two groups of rats treated with 2 and 18 mg/kg/day of caffeine treated rats, the percent cytotoxicities were not significantly different from the control group. The limited data performed suggests that chronic consumption of caffeine at the dosage of 6 mg/kg/day in Sprague-Dawley rats decreased the NK cell activity.

Table 1 The effect of various doses of chronic caffeine consumption on rat natural killer cell activity.

Caffeine dose (mg/kg/day)	**Group size(n)**	**% Cytotoxicity[a] (Mean ± SEM)**	**p-value[b]**
0	10	32.6±1.8	
2	7	28.9±2.0	> 0.05
6	6	25.2±1.5	< 0.05
18	7	34.1±2.3	> 0.05

[a] effector : target = 50:1

[b] Statistical analysis against non-caffeine treated rats (0 mg/kg/day) by Mann-Whitney U test at p-value = 0.05

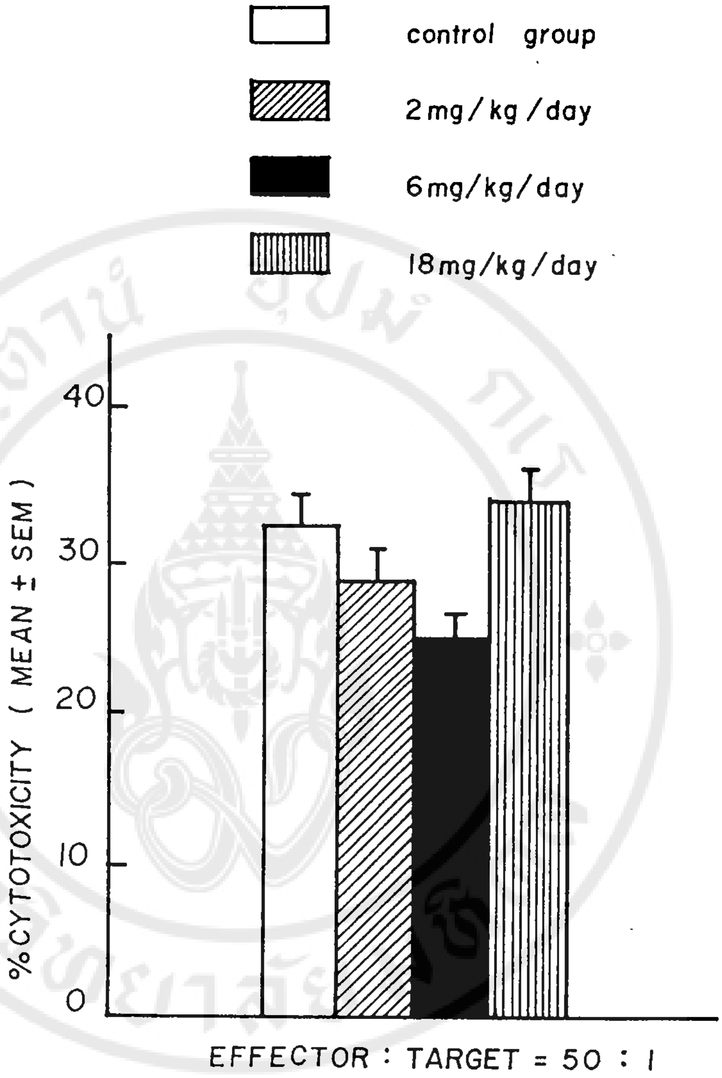


Figure 1 The percent cytotoxicity of control and chronic caffeine treated rat NK cell activities against YAC-1 in a 4-hour cytotoxicity assay.

1.2 Splenic lymphocyte proliferative response

Mononuclear cells from spleens of caffeine treated rats (more than 4 rats per group) were cultured with 10 µg/ml of PWM to study B lymphocyte function. The stimulation indices are shown in Table 2 and Figure 2. The pattern of the stimulation indices with respect to dose was similar to that of splenic NK cell activity. That is, statistical analysis showed that the stimulation index of the 6 mg/kg/day caffeine treated rats was decreased significantly (36.0±8.1 vs 11.1±7.2, $p < 0.05$). No significant difference was observed in rats which received caffeine at the other two doses (2 and 18 mg/kg/day).

The proliferative response after stimulation with PHA-P was measured to study T cell functions. Three different concentrations of PHA-P were used, i.e., 1, 50 and 100 µg/ml. As shown in Table 3 and Figure 3, the PHA-P concentration of 50 µg/ml gave the highest stimulation indices of all groups of rats. At the lowest concentration of PHA-P, 1 µg/ml, the proliferative responses were not significantly different between caffeine treated rats and control rats. At a PHA-P concentration of 50 µg/ml, the proliferative response of T cells from 18 mg/kg/day caffeine treated rats was significantly higher than that of control rats (59.8±9.2 vs 29.8±5.1, $p < 0.05$). A significant increase of the stimulation index was also observed in the same group of rats stimulated with PHA-P at 100 µg/ml (38.0±8.0 vs 15.7±3.3, $p < 0.05$). The proliferative

Table 2 The effects of chronic caffeine consumption on rat B lymphocyte proliferative response to PWM stimulation (10 μg/ml).

Caffeine dose (mg/kg/day)	**Group size(n)**	**Stimulation index (Mean ± SEM)**	**p-value**[a]
0	11	36.0±8.1	
2	6	35.0±5.3	> 0.05
6	5	11.1±7.2	< 0.05
18	6	32.7±8.7	> 0.05

[a] Statistical analysis against non-caffeine treated rats (0 mg/kg/day) by Mann-Whitney U test at p-value = 0.05

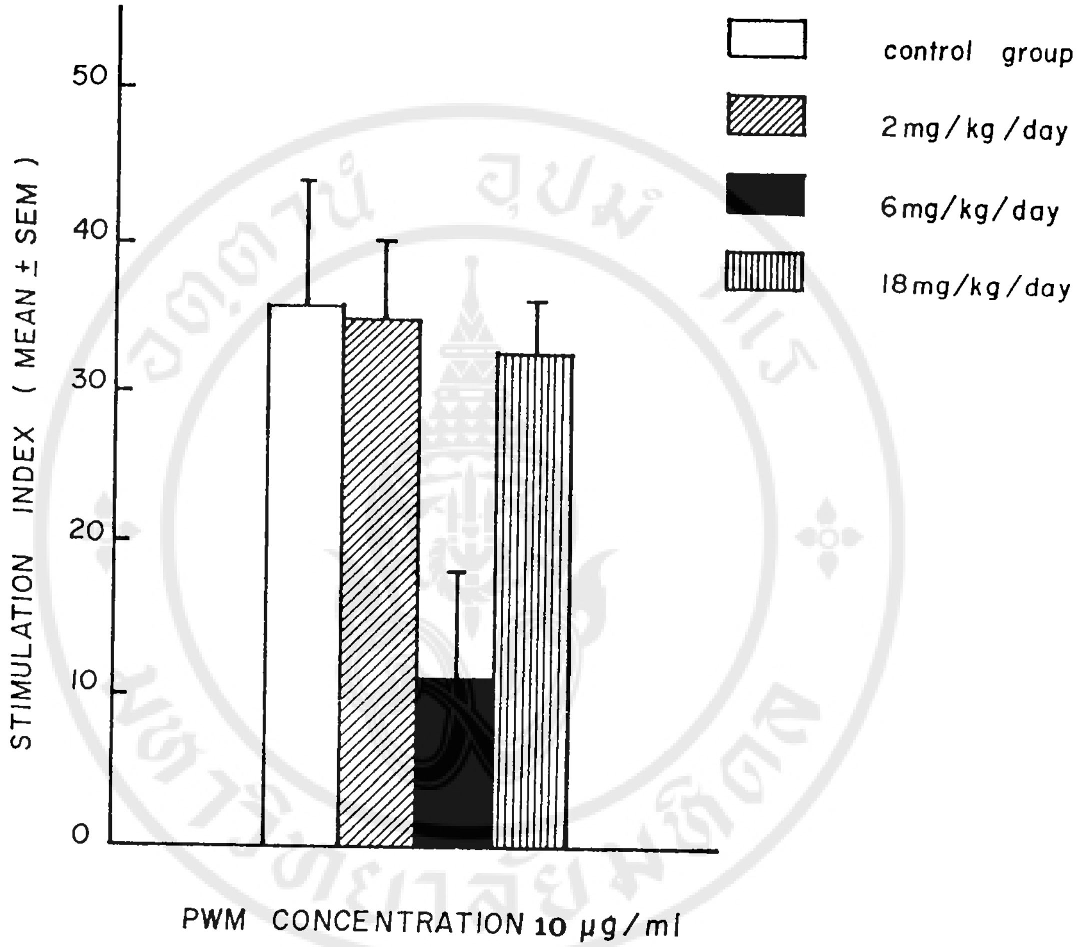


Figure 2 The mean (± SEM) stimulation indices of control and chronic caffeine treated rat splenic mononuclear cells stimulated by PWM.

Table 3 The effects of chronic caffeine consumption on rat T lymphocyte proliferative responses to PHA-P stimulation.

Caffeine dose (mg/kg/day)	Group size(n)	PHA-P concentration (μg/ml)[a]		
		1	50	100
0	10	1.6±0.5	29.8±5.1	15.7±3.3
2	6	3.4±1.5	43.5±9.8	20.4±4.8
6	5	1.6±0.3	43.8±8.4	19.7±4.0
18	7	2.3±0.5	59.8±9.2*	38.0±8.0*

[a] Stimulation indices at three concentrations of PHA-P; Mean ± SEM

* Statistically significant by Mann-Whitney U test against non-caffeine treated rats, $p < 0.05$)

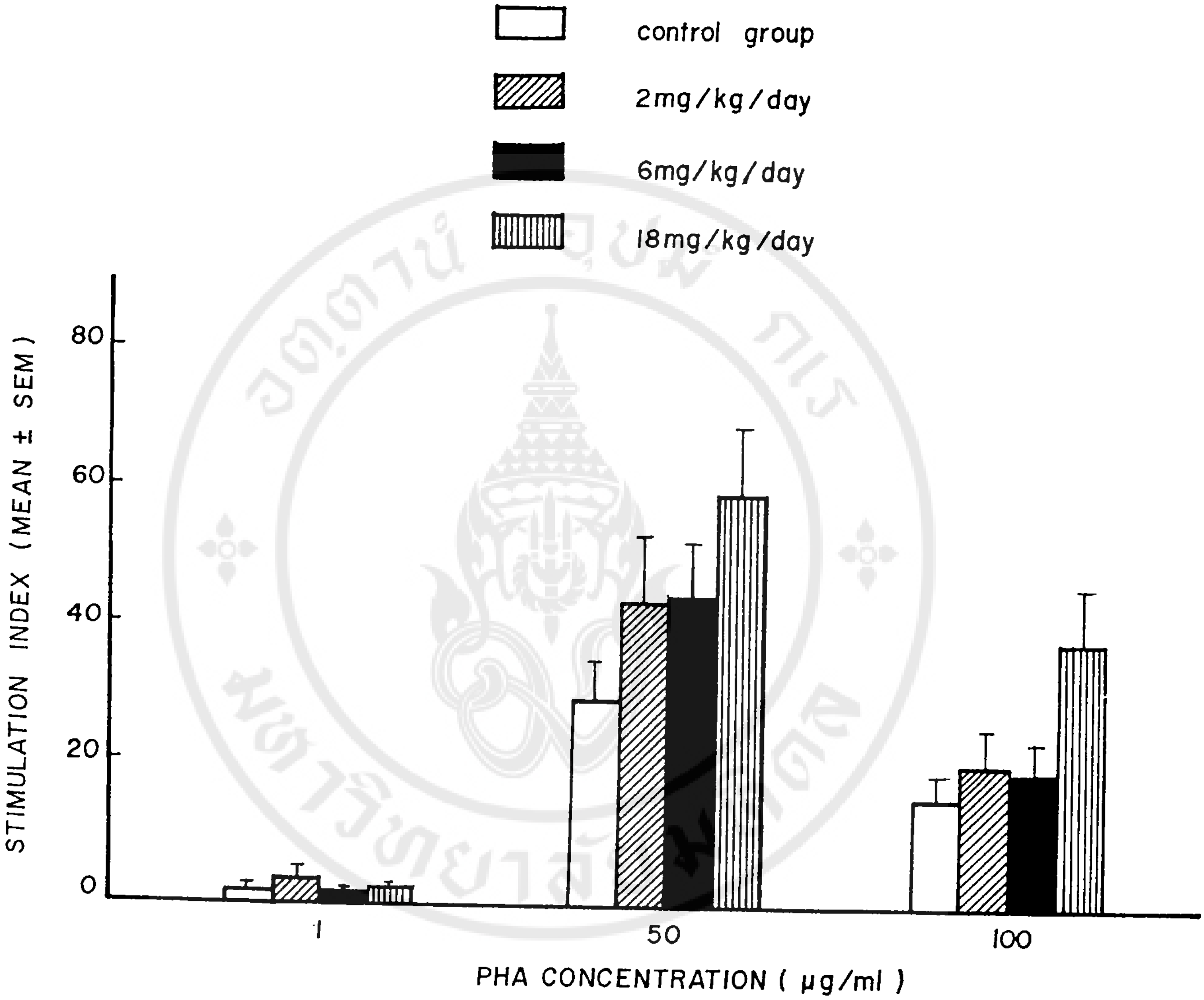


Figure 3 Histogram shows stimulation indices of control and chronic caffeine treated rat splenic mononuclear cells by different doses of PHA-P.

responses of the other two groups of caffeine treated rats (2 and 6 mg/kg/day) showed slight but insignificant increase ($p > 0.05$).

1.3 Total and differential leukocyte count

Total and differential leukocyte count were investigated in all groups of rats. Data in Tables 4-7 and Figure 4 show some changes of total leukocytes in caffeine treated rats. For leukocyte differentiation (Tables 4-7, Figures 5, 6), the percentage of various leukocytes (small lymphocytes, large lymphocytes, LGL, neutrophils, monocytes and eosinophils) showed slight differences between groups of rats. But no significant differences were observed between caffeine treated rats and control rats.

2. Direct effect of various caffeine concentrations on rat NK cell activity and lymphocyte proliferative response in in vitro assay system

The possible direct effects of caffeine on Sprague-Dawley rat NK cells and B and T lymphocyte functions were investigated. Various concentrations of caffeine, i.e., 5, 10, 20 and 40 µg/ml were added directly into the cultures. The results are shown in Tables 8-10 and Figures 7-9.

2.1 Splenic NK cell activity

The direct effect of caffeine (various concentrations) on YAC-1 lysis was studied concurrently with

Table 4 Total and differential leukocyte count of control rats.

Rat no.	Total leukocytes/mm^3	Lymphocyte		LGL[a]	Neutrophil[a]	Monocyte[a]	Eosinophil[a]
		Small	Large				
1	5900	228	26	2	40	3	1
2	3200	217	26	0	53	2	2
3	3900	220	29	0	51	0	0
4	6900	213	39	2	40	4	2
5	4350	213	30	1	50	3	3
6	4400	222	29	2	45	2	0
7	4600	205	33	0	60	1	1
8	5700	196	28	2	66	5	3
9	5250	233	20	2	40	3	2
10	6050	219	29	0	45	5	2
11	5170	229	20	1	48	2	0
$\bar{X}$±SEM	5038±323	217.7±3.3	28.1±1.6	1.1±0.3	48.9±2.5	2.7±0.5	1.4±0.3
%$\bar{X}$±SEM		72.6±1.1	9.4±0.5	0.4±0.1	16.3±0.8	0.9±0.2	0.5±0.1

---

[a] per 300 leukocytes

Table 5 Total and differential leukocyte count of 2 mg/kg/day chronic caffeine treated rats.

Rat no.	Total leukocytes/mm^3	Lymphocyte^a		LGL^a	Neutrophil^a	Monocyte^a	Eosinophil^a
		small	large				
1	3700	228	30	0	38	1	3
2	5250	201	47	1	42	5	4
3	6300	214	31	1	53	0	1
4	5700	219	29	0	50	2	0
5	3500	198	32	1	66	1	2
6	4650	224	24	3	47	2	0
7	3450	206	23	0	65	4	2
$\bar{X}\pm$SEM	4650±432	212.9±4.4	30.9±3.0	0.9±0.4	51.6±4.0	2.1±0.7	1.7±0.6
%$\bar{X}\pm$SEM		71.0±1.5	10.3±1.0	0.3±0.1	17.2±1.3	0.7±0.2	0.6±0.2

[a] per 300 leukocytes

Table 6 Total and differential leukocyte count of 6 mg/kg/day chronic caffeine treated rats.

Rat no.	Total leukocytes/mm^3	Lymphocyte[a]		LGL[a]	Neutrophil[a]	Monocyte[a]	Eosinophil[a]
		small	large				
1	4800	198	38	2	58	3	1
2	5000	201	33	0	63	3	0
3	4850	204	25	2	65	2	2
4	5400	236	21	1	38	3	1
5	3950	202	29	0	67	0	2
6	3400	217	29	2	50	1	1
$\bar{X}$±SEM	4566±303	209.7±5.9	29.2±2.4	1.2±0.4	56.8±4.5	2.0±0.5	1.2±0.3
%$\bar{X}$±SEM		69.9±2.0	9.7±0.8	0.4±0.1	18.9±1.5	0.7±0.2	0.4±0.1

[a] per 300 leukocytes

Table 7 Total and differential leukocyte count of 18 mg/kg/day chronic caffeine treated rats.

Rat no.	Total leukocytes/mm^3	Lymphocyte[a]		LGL[a]	Neutrophil[a]	Monocyte[a]	Eosinophil[a]
		small	large				
1	3900	241	16	0	39	2	2
2	4450	207	27	1	57	6	2
3	4900	207	28	2	60	1	2
4	5800	215	14	0	66	3	2
5	5550	215	16	1	64	3	1
6	6550	189	35	2	70	2	2
7	5200	200	38	1	60	0	1
$\bar{X}$±SEM	5192±332	210.6±6.1	24.9±3.7	1.0±0.3	59.4±3.8	2.4±0.7	1.7±0.2
%$\bar{X}$±SEM		70.2±2.0	8.3±1.2	0.3±0.1	19.8±1.3	0.8±0.2	0.6±0.1

[a] per 300 leukocytes

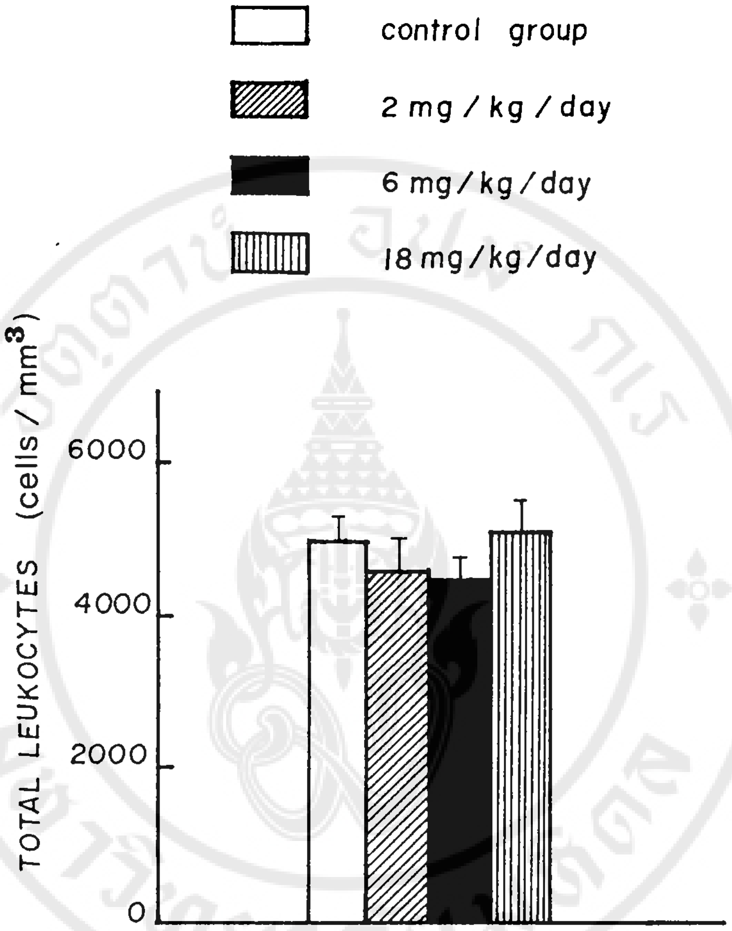


Figure 4 Total leukocytes of control and chronic caffeine treated rats (various doses).

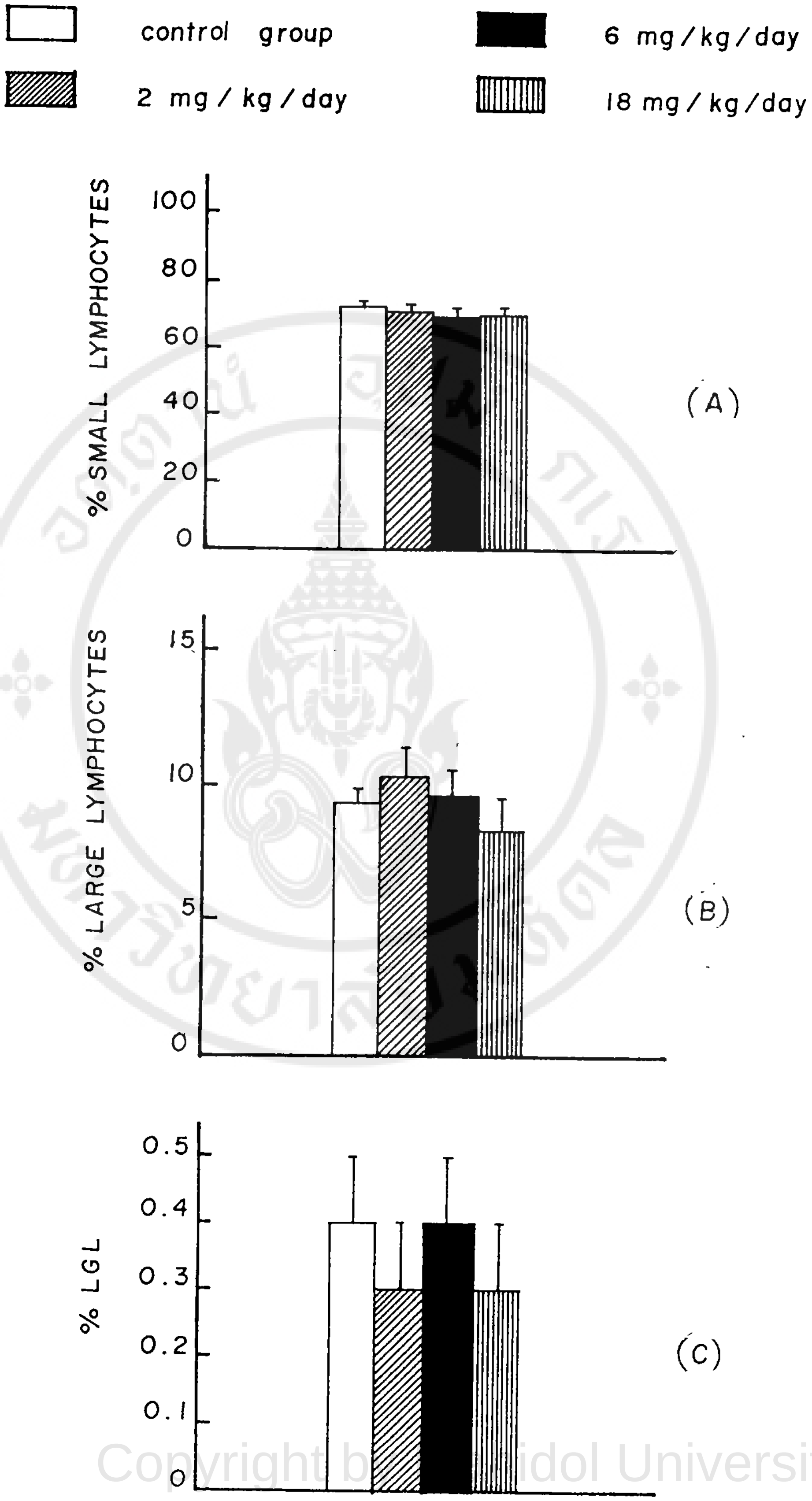


Figure 5 Percentages of small(A), large(B) and large granular(C), lymphocytes of control and chronic caffeine treated rats (various doses).

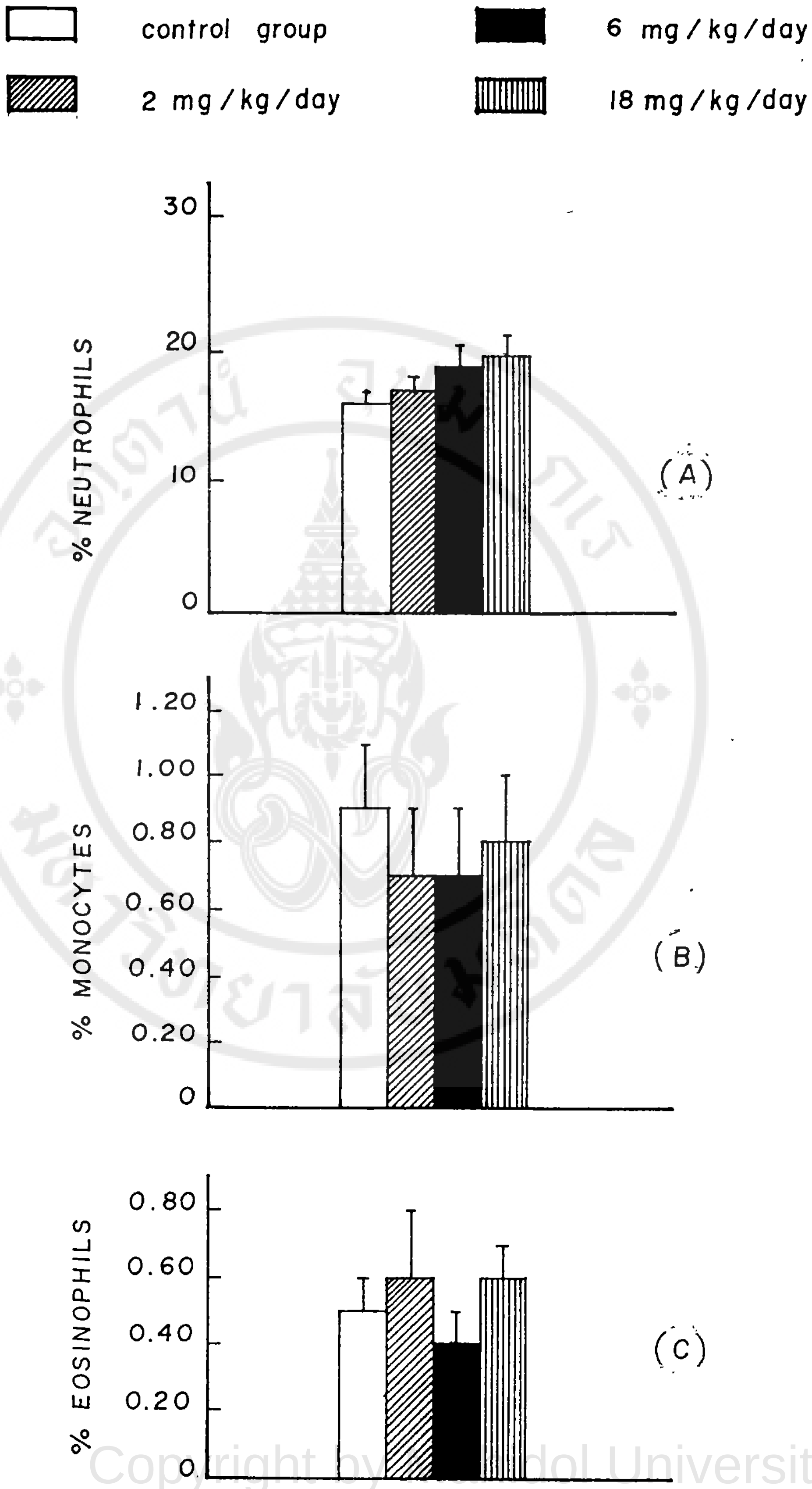


Figure 6 Percentages of neutrophils(A), monocytes(B) and eosinophils(C) of control and chronic caffeine treated rats (various doses).

conventional NK cytotoxicity assay. The results (n = 10) from Table 8 and Figure 7 showed that percent labelled target cell (YAC-1) lysis and cpm of caffeine-target cell culture were not different from that of labelled target cell culture alone (spontaneous lysis). This indicates that caffeine itself does not have any effect on labelled target cells (YAC-1) lysis.

Table 9 and Figure 8 show percent cytotoxicity of rat NK cells to labelled target cells in a 4 hr, ^{51}Cr release cytotoxicity assay. NK cell activities with all various caffeine concentrations in the <u>in vitro</u> assay system were not different from that of the control condition (culture of effectors and labelled target cells without caffeine).

2.2 Lymphocyte proliferative response

The effect of four different concentrations of caffeine were studied in groups of rats (13 rats). The results are shown in Table 10. In the culture without mitogen, it was demonstrated that proliferative response cpm as well as stimulation indices of all five concentrations of caffeine (0, 5, 10, 20, 40 μg/ml) were not different. Caffeine alone therefore, does not have any effect on splenic mononuclear cell culture.

In the PWM stimulation assay system, as shown in Table 10 and Figure 9, caffeine shows an interesting effect on B lymphocyte proliferative response. At all three high doses of caffeine (10, 20 and 40 μg/ml) in the assay, there

Table 8 All concentrations of caffeine have no effect on the lysis of labelled target cells.

Caffeine conc. (μg/ml)	Group size(n)	cpm[a] (Mean ± SEM)	% labelled target cell lysis[b]	p-value[c]
0	10	49.1±8.3	18.9±2.3	
5	10	50.9±7.6	19.2±1.9	> 0.05
10	10	52.4±7.3	19.4±1.5	> 0.05
20	10	48.7±7.0	18.2±2.1	> 0.05
40	10	47.5±7.9	17.0±1.8	> 0.05

[a] effector : target = 50:1

[b] % labelled target cell lysis

$$= \frac{\text{cpm of labelled target cell culture}}{\text{cpm of maximum lysis}} \times 100$$

[c] Statistical analysis against of labelled target cells (spontaneous lysis) at p-value = 0.05

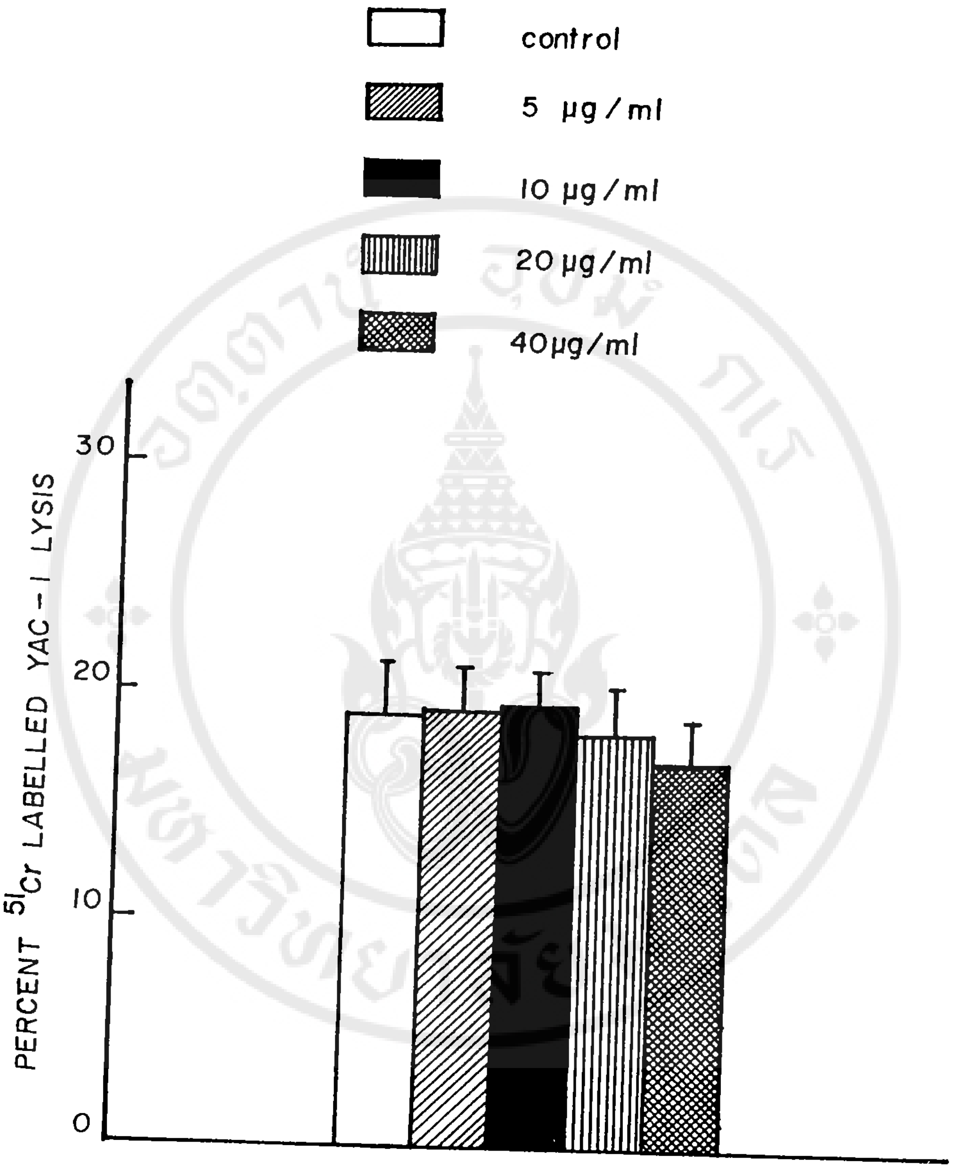


Figure 7 Percentage ^{51}Cr-labelled YAC-1 lysis in the absence and presence of various concentrations of caffeine.

Table 9 The effects of various concentrations of caffeine on rat NK cell activities in the *in vitro* assay system.

Caffeine conc. (µg/ml)	Group size(n)	% Cytotoxicity[a] (Mean ± SEM)	p value[b]
0	13	25.4±2.2	
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] Statistical analysis against the culture of effector and target cells by Mann-Whitney U test at p-value = 0.05

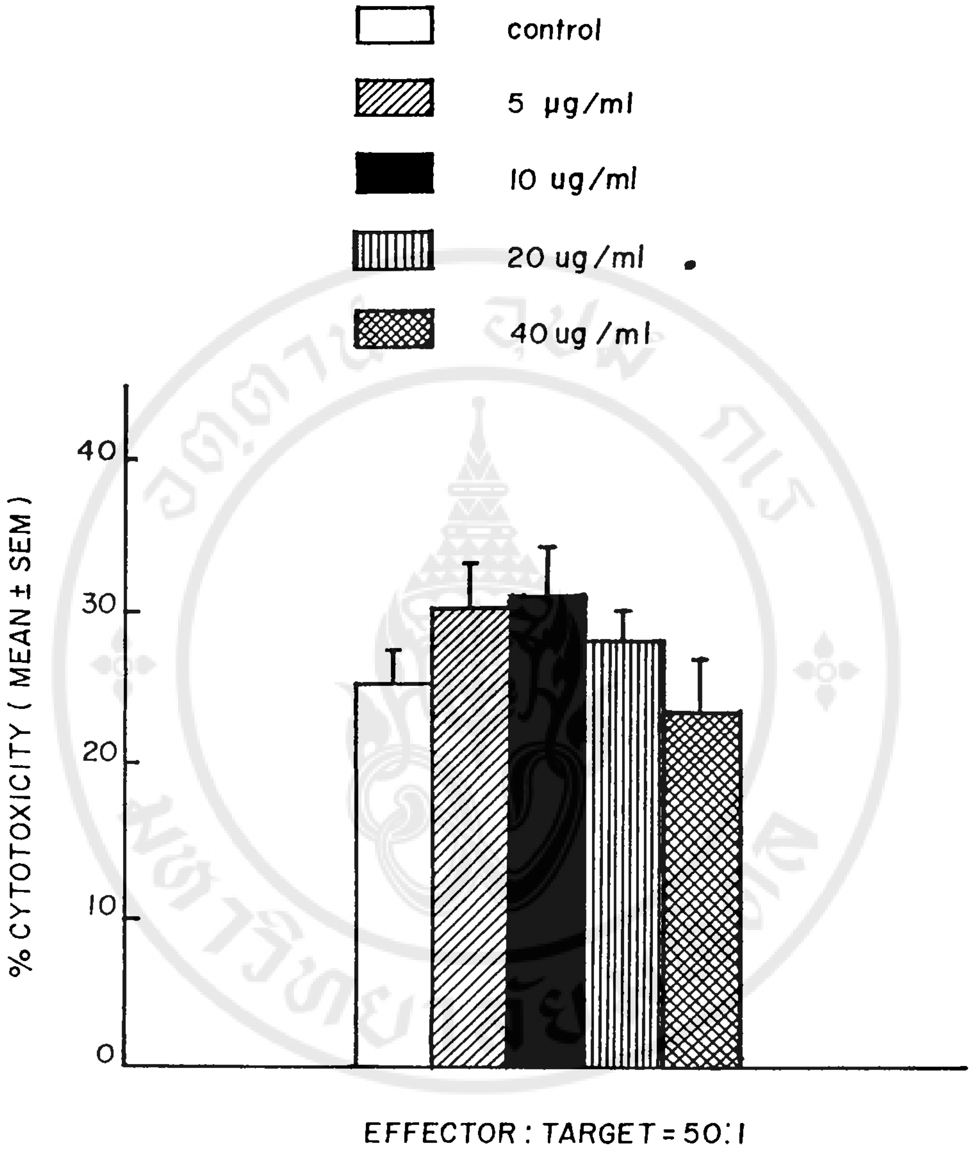


Figure 8 The percent cytotoxicity of rat NK cells on YAC-1 in the absence and presence of various concentrations of caffeine.

Table 10 The effects of various concentrations of caffeine in the assay system on rat lymphocyte proliferative responses.

Caffeine conc. (µg/ml)	- PWM, - PHA			+ PWM		+ PHA	
	Group size(n)	cpm[a]	SI[a]	Group size(n)	SI	Group size(n)	SI
0	13	437.7±44.9	1.00±0.00	13	40.2±4.4	13	31.6±4.9
5	13	340.2±46.8	0.84±0.11	12	28.8±3.8	13	20.8±4.3
10	13	323.9±53.5	0.76±0.13	12	27.3±3.4*	13	18.3±3.7*
20	13	325.5±42.9	0.80±0.10	12	28.1±2.6*	13	19.5±2.6*
40	13	312.4±46.0	0.75±0.11	13	24.5±3.1*	13	17.1±3.2*

Final conc. PWM = 1 µg/ml; PHA-P 50 µg/ml

[a] Mean ± SEM

* Statistically signifioant by Mann-Whitney U test against the mitogen-mononuclear cell culture at p-value = 0.05

were significant reductions of the stimulation indices (27.3±3.4, 28.1±2.6, 24.5±3.1 vs 40.2±4.4, $p < 0.05$). The low dose of caffeine (5 µg/ml) in PWM stimulation assay, showed some reduction in the stimulation index but it was not significant.

Caffeine also induced reductions of the stimulation indices similar to those of B lymphocytes on the proliferations of T lymphocytes in response to PHA-P stimulation (Table 10, Figure 9). In the presence of three high concentrations of caffeine, the significant reductions of the PHA-P stimulation indices were observed (18.3±3.7, 19.5±3.2, 17.1±3.2 vs 31.6±4.9, p value < 0.05). The low caffeine concentration (5 µg/ml) gave some reduction of the stimulation index but it was not significant. Thus, the results indicate that certain concentrations of caffeine present in the assay system, produce significant reductions in the proliferative responses of Sprague-Dawley rat splenic mononuclear cells to both PWM and PHA-P.

3. The dose relationship of caffeine on rat splenic mononuclear cell proliferative response.

The previous study of the effect of various caffeine concentrations on rat splenic mononuclear cell proliferative response in *in vitro* assay system in 2.2 showed that caffeine inhibits the proliferative responses. It had the inhibiting effect on rat lymphocyte proliferative responses to both PWM and PHA-P stimulation. In this experiment, the dose

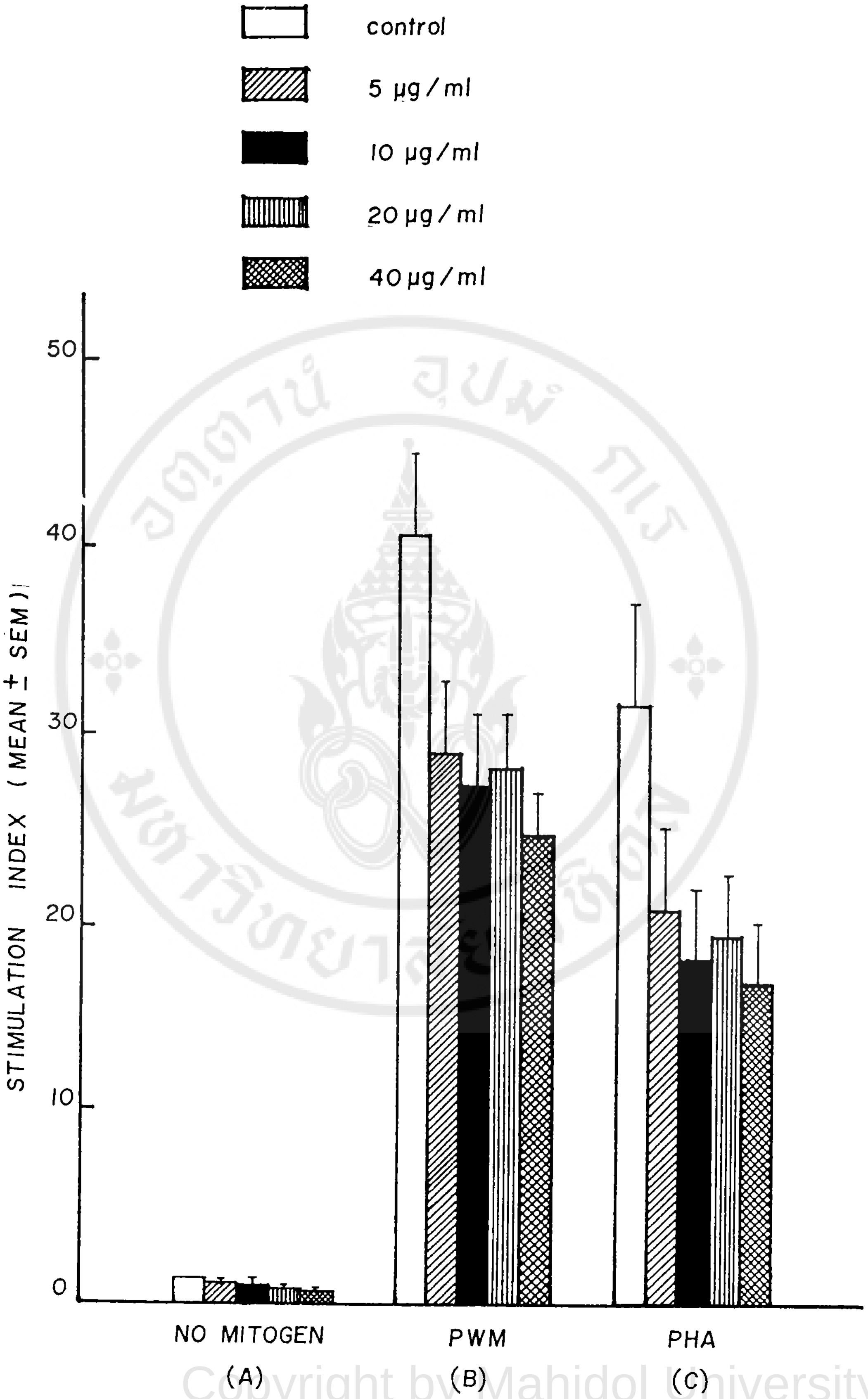


Figure 9 The stimulation indices of splenic rat mononuclear cells in the absence and presence of various concentrations of caffeine. (A) no mitogen, (B) PWM stimulation and (C) PHA-P stimulation.

relationship of a broad range of concentrations of caffeine on rat lymphocyte proliferative responses was determined. The final concentrations of caffeine used in this study were 1, 10, 100 and 1,000 µg/ml. Caffeine at all concentrations did not have any effect on stimulation indices of splenic mononuclear cell cultures without mitogen (Table 11). The interfering (inhibiting) effect on proliferative response to both PWM and PHA-P could be observed at the caffeine concentrations of 10, 100 and 1,000 µg/ml. These inhibiting effects were statistically significant (31.5±4.7, 8.7±1.3, 1.6±0.3 vs 49.2±5.2 for PWM stimulation and 35.8±5.0, 12.2±1.6, 1.3±0.2 vs 50.2±6.0 for PHA-P stimulation, $p < 0.05$).

To demonstrate the inhibiting effect of caffeine, the percent inhibition was calculated as shown in Table 12. The log dose-efffect relationship curve between various concentrations of caffeine in the assay system and percent inhibition of stimulation indicies is shown in Figure 10. It was demonstrated that there was relationship between concentration of caffeine and the percent inhibition of the proliferative response in the dose dependent pattern.

Table 11 Stimulation indices of splenic mononuclear cell culture with various concentrations of caffeine in the absence and presence of mitogens (PWM or PHA-P).

Caffeine conc. (µg/ml)	Group size(n)	Stimulation index[a]			
		- PWM	+ PWM	- PHA	+ PHA
0	10	1.00±0.00	49.2±5.2	1.00±0.00	50.7±6.[illegible]
1	10	1.02±0.15	42.8±6.0	1.31±0.13	45.[illegible]±5.[illegible]
10	10	1.41±0.22	31.5±4.7*	1.51±0.20	35.8±[illegible]*
100	10	0.93±0.14	8.7±1.3*	1.21±0.09	12.[illegible]*
1000	10	1.23±0.22	1.6±0.3*	1.29±0.[illegible]	1.3±[illegible]*

[a] Mean ± SEM

* Statistically significant by Mann-Whitney U test against the no caffeine mitogen-mononuclear cell culture at p-value = 0.05

Table 12 The percent inhibition of stimulation indices of various concentrations of caffeine on 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

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

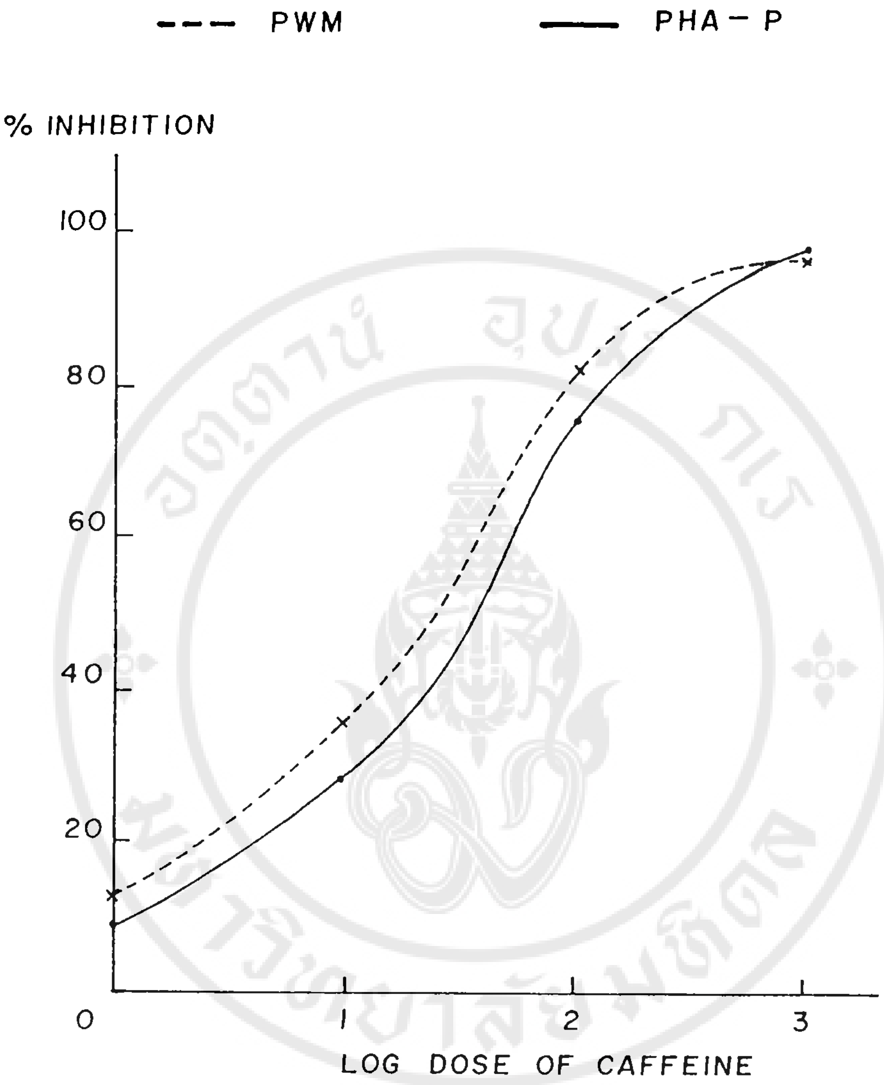


Figure 10 Log dose-effect relationship between percent inhibition of stimulation indices and various concentrations of caffeine (µg/ml).

CHAPTER V

DISCUSSION

Immune cell functions are the results of various biological reactions which occur inside the cells. These biological reactions happen when the surface receptors are stimulated properly. There are many kinds of surface receptors on immune cells, for example, antigen receptor, mitogen receptor, hormone and growth factor receptors. The surface receptors on immune cells are also called cell surface antigen, antigenic marker, phenotypic marker, etc. Many monoclonal antibodies have been produced against well-characterized cells in the immune system and show patterns of reactivities with subpopulations determined by expression of highly specific surface receptors. Some phenotypic markers or surface markers are commonly found on many kinds of immune cells, e.g., CD45, a human leukocyte common antigen expressed on all leukocytes: T lymphocytes, B lymphocytes, granulocytes and macrophages (44). However, some are unique, e.g., CD4 on T helper/inducer lymphocytes, CD8 on T cytotoxic/suppressor lymphocytes and CD25 on NK cells. It is not only the presence or absence of these surface receptors on the cells that play roles in immune cell functions, but the quantities of these surface receptors are also important.

Adenosine receptor is found in most cells including lymphocytes (119, 120). There are at least two subclasses of adenosine receptors that modulate either inhibition or stimulation of adenylate cyclase; the inhibition receptor (R_i or A_1) and the stimulatory receptor (R_a or A_2). In addition, an inhibitory adenosine-related P-site has also been identified. When adenosine bind to P-site it inhibits adenylate cyclase activity (121). Adenylate cyclase is a plasma-membrane-bound enzyme involved in cyclic 3′, 5′-adenosine monophosphate (cAMP) synthesis.

$$\text{ATP} \xrightarrow[\searrow \text{Ⓟ-Ⓟ}]{\text{adenylate cyclase}} \text{cAMP} \xrightarrow{\text{phosphodiesterase}} 5' - \text{AMP} \quad (122)$$

Caffeine (1, 3, 7-trimethylxanthine), theophylline (1, 3-dimethylxanthine) and theobromine (3, 7-dimethylxanthine) are naturally occurring xanthine derivatives. These compounds are called methylxanthines and are classified as central nervous system stimulants. Although caffeine is used in combination with other drugs, the safety of caffeine is still controversial (123, 124). Many investigators have studied the mechanism(s) of action of methylxanthine on cells (125, 126). At present, it is generally accepted that the effects of caffeine are mediated through antagonistic actions on A_1adenosine receptor (127, 128).

To learn whether or not caffeine has any effect on immune cell functions, an animal model was used (adult male Sprague-Dawley rats) in this study. Some advantages achieved in using adult male rats were: First, the caffeine consumption was more controllable; both times and doses could be manipulated (see appendix). Second, undesirable hormonal effects such as those produced by estrogen (129), a female sex hormone, were excluded. The effects of caffeine on immune cell functions were shown under conditions of both chronic administration (*in vivo*) and the direct addition (*in vitro*). The chronic conditions were studied by feeding caffeine to rats for 120 consecutive days. Three different doses of chronic caffeine given coresponded to the amounts of coffee intake in humans of 1-2, 3-4 and 9-10 cups/day (approximately 60-80 mg caffeine/cup). Four different concentrations of caffeine were tested for the direct effects (*in vitro*) on immune cell functions. Another four broad concentrations of caffeine were used to test for the dose relationship pattern.

In chronic caffeine consumption (*in vivo*), there were two opposing effects observed on rat NK, B and T cell functions. Both effects were shown at different caffeine concentrations and on different immune cells (Tables 1-3, Figures 1-3). Under these circumstances, at least in the rat model, caffeine had effects on immune B, T and NK cell activities. At the caffeine dose of 6 mg/kg/day, the activities of B and NK cells were significantly decreased ($p < 0.05$), while their activities were unaffected at low (2 mg/kg/day) and high (18 mg/kg/day) caffeine dose. This pattern of the effect of chronic caffeine consumption on B and NK cells concur with the results from the study of Mohr *et al*. (130) who reported an inverted dose-response relationship of chronic caffeine treatment in Sprague-Dawley rats and tumour incidence with respect to both frequency and multiplicity. The possible explanations for this decreasing (reducing) effect of chronic caffeine consumption may involve either the quantity or the distribution of surface membrane receptors especially receptors on NK cells involved in target cell recognition and receptors on B cells for PWM stimulation. If there are changes of surface receptors for target cell recognition on NK cells, the enumeration for NK and target cell binding might help to clarify this. Alternatively, there may be a deviation of intracelluar Ca^{2+} concentration. Caffeine might interfere with the level of Ca^{2+} storage since it induces an increase in intracellular Ca^{2+} in certain types of cells, e.g., skeletal and cardiac muscle cells (21, 131). So in the chronic condition, caffeine may alter intracellular Ca^{2+} concentration which in turn affect the NK and B cell activities since the optimal Ca^{2+} concentration is needed for the NK cell cytolysis (90) and for the proliferation of B cell (132).

In contrast to this inhibition of NK and B cell activities, the intermediate caffeine concentration had no effect on the T cell proliferative response to PHA-P. However, at higher dose (18 mg/kg/day) a significant increase of T cell proliferative response was observed, particularly at the PHA-P concentration of 50 µg/ml. Again, such an effect could be explained on the basis of changes of the membrane receptors, on different subpopulation of T lymphocytes. In general, the optimal PHA concentration stimulates all subpopulations of T lymphocytes while the high concentration is suggested to stimulate preferentially some subpopulation (T suppressor ?). An alternative explanation could be that the T lymphocytes have entered a (potentially) hyperreactive state as a result of the treatment at high caffeine dose for 120 days; this in turn could reflect in an altered density and distribution of PHA-P receptors. These discordant effects of chronic caffeine treatment on B, NK and T lymphocyte demonstrated requirements for different optimal caffeine concentrations.

The direct (<u>in vitro</u>) effects of caffeine on NK cell and B and T lymphocyte activities demonstrated different of results on the different populations. On NK cell activity measured in a 4 hr, ^{51}Cr cytotoxicity assay, the NK cell activity was normal, <u>i</u>.<u>e</u>., no interfere, at 4 different concentrations of caffeine (5, 10, 20 and 40 μg/ml). Thus, this study suggested that caffeine itself (5-40 μg/ml) did not have any direct interfering effect on any step of NK cell cytolysis. Nonetheless caffeine at optimal intermediate dose in the chronic condition (<u>in vivo</u>) had a decreasing effect on NK cell activity. This supports an explanation in terms of changes in the quantity and distribution of surface receptor (or target cell binding receptors) on NK cells. However, these changes need a period of time more than 4 hrs to occur. On the other hand, caffeine added directly to cultures exerted similar (significant) inhibiting effects on both the B and T cell proliferative responses to the mitogens PWM and PHA-P, respectively. The mechanisms proposed for this effect are first, changes of intracellular Ca^{2+} level inside B and T lymphocytes by caffeine. PWM and PHA appear to activate B and T lymphocyte proliferation by regulation of Ca^{2+} channel opening through the generation of inositol polyphosphate. The increase of intracellular Ca^{2+} concentration and the activation of protein kinase C appear to play important roles in the initiation of immune response. Freedman _et al_. (133) reported an abrupt two- to five-fold increase in rate of $^{45}Ca^{2+}$ uptake into lymphocyte within 1 to 5 minutes after mitogen contact. In addition, Whitney and Sutherland (134) reported up to 70 to 85% inhibition of DNA and RNA synthesis in PHA stimulation if external Ca^{2+} was low 2 hours before stimulation and 25% inhibition after 12 hours incubation. The possible interfering effect of caffeine (_in vitro_) on B and T lymphocytes might be at the level of membrane polyphosphoinositide hydrolysis that interferes with the generation of inositol polyphosphate. However, this reaction may require a period of time (72 hours) for the effect to be observed.

A second possible mechanism may involve cAMP. It has been shown that cAMP plays an important role in cell proliferation. Caffeine interacts with A_1 adenosine receptor on the membrane of lymphocytes and functiones as adenosine receptor antagonist. resulting in the accumulation of cAMP (135-138). In addition, higher concentration of caffeine acts as a phosphodiesterase inhibitor. Therefore, more accumulation of cAMP is observed (128). Most reports indicate that cAMP generally inhibites immune responses, for example, PHA mitogenesis can be prevented by cAMP and also by agents which induce intracellular cAMP synthesis (120, 139). These evidences agree with our study. It is likely that one mechanism whereby caffeine inhibits mitogen stimulated lymphocyte proliferative response(s) is through the accumulation of intracellular cAMP.

At certain concentration, caffeine may interfere with the biological activity of macrophage, such as the release of interleukin-1 (IL-1) or it may induce the secretion of prostaglandin (PGE_2). In the proliferation of immune cells, various factors are necessary. In in vitro assay, caffeine may affect the release of IL-1 from macrophage, which may also decrease the synthesis of IL-2 from the T_H subpopulation. The decreased IL-1, IL-2 or both or the accumulation of PGE_2 in the culture condition may decrease or inhibit the proliferation capacities of B and T lymphocytes to mitogen stimulation.

In conclusion, caffeine showed different effects on rat immune cell functions. The differences were also observed in chronic (in vivo) and in vitro (direct) conditions. However, all effects of caffeine were dose dependent in both conditions and there was a dose relationship of caffeine concentration and the inhibition of B and T lymphocyte proliferative responses in *in vitro*. There were different effects of caffeine observed in this study: 1) An enhancing effect was demonstrated in T lymphocyte proliferation to PHA-P stimulation (50 and 100 µg/ml) in chronic caffeine treated rats 18 mg/kg/day. 2) inhibitory effects were observed on NK cell cytotoxic activity and B lymphocyte mitogen-stimulated proliferations from chronic caffeine treated rats administered an intermediate 6 mg/kg/day. In contrast, both B and T lymphocyte mitogen-stimulated proliferations decrease by *in vitro* (direct) caffeine addition. However, no effect could be demonstrated on NK cell cytotoxic activity in the *in vitro* (direct) caffeine addition. The mechanisms proposed were: 1) the changes of distribution and quantities of surface receptors on NK, B and T cells in chronic caffeine administration, 2) the interfering effect of caffeine on intracellular Ca^{2+} level, 3) the accumulation of cAMP inside the cell, 4) the reduction of IL-1 secreted from macrophages and IL-2 synthesized by T cells, and 5) the increased product of PGE_2 secreted from macrophage.

This study showed that caffeine had the effects on NK, B and T cell activities both in the chronic (*in vivo*) and *in vitro* (direct) conditions. The *in vitro* (direct) effect of caffeine on B and T cells proliferation activities showed the pattern of log dose-effect relationship. Thus, the future studies for the exact mechamism(s) and dose effect relationship are needed which may be applied for the manipulation of B and T cell activities in some immunopathological conditions.

CHAPTER VI

SUMMARY

The conventional 4-hr ^{51}Cr release cytotoxicity assay for NK cell activities on YAC-1 target cell and the assay for proliferative responses of B and T cells to mitogen stimulation (PWM, PHA-P) were performed to study the effect of caffeine on Sprague-Dawley rat immune cells. Both chronic (in vivo) and direct (in vitro) effects of caffeine were studied.

1. Both the NK cell activity and B lymphocyte proliferative response to PWM of 6 mg/kg/day chronic caffeine (in vivo) treated rats (120 days) were significantly decreased ($p < 0.05$) from control rats. However, there were no significant differences ($p > 0.05$) in the 2 and 18 mg/kg/day chronic caffeine treated rats nor among each group of rats.

2. At 50 and 100 μg/ml PHA-P stimulation, T lymphocyte proliferative responses of 18 mg/kg/day chronic caffeine treated rats were significantly increased ($p < 0.05$). But the increases were not significant in the other two groups (2 and 6 mg/kg/day).

3. Total leukocytes and leukocyte differentiation of all three groups (2, 6 and 18 mg/kg/day) of chronic caffeine treated rats were not different ($p > 0.05$) from the

control rats.

4. No significant differences ($p > 0.05$) in NK cell activities were observed in the absence or presence of various concentrations of caffeine (5, 10, 20, 40 µg/ml) in the assay system.

5. Caffeine at any concentration studied did not have any lytic effect on YAC-1 cells nor any proliferative effect on splenic mononuclear cells.

6. The direct (in vitro) effect of caffeine on both B and T lymphocyte mitogen-stimulated proliferative responses showed similar results. There were significantly decreased ($p < 0.05$) B and T lymphocyte proliferative responses at 10, 20 and 40 µg/ml caffeine.

7. Positive relationships were found between percent inhibition of B and T lymphocyte proliferative responses and the various concentrations of caffeine (1, 10, 100 and 1,000 µg/ml) in the assay system.

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APPENDIX

RPMI 1640 culture medium (Gibco)

RPMI 1640 (instant powder)	1 package/litre
$NaHCO_3$	0.2 g
Penicillin-Streptomycin (10^5 U, 10^5 μg/ml) solution	1.0 ml
1 M HEPES	10.0 ml
L-glutamine	350.0 mg

Adjust pH with 1 N NaOH or 1 N HCl to 0.2-0.3 below final working pH (pH 7.2-7.4). Add non-ionized distilled water to 1000 ml. Sterilization by 0.45 μ millipore membrane filtration.

Ficoll-Hypaque mixture (density 1.077 g/cm^3)

9% Ficoll	: Ficoll 400 (Phamacia Fine Chemicals)	9.0 ml
	Distilled water to	100.0 ml
	Sterilization by autoclaving	
To use	: 9% Ficoll	20 volume
	33.3% Hypaque (Wintrop Laboratories)	10 volume

Scintillation fluid

PPO	5.0 g
M_2POPOP	0.1 g
Toluene	1.0 l

EDTA

Dipotassium EDTA	7.5 g
Distilled water to	100.0 ml

Put 0.1 ml of EDTA solution into 5-6 ml test tube, then dry it in 40-50 °C oven. This amount of EDTA can act as

anticoaggulant of 2-5 ml of blood.

Phosphate buffer (pH 7.2, 1/15 M)

Stock A 1/15 M Na_2HPO_4

Na_2HPO_4	9.5 g
Distilled water to	1,000 ml

Stock B 1/15 M NaH_2PO_4 $2H_2O$

NaH_2PO_4 $2H_2O$	9.2 g
Distilled water to	1,000 ml

Working buffer

Stock A	72.0 ml
Stock B	28.0 ml
Distilled water	900 ml
Final pH 7.2	

Phosphate buffer saline (pH 7.2, 0.15 M)

Sodium chloride	8.5 g
Phosphate buffer to	1,000 ml
Final pH 7.2	

Calculation of caffeine doses for rat feeding

It is important to design appropriate amount of test compound feeding laboratory animals. In general, the doses base on the amount of substances per body weight of animal, e.g., mg/kg. Pinkel (140) showed that to determine the appropriate amount of test compound to administer to patient or laboratory animal, it should be based on body surface area. On the basis that small animals had relatively more surface and used relatively more oxygen than large animals.

Doses in rats can be roughly equivalent to the dosage used in human. The doses can be converted from mg/kg to mg/m^2 by the following formula.

$$\text{dose in mg/m}^2 = \text{km} \times \text{dose in mg/kg}$$

Where km is appropriate factor derived from standard relationship between weight and surface area as given by Sendory and Cecchini (141)

$$\text{km} = \frac{10^2 \times (\text{kg})^{1/3}}{k}$$

where k values were given for each species (mouse=9.0, rat=9.0, monkey=11.8, dog=10.1 and man=1.0).