SUZI HONG

The Effect of Voluntary Wheel Running on Natural Killer Cell Cytotoxicity after Olfactory Bulbectomy

(Under the Direction of ROD K. DISHMAN)

This dissertation summarized and quantitatively evaluated the published literature on the effect of exercise on natural killer (NK) cell cytotoxic function in humans using meta-analysis. It, also investigated the putative role of exercise in the modulation of splenic NK cell cytotoxicity after olfactory bulbectomy (OBX), a rat model of human depression which has immunosuppressive features.

Results from a quantitative synthesis of the literature in humans indicated that there were moderate effects of acute exercise and exercise training and a large effect of fitness/training status on NK cell cytotoxicity. Moderator analyses showed that acute exercise produced a distinct pattern of alteration in NK cell cytotoxicity. NK cell cytotoxicity is markedly increased during and soon after exercise, decreased below the pre-exercise baseline 1-3 hours after termination of exercise, and returned to the baseline value within 24 hours of recovery. Also, effects of acute exercise were larger for sedentary or female participants and when an exercise session lasted 20 minutes to 2 hours, compared to exercise sessions that were longer than 2 hours. Therefore, the synthesis of literature on the effect of exercise on NK cell activity revealed that NK cell activity is influenced by both acute exercise and exercise training, but this effect varied according to characteristics of the exercise and participants.

The effects of OBX on splenic NK cell cytotoxicity *in vitro,* plasma corticosterone level and NK cell apoptosis in Long-Evans rats were examined. Whether activity wheel running and imipramine would moderate those effects also was examined. A 2 Condition (OBX vs. sham) x 2 Group (activity wheel vs. sedentary) x 2 Treatment (imipramine vs. saline) factorial design was used. Male Long-Evans rats were randomly assigned to groups 24 hours after OBX or sham surgery. After 3 weeks, animals were tested for open-field behavior. Blood and spleens were collected 24-72 hours later and assayed for NK cell cytotoxicity using standard chromium release and for plasma corticosterone using radioimmunoassay. Analysis of

NK cell apoptosis was done by flow cytometry. Incubation with dexamethasone (100 nM) resulted in apoptosis of NK cells as expected. NK cell activity was not affected by OBX or 3 weeks of voluntary wheel running or imipramine. Also, there was no difference in the percentage of apoptotic cells between OBX (4.58 ± 3.69 %) and sham (4.93 ± 5.09 %) animals. In contrast to expected results, plasma corticosterone level was lower in OBX rats (6.52 ± 6.0 µg/dL) compared to sham surgery animals (10.30 ± 8.70 µg/dL), $p = 0.03$. Although olfactory bulbectomy resulted in hyperactive behavior, it did not result in immunosuppression of splenic NK cell activity through the hypothesized mechanism of glucocorticoid-induced cell apoptosis.

INDEX WORDS: Apoptosis, Corticosterone, Exercise, Open-field behavior, Rat

THE EFFECT OF VOLUNTARY WHEEL RUNNING ON NATURAL KILLER CELL CYTOTOXICITY AFTER OLFACTORY BULBECTOMY

by

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DEDICATION

I would like to dedicate my dissertation to my family who has been always supportive and understanding:

I thank my Mom and Dad who always encouraged me to be ambitious and confident in myself, for their constant prayers and love.

I thank my sister, Shinji for being a terrific sis and a friend and brother, Seungki for being Seungki.

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INTRODUCTION AND LITERATURE REVIEW

Historically, the immune system has been considered to be self-regulating and independent of other regulatory mechanisms. Hence, the interaction of the immune system with other systems of the organism did not receive much attention among immunologists until the past decade. Communication between the nervous and immune systems began to draw attention in the area of psychoneuroimmunology. The term, 'psychoimmunology' appeared in a paper by Solomon and Moos in 1964 (Solomon & Moos, 1964) and was modified to 'psychoneuroimmunology' by Ader in 1981 (Ader, 1981). Psychoneuroimmunology is the study of the reciprocal interactions between the behavioral-neural-endocrine-immune systems (Ader, Felten, & Cohen, 1995; Solomon, 1969), which would be described more accurately by the term, 'psychoneuroendocrinoimmunology' (Solomon, 1987). Research in the area of psychoneuroimmunology has flourished since the early 1980s. However, the issue of communication between the brain and the immune system began to receive attention in the 1960s. Solomon (1968) reported stress-induced suppression of immune responses in rats. Higher serum antibody titers were found in a group of rats handled neo-natally compared to control group (Solomon, 1969).

Findings of the early research on psychoneuroimmunology were based on investigations of psychosocial influences on immunity. Among humans, immunosuppression (e.g., decreased NK cell function, interferon (INF) production, lymphocyte proliferation, etc.) was observed during or after acute stress (e.g., examinations in medical school students) and chronic stress (i.e., depression caused by prolonged or repeated stressful events) (Kiecolt-Glaser & Glaser, 1987). The relationship between social and/or emotional factors and immunomodulation has

been studied extensively in the area of psychoneuroimmunology. Various functional and enumerative features of the immune system (e.g., NK cell cytotoxicity and circulating number, lymphocyte proliferation, etc.) were compromised under psychological and physical stress, which led to an increased disease susceptibility (Glaser, Kiecolt-Glaser, Malarkey, & Sheridan,1998; Herbert & Cohen, 1993; Kemeny & Gruenewald, 1999; Weisse, 1992). More recently, the bidirectional communication between brain and the immune system has drawn attention in the area of psychoneuroimmunology (Maier & Watkins, 1998), including the role of cytokines in human behavior and affective disorders. However, this dissertation will only address the influence of psychological factors on immune measures, NK cell cytotoxic function in particular, will be discussed.

In this review, psychosocial factors that have been found to affect various components of immunity will be divided into two categories: 1) acute stress, including laboratory stressors (e.g., mental arithmetic) and real life stressors (e.g., examination, public speaking) in humans, or physical stressors (e.g., electric footshock, forced swimming) in rats and mice, and 2) major depression that has prolonged effects on immunity.

The NK cell is a critical component of innate cellular immunity in host defense against viral infections and tumor cells. Both functional and enumerative aspects of NK cells are among the most responsive parameters of the immune system to psychological and physical challenge. NK cell cytotoxic function against tumor cells *in vitro* has been found to be lower in individuals experiencing psychological stress (Ader et al., 1991; Black, 1995; Kiecolt-Glaser & Glaser, 1987) and in patients with major depression (Bauer et al., 1995; Cover & Irwin, 1994; Herbert & Cohen, 1993). Changes in the function and circulating number of NK cells are reliable indices of immunomodulation induced by stress.

The effect of exercise on immunity has been extensively studied during the past 20 years. However, the findings in the area of "exercise immunology" have not been consistent, and the mechanisms underlying immunomodulation during or after acute exercise or exercise training are

not clear. The mixed findings are thought to depend upon different exercise stimuli (e.g., various exercise intensities, length, mode, etc.), individual characteristics of participants (e.g., age, fitness level/training status, etc.), and training regimen (e.g., frequency, duration, etc.). An acute bout of exercise either enhanced or reduced functions of the immune system, and exercise intensity appeared to be a main factor accounting for differential effects of exercise on NK cell number and function in peripheral blood (Pedersen & Hoffman-Goetz, 2000). Hoffman-Goetz & Pedersen (1994) concluded in their review that an exercise bout of moderate intensity enhanced NK cell activity, but a heavy exercise bout resulted in decreased NK cell cytotoxicity and increased susceptibility to infections, such as upper respiratory tract infection. Also, different intensities of exercise have been shown to induce different patterns of leukocyte circulation and migration in a leukocyte-specific manner (e.g., leukocytosis after moderate exercise, but lymphocytopenia after heavy exercise) (Mars et al., 1998).

There is a relatively small number of studies examining the effects of exercise training on immunity. Furthermore, there has been little effort given to examining possible adaptations at the level of the nervous and endocrine systems after exercise training that might explain immunomodulation after exercise. Therefore, a systematic and quantitative review of literature will enable researchers to evaluate the magnitude of the effect of exercise on immunity. And, investigations to examine the role of the neuroendocrine system in immune function will provide evidence about putative neuroendocrine-immune communication and its role in immunomodulation.

Purposes

This dissertation 1) describes the literature in psychoneuroimmunology with an emphasis on NK cell cytotoxicity after acute or chronic psychological and physical stressors in humans and rats and mice, 2) describes the linkage between the sympathetic and neuroendocrine systems with the immune system as plausible mechanisms of stress- induced immunomodulation, 3) summerizes and quantitatively evaluates the published literature on the effects of exercise on

NK cell cytotoxic function among humans using meta-analysis, and 4) investigates a putative role of exercise in the modulation of NK cell activity after olfactory bulbectomy, which is a rat model of human depression that has immunosuppressive features.

Psychosocial Stress and the Immune System

Stress and NK Cells in Humans

Suppressed NK cell cytolytic function against tumor cells measured *in vitro* has been consistently observed among individuals experiencing psychological stress (Ader et al., 1991; Kiecolt-Glaser et al., 1987). A meta-analysis of 8 studies estimated an average increase in circulating NK cell number that was large (effect size of about .80 SD; Cohen's d, weighted) after acute laboratory stressors (e.g., Stroop test, speech, mental arithmetic, etc.) among women (Benschop, geenen, Mills, Naliboff, Kiecolt-Glaser, Herbert, Van der Pompe, Miller, Matthews, Godaert, Heijnen, dopp, Bijlsma, Solomon & Cacioppo, 1997). However, changes in peripheral NK cell activity during or after acute stressors (e.g., exams , laboratory stressors, etc.) were inconsistent across studies (Van Rood, Bogaards, Goulmy & van Houwelinger, 1993).

Mental arithmetic stress increased peripheral blood NK cell cytolysis measured *in vitro* in young, but not in old women (Naliboff, Benton, Solomon, Morley, fahey, Bloom, Makinodan & Gilmore, 1991). NK cell activity was enhanced during and at the end of a 12-min stress session of anticipating electric-shock and lower than the baseline value during 12-min of relaxation in young males (Breznitz, Ben-Zur, Berzon, Weiss, levitan, tarcic, Lischinski, greenberg, levi & Zinder, 1998). Reduced NK cell activity was observed in a group of high trait-anxious students on an examination day compared to baseline activity (Borella, Bargellini, Rovesti, Pirielli, Vivoli, Solfrini & Vivoli, 1999). In contrast, a low trait-anxiety group of students demonstrated increased NK cell activity under the exam stress.

Psychological and physical stress and NK cell in Rats and Mice

Various emotional and physical stressors have influenced NK cell activity and host defense against viral infections in rodents. Acute footshock reliably leads to a 25-50% suppression *in vitro* of splenic NK cell cytolysis in rats that persists for 24 to 96 hours (Cunnick, Lysle, Armfield & Rabin, 1988; Shavit, Terman & Lewis, 1986). Natural cytotoxicity measured *in vivo* by lung-clearance of ^{51}Cr- labeled YAC-1 cells, increased during 25 minutes of air jet stress correlated with increased level of plasma norepinephrine level and returned to the baseline value two hours after the stress session (Jonsdottir, Johansson, Asea, Hellstrand & Hoffman, 1996).

Mixed findings in immune responses against viruses have been reported in studies which used various stressors of different intensities. Mice physically restrained by immobilization showed a significantly higher mortality rate after infection with influenza virus compared to a control group, although the ability to clear virus from the respiratory tract was not different between the two groups (Hermann, Tovar, Beck, Allen & Sheridan, 1993). Sleep deprived mice showed complete abrogation of immunization with influenza virus, while immunized mice with normal sleep had total virus clearance (Brown, Pang, Husband & King, 1989). However, either 7 or 12 hours of sleep deprivation did not significantly influence lung viral clearance in naive and immunized mice (Toth & Rehg, 1998). The mixed data illustrate the important issue of developing reliable stress models that are appropriate for investigating the immuno-modulating effect of physical or emotional stressors.

Underlying Mechanisms of Stress-Induced Immunomodulation

Brain-lesion studies have been conducted to examine neural links between the nervous and immune systems. Lesions in brain areas such as the hypothalamus, limbic system, and cortex resulted in decreased immune function (e.g., NK cell cytotoxicity, T cell proliferation, etc.) (Ader et al., 1995). There have been two main pathways investigated as outflow from the brain to the immune system under psychosocial stress: activation of the sympathetic nervous system (SNS) and the hypothalamic-pituitary-adreno-cortical (HPAC) axis.

Sympathetic Innervation and Immunomodulation. There are neuronal connections found between the brain and immune organs. Noradrenergic projections to the rat spleen and norepinephrine (NE) containing terminals have been found in the periarteriolar lymphatic sheath of rat spleen (Felten, Bellinger, Carlson, Ackerman, madden, Olschowka & Livnat, 1987; Felten & Olschowka, 1987). In humans, $-adrenergic receptors have been identified on lymphocytes including NK cells (Maisel, harris & Rearden, 1990). NE release from splenic sympathetic nerves has been correlated with inhibition of NK cell cytotoxicity (Hellstrand et al., 1987).

The $-adrenergic agonist, isoproterenol, and other cAMP inducers inhibit NK cell activity of human peripheral blood monocytes measured *in vitro* (Katz et al., 1982). Suppression of splenic NK cell activity after surgical laparotomy stress in rats was blunted after splenic denervation and restored by electrical stimulation of the cut nerve (Katafuchi, take & Hori, 1993). The suppression by electrical stimulation was blunted by non-selective adrenergic blockade, prazosin. In-vitro preincubation with different concentrations of epinephrine resulted in mixed effects on NK cell activity; 10^{-6} M inhibited, but 10^{-8} M enhanced NK cell activity (Hellstrand et al., 1985). Infusion of epinephrine (1 mg/ml infused at 500ng/min) for 1 hour resulted in increased lymphokine activated (LAK) cell activity accompanied by an increased percentage of circulating NK cells, whereas NE infusion (1 mg/ml infused at 1100 ng/min) for 1 hour did not affect NK cell activity or percentage (Kappel et al., 1997). Meanwhile, administration of epinephrine resulted in an immediate but transient increase in NK cell activity (Tonnesen , Christensen & Brinklov, 1987).

The regulatory role of the SNS on the immune system also has been also investigated by destruction or impairment of sympathetic outflow to the immune tissues. Chemical sympathectomy has been shown to positively influence the immune system, especially splenic NK cell cytotoxicity. Noradrenergic nerve destruction by the neurotoxin, 6-hydroxydopamine (6-OHDA), which causes dendritic killing of peripheral sympathetic nerve cells, has resulted in

NE depletion in spleen by 70-95 % in rats and mice (e.g., Dishman, Hong, Soares, Bunnell & Evans, 2000; Irwin, Hauger & Brown, 1992; Madden, Ackerman, Livnat, Felten & Felten, 1993). Livnat and colleagues (1987) showed an increased basal NK cell activity after sympathectomy, assessed *in vivo* by lung-clearance of YAC-1 lymphoma cells and *in vitro* by ^{51}Cr-release assays with splenic NK cells (Livnat, madden, felten & Felten, 1987). Similarly, suppressed splenic NK cell activity after intracerebroventricular injection of corticotrophin releasing factor (CRF), was restored by sympathectomy using chlorisondamine, a ganglionic-blocker (Irwin, 1988) and 6-OHDA (Irwin, Hauger, Jones, provencio & Britton, 1990). Therefore, it was hypothesized that sympathectomy offset the immunusuppression caused by sympathetic outflow to immune cells and organs.

There is mixed evidence in the *in vivo* rat studies. Cervical sympathetic denervation of the stellate ganglion suppressed splenic NK cell activity compared to sham operation (Okada, Guo & Hisamitsu, 1996). However, preganglionic sympathectomy using an acetylcholinesterase (AchE) monoclonal antibody did not alter morphine-induced suppression of splenic NK cell activity (Fecho, Masloek, Dykstra & Lysle, 1996), and neither $1-selective nor $2-selective blockade blunted suppression of splenic NK cell activity conditioned to electric shock (Luecken & Lysle, 1992). Peripheral chemical sympathectomy was also shown to activate brain regions, especially, the paraventricular nucleus (PVN) of the hypothalamus and CRF-containing neurons in the PVN, which may offer an alternative explanation for sympathectomy-induced immunomodulation (Callahan, Moynihan & Piekut, 1998).

Activation of the HPAC Axis and Immunomodulation. Neuroendocrine responses to stressors include increased levels of glucocorticoids in circulating blood as a result of the activated HPAC axis. Activation of the hypothalamus under stress is another putative pathway for a brain-immune system interaction. Increased levels of circulating glucocorticoids regulate organs and cells of the immune system which possess receptors for these hormones (Maier & Watkins, 1998).

The effects of cortisol on immunity have been inconsistent in humans. Intravenous (i.v.) administration of hydrocortisone (300 mg) resulted in increased NK cell activity against K562 target cells *in vitro* at 4 hours, decreased activity at 24 hours, and a return to baseline activity at 48 hours (Onsrud & Thorsby, 1981). However, no change in NK cell activity was observed during or after continuous 5 hour i.v. infusion of cortisol (Tonnesen et al., 1987) or upon decreased and increased cortisol levels manipulated by dexamethasone treatment and i.v. injection of ACTH (1 mg), respectively (Bodner, Ho & Kreek, 1998).

Elevated corticosterone level in plasma during immobilization stress has been shown to modulate immunity associated with increased susceptibility to or severity of a viral infection. The immunity against infections was measured by homing of cells and the clearance of viruses in the areas of infection such as lungs. Enhanced immune responses to a viral infection was shown in mice after adrenalectomy (Semenkov, Levina, Lavrov & Ageena, 1990). Infiltration and lung consolidation of mononuclear cells during an experimental influenza viral infection were reduced in physically restrained mice, and depressed anti-viral cellular immunity was correlated with elevated plasma corticosterone levels (Hermann et al., 1993; Sheridan, Feng, Bonneau, Allen, Huneycutt & Glaser, 1991). Similarly, immobilization stress resulted in elevated plasma corticosterone levels and reduced accumulation of mononuclear cells in the lungs, which were blunted by the glucocorticoid antagonist, RU486 (Hermann, Beck & Sheridan, 1995). The enhanced survival rate during influenza viral infection in restrained mice was negatively correlated with corticosterone levels and was dependent on the strain of mice (Hermann, Beck, Tovar, Malarkey, Allen & Sheridan, 1994). Mortality was decreased in DBA/2 mice but unaffected in C57BL/6 mice after influenza virus infection.

Mice under physical restraint, also demonstrated reduced production of the virus-specific cytokines such as IL-2, IFN-(, and IL-10 by mononuclear cells and splenocytes after infection with influenza virus. Also, *in vitro* culture with corticosterone in the range of 10^{-10} to

10^{-6} M resulted in inhibition of virus-specific cytokine production by splenic T_H cells in a dose-dependent manner (Dobbs, feng, Beck & Sheridan, 1996).

Other Mechanisms. In addition to the activated SNS innervation of immune tissues and increased level of adrenal hormones, opiates and endogenous opioid peptides appear to play a role in the regulation of immunity during stress. Opioid receptors have been found on large granular lymphocytes, which include NK cells (Mandler, Mandler & Serrate, 1986). Intermittent electric footshock activates the opioid system and suppresses NK cell activity. That suppression is blunted by opioid antagonists. Continuous footshock stress which induces nonopioid analgesia did not induce suppression of NK cell activity (see Shavit & Martin, 1987 for review). However, five consecutive 3-minute forced swimming bouts separated by 3-minute rest periods significantly suppressed splenic NK cell activity, but the suppression was not blocked by the opioid antagonist, naltrexone (Eliyahu, Yirmiya, Shavit & Liebeskind, 1990). Reduced NK cell activity was observed after aversive conditioned stimulus paired with electric shock which was blocked by naltrexone or a µ1-selective opioid antagonist (Perez & Lysle, 1997). Also, chronic i.v. administration of $-endorphin has been reported to augment NK cell activity (Jonsdottir, asea, Johansson, Hellstrand, Thoren & Hoffman, 1996).

Depression and Immunomodulation

It has been suggested in the literature that there is a relationship between depression and immune function. Depression, a major affective disorder characterized by a wide range of symptoms (i.e. feelings of sadness, helplessness, guilt, agitation, hypercortisolism, etc.) and signs (i.e. sleep disturbances, weight loss, etc.), has been shown to suppress immunity which leads to heightened disease susceptibility. Weisse (1992) reviewed the literature on altered immunity in depressed patients and reported that depressed patients diagnosed by various diagnostic criteria (i.e. DSR-III, Hamilton Rating Scale for Depression, RDC, etc.) exhibited lower lymphocyte responses to mitogens, altered numbers or percentages of lymphocytes, decreased neutrophil function, lowered antibody production and lower NK cell activity compared to

normal individuals. Lymphocyte proliferative responses and NK cell activity have been consistently shown to be low in depressed patients. Depressed patients have lower antibody production, and neutrophil activity has shown to be negatively correlated with severity of depressive symptoms. However, the evidence on the numbers or percentages of leukocytes or lymphocytes in depressed patients is not consistent (Weisse, 1992).

Herbert and Cohen (1993) conducted a meta-analysis to investigate the relationship between clinical depression and both the enumerative (i.e. T helper and cytotoxic cell ratio, antibody titers to virus, and numbers of leukocytes) and functional (i.e. lymphocyte proliferative response and NK cell activity) aspects of immunity. There was an association between enumerative parameters of immunity and depression in 14 studies with sound experimental design and methodology as Pearson's product-moment correlation coefficient (r) was used as the effect size estimate; higher number of total leukocytes (0.38; CI: 0.26 to 0.49), higher number of monocytes (0.26; CI: 0.10 to 0.41) and the percentage of neutrophils (0.80; CI: .75 ~ .84), lower numbers of lymphocytes (-0.14; CI: -0.26 to -0.05), lower numbers or percentages of B cells (-0.2; CI: -0.29 to
-0.05), T cells (-0.43; CI: -0.51 to -0.34), and NK cells (-0.21; CI: -0.37 to -0.03). Herbert and Cohen (1993) concluded that there was a overall association between depression and functional measures of immunity. Effect sizes (r) for proliferative measures ranged from -0.24 to -0.45 according to different mitogens used, and lower natural killer cell activity (-0.25; CI: -0.34 to -0.16) was shown in depressed population.

Since Weisse (1992) and Herbert and Cohen (1993) have reviewed the studies on depression and immunity more recent studies have shown a higher T helper to suppressor/cytotoxic (T_H :$T_{S/C}$) cell ratio, leukocytosis, monocytosis and neutrophilia (Charles et al., 1992; Maes, Bosmans & Meltzer, 1995; Maes, Lambrechts, Suy, Vandervorst & Bosmans, 1994; Maes, Vandoolaeghe, Ranjan, Bosmans, van Gastel, bergmans & Desnyder, 1996), higher NK cell count reversed following antidepressant treatment (Ravindran, Griffiths,

Merali & Anisman, 1998). Also, reduced lymphocyte proliferative responses (Spurrell & Creed, 1993) to mitogens and neutrophil phagocytosis (McAdams & Leonard, 1993) were found in depressed patients. However, monocyte function was found to be higher (McAdams & Leonard, 1993) or similar (Landmann, Schaub, Link & Wacker, 1997) in depressed individuals compared to control groups. In addition, depressed patients demonstrated decreased delayed-type hypersensitivity (DTH) skin responses (Hickie et al., 1993), increased levels of acute phase proteins such as C4, IL-6, and C-reactive protein (Berk, Wadee, Kuschke & O'Neill-Kerr, 1997), and cytokine hypersecretion (Connor & Leonard, 1998).

Evidence showed that individuals suffering from major depression have exhibited increased susceptibility to viral infections and a higher risk for prolonged chronic illnesses such as cancer (Ballin et al., 1997; LaPerriere Antoni, Schneiderman, Ironson, Kilmas, Caralis, & Fletcher, 1990). Reduced NK cell activity has been found in clinical populations suffering from chronic illnesses such as cancer (Levy, Herberman, Whiteside, Sanzo, lee & Kirkwood, 1990; Ballin et al., 1997) and acquired immunodeficiency syndrom (AIDS) (LaPerriere et al., 1990), that were accompanied by a high level of distress and depressive symptoms. Due to the anxiety and stress that patients with chronic illness experience, a large percentage of cancer patients suffer from psychiatric disorders, especially depression. Immunosuppressing effects of depression influence the course of cancer and a longer survival period was reported in cancer patients who received psychotherapy (Spiegel, 1996).

Depression and NK Cell Function

Activity of NK cell against tumor cells is one of the major functional immune parameters studied in the area of psychoneuroimmunology and has been found to be significantly reduced in clinically depressed patients as well as individuals undergoing traumatic life events (i.e. divorce, bereavement, etc.).

Irwin and colleagues conducted a series of studies on NK cell activity in individuals undergoing stressful life events and experiencing depressive symptoms. NK cell cytotoxicity

was investigated in women during bereavement and was significantly lower in women who lost their spouses compared to an age-matched control group. Depressive symptoms before and after the death of their spouses were shown to be related to decreased NK cell activity (Irwin et al., 1987). Twenty-one middle aged widows who recently lost their spouses were found to exhibit similar immune functions with controls. However, those who experienced major depressive symptoms by DSM-III-R criteria showed reduced NK cell activity (Zisook, Shuchter, Irwin, Darko, Sledge & Resovsky, 1994). The relationship between NK cytotoxicity and severe life stress and depression was also examined in hospitalized depressed patients. It was shown that major depression and stressful life events were associated with 50% decrease of NK cell activity, independent of hospitalization itself (Irwin et al., 1990, Caldwell, Irwin, & Lohr, 1991).

Decreased NK cell activity was shown in various age groups. Young adults with unipolar major depression determined by DSM-III-R showed lower number of circulating NK cells and NK cell activity compared to the matched control subjects (Schleifer, Keller, Bartlett, Eckholdt & Delaney, 1996). The immunological consequences of chronic stressors were examined in older adults. Former and current Alzheimer caregiver individuals demonstrated depressive symptoms and lower reponsiveness of NK cells to cytokines such as INF-(and IL-2 that activate NK cells (Esterling, Kiecolt-Glaser, Bodnar & Glaser, 1994).

The leukocytosis found in depressed patients is mainly caused by monocytosis and neutrophilia, while decreases in NK cell number has been reported in individuals with major depression in many studies. Moreover, there is empirical evidence showing that reduced NK cell cytotoxicity found in depressed patients is independent of alterations in the number or percentage of NK cells. Maes et al. (1994) argued that depression-associated reduced NK cell activity in 36 depressed in patients was not related to the altered peripheral NK cell number or percentage. Regression analyses revealed that depressive mood and somatic anxiety contributed to 43% of the variance in NK cell cytotoxicity in depressed patients

compared to normal controls (Maes et al., 1994). It was also argued that decreased NK cell activity was not due to lower circulating numbers of NK cells in depressed patients (Evans, Leserman, Perkins, Stern, Murphy, Tamul, liao, van der Horst, hall & Folds, 1995).

Neuroendocrine Response in Depression

Monoamines. Hypotheses of brain monoamine dysregulation has been investigated in the neurobiology of depression since reserpine, the rauwolfia alkaloid, was found in the 1950s to deplete brain catecholamines and serotonin (5-HT) resulting in depressive states in patients who were treated for hypertension (Maas, 1979; Nemeroff, 1998). Researchers have studied the abnormalities in the brain monoamine system in depressed individuals. In general, abnormally low levels of monoamines in the brain were found to cause the depression which led to development of monoamine oxidase inhibitors as antidepressant agents. The catecholamine hypothesis of mood disorder was proposed in which a norepinephrine (NE) deficiency or abundance in certain brain circuits was the cause of depression and mania respectively by Joseph Schildkraut in the 1960s. The NE hypothesis of depression has been recognized, and NE reuptake inhibitor has been shown to be an effective antidepressant in many depressed individuals.

More recently, research on the serotonergic system of depression has flourished. It was hypothesized that synaptic depletion of serotonin (5-HT) caused depression by promoting a decrease in the level of NE. Serotonin-producing neurons project from the raphe nuclei to various regions of the brain including the amygdala, hypothalamus, and those that secrete or control the release of NE, locus coeruleus. It has been shown that the drugs that block presynaptic reuptake transporters have significant therapeutic effectiveness in treating depression which led to the development of antidepressants that are collectively called selective serotonin reuptake inhibitor (SSRI) (Nemeroff, 1998).

Researchers have attempted to resolve whether excessive or depleted neurotransmitters in the brain was responsible for depressive states. Evidence showed that both reserpine and

isoniazid administration resulted in depressive symptoms although reserpine is a catecholamine depleting agent and isoniazid is a monoamine oxidase (MAO) inhibitor which increases the level of those neurotransmitters. However, Maas (1979) pointed out that static measures of neurotransmitter levels did not provide the information regarding the integrated alteration in the functioning of the system (i.e. turnover, reuptake, sensitivity of receptors, etc.). Therefore, investigating the ability to regulate functioning of the neurotransmitter system appears to be more critical than the absolute levels of neurotransmitters (Maas, 1979).

HPAC system. Hormonal abnormalities have been reported in depressed individuals. Depressed patients have demonstrated reduced responses to the hypothalamic substances that stimulate the release of pituitary hormones such as growth hormone and thyroid-stimulating hormone. The majority of empirical evidence is pointing toward dysregulation of the HPAC axis as one of possible underlying mechanisms of depression. Chronic activation of The HPAC axis that regulates the body's response to the stressor, has been suggested to result in depression and increased activity of HPAC axis has bee found in depressed patients as measured by higher level of corticotropin-releasing factor (CRF) in cerebrospinal fluid and cortisol in urine, blood and cerebrospinal fluid. Also, enlargement of adrenal gland and pituitary was found in depressed individuals (Nemeroff, 1998).

Mechanisms of Depression & Immunomodulation

Monoamines. Serotonergic dysregulation in depression was also examined as a possible mechanism of immunosuppression in depressed patients using various classes of SSRI or other antidepressant drugs. Rabkin and colleagues (1994) investigated the effect of imipramine on $CD4^+$ cells in HIV positive individuals with major depression. Twelve weeks of imipramine treatment blunted the decline in T cells (Rabkin, Rabkin, Harrison & Wagner, 1994). However, 12 weeks of SSRI, fluoxetine, treatment did not influence CD4 cell counts (Rabkin, Wagner & Rabkin, 1994). Meanwhile, another SSRI, fluvoxamine was shown to increase NK cell counts in cancer patients with major depression after 6 months of treatment

(Ballin et al., 1997). Also, 4 weeks of fluoxetine treatment resulted in decreased depressive symptoms and enhanced NK cell activity in patients who demonstrated low NK cell activity at baseline (Frank, Hendricks, Johnson, Wieseler & Burke, 1999).

The association between activation of the SNS and decreased NK cell activity in patients with major depressive disorder was also investigated. The concentrations of epinephrine, norepinephrine, and neuropeptide-Y were measured in plasma in depressed individuals. NK cell activity was significantly lower in depressed patients and was negatively correlated with the plasma level of neuropeptide-Y (Irwin et al., 1991).

HPAC system. Maes et al. (1994) investigated the relationship between the activity of the HPAC axis and in vivo immune function in depression. Twenty-four hour urinary and plasma cortisol, beta-endorphin, and lymphocyte subsets were examined pre to post dexamethasone administration. Alterations in leukocyte subset populations such as leukocytosis, lymphocytopaenia, monocytopaenia, and neutrophilia were observed after dexamethasone treatment and were significantly correlated with urinary cortisol levels (Maes et al., 1994). Also, there were significant positive correlations between plasma cortisol and levels of IL-6 or IL-2 receptors (Maes et al., 1995) and CD8 cells (Maes et al., 1996) in depressed patients. Plasma prolactin, which is a pituitary hormone known to enhance immune function, was positively correlated with soluble IL-2 receptor concentrations (Maes et al., 1995) and T-lymphocyte proliferation to mitogens (Darko, Gillin, Risch, Bulloch, Golshan, tasevka, & Hamburger, 1989).

Leukocytosis, lymphocytopenia, monocytopenia and neutrophilia following dexamethasone treatment were observed, and they were correlated with urinary cortisol levels (Maes et al., 1994). Also, there were significant positive correlations between plasma prolactin and soluble IL-2 receptor concentrations, and between plasma cortisol and IL-6 or IL-2R (Maes et al., 1995), and CD8 cell concentration (Maes et al., 1996) were found in depressed patients. Cellular immune status and the plasma levels of hypothalamic-pituitary hormones were

assessed in patients with major depression. T-lymphocyte proliferation to mitogens was lower than control subjects at the lowest concentration of ConA and plasma prolactin was significantly correlated with lymphocyte proliferation (Darko et al., 1989). However, alterations in immune parameters in relation to activation of the HPAC axis is characteristics of both stress response and major depression which might be problematic in investigating possible mechanisms of immunosuppressive effect of major depression.

Animal Model of Depression: Olfactory Bulbectomy (OBX) Model

Various animal models of depression have been developed for the purposes of antidepressant screening tests and investigating the neurobiology of human depression. The animal models are based on the behaviors or signs in animals that resemble the symptoms of human depression. Those behaviors and signs are induced by different environmental manipulations such as chronic exposure to a stressor(s) and pharmacological manipulations. As a test for the antidepressant effects of various drugs, the models that are based on pharmacological manipulations used the agents that are shown to affect catecholamine or indolamine systems: reserpine antagonism, tetrabenazine antagonism, potentiation of $_{L}$-Dopa, amphetamine, and the tryptophan models. Chronic exposure to stressors that has been shown to induce depressive signs in animals includes separation, learned helplessness, behavioral despair, and chronic intermittent stress. There are other models that were proposed such as muricide model and electrophysiological model (Jesberger & Richardson, 1985).

The majority of animal models of depression suffer from a lack of validity as a simulation of human depression. The responsiveness to antidepressant drugs and the behavioral features that resemble core symptoms of human depression provide the predictive and face validity of the model (Willner, 1991). Most of the animal models have disadvantages regarding the response to antidepressants because they were shown to be reversed following acute administration of drugs which is not the case in human depression. Most antidepressant drugs were found to be effective only after a minimum of 10-14 days of administration in humans

(Leonard & Tuite, 1981). The olfactory bulbectomy (OBX) model has the advantage in which the depression-like signs induced by bilateral olfactory bulb ablation (i.e. hyperactivity in a novel environment) have been shown to be reversed by various classes of drugs only after chronic administration (Leonard & Tuite, 1981; Richardson, 1991). In the OBX model, the depression-like signs induced by bilateral olfactory bulb ablation such as altered levels of monoamines in the brain (Redmond, Kelly, & Leonard, 1997), elevated plasma levels of corticosterone (Cairncross, Cox, Forster, & Wren, 1979; Cairncross, 1984), hyperactivity in a novel environment (O'Connor & Leonard, 1986; Redmond, Kelly & Loenard, 1999) have been shown to be reversed only after chronic administration of various classes of anti-depressant drugs in rats (Leonard & Tuite, 1981; Richardson, 1991).

OBX and Immune Function

As an established animal model of depression, immunomodulatory effects of olfactory bulbectomy (OBX) in rats have been found to be similar to those shown in depressed patients (Kelly, Wrynn, & Leonard, 1997; Song & Leonard, 1995). The pharmacological manipulation in olfactory bulbectomized rats using antidepressant drugs reversed the immunosuppressive effects of OBX.

OBX resulted in decreased neutrophil phagocytosis (O'Neill et al., 1987; Song & Leonard, 1997), increased leukocyte adhesiveness (Song & Leonard, 1993), lower mitogen-stimulated lymphocyte proliferation (Song & Leonard, 1992, 1994), and an inhibition of plaque-forming cell count accompanied by thymic involution (Komori, Fujiwara, nomura, & Yokoyama, 1991).

Although it was suggested in the literature that olfactory bulbectomy in rats results in impaired immune functions in general, NK cell cytotoxicity in OBX rats has not been examined, to our knowledge. Furthermore, the possible mechanisms of suppressed NK cell function in OBX rats are not known.

Nueroendocrine responses in OBX rats

Monoamines. The olfactory bulbs have cell bodies and nerve terminals for monoamines, neuropeptides, and neuromodulators and extensive neural connections with various other brain regions including the limbic system (e.g., amygdala). Therefore, disruption of neural connections between the olfactory bulbs and other brain areas by removing the olfactory bulbs results in degeneration of neurofibers in the anterior hippocampus, amygdala, the bed nucleus of stria terminalis and the preoptic area of hypothalamus (Cairncross et al., 1979). Alterations in levels of neurochemicals such as decreased norepinephrine (NE) and NE turnover and increased $-adrenoreceptor density have been observed. Reduced brain levels of 5-hydroxytryptamine (5-HT) has been accompanied by increased 5-hydroxy tryptamine (5-HIAA) in brain regions, decreased platelet serotonin uptake, and altered serotonin receptors have also been shown in addition to changes in other substances such as glutamate, glycine, acetylcholine, and GABA (Kelly et al., 1997).

HPAC system. Hypercortisolism has been shown to be a common feature in depressed humans (Nemeroff, 1998). Elevated serum corticosterone level has been found in bulbectomized rats, suggesting chronic activation of the HPAC axis. Increased mean levels of corticosterone and significantly higher plasma corticosterone morning values were shown in male rats after olfactory bulbectomy (Marcilhac, Maurel, Anglade, Ixart, Mekaouche, Hery & Siaud, 1997). Hyperresponsiveness and dysregulation of the HPAC system has been shown to contribute to the etiology of suppressed natural immunity (Bernton et al., 1991).

Apoptosis. It could be hypothesized that an increased level of corticosterone resulting from an activation of the HPAC axis might modulate the activity of NK cells via several pathways. Elevated plasma glucocorticoid levels have been shown to cause apoptotic cell death of leukocytes including NK cells. Apoptosis is a morphological process of programmed cell death that involves chromatin condensation and margination, cell shrinkage, membrane blebbing and formation of nuclear fragments or apoptotic bodies. In addition, enzymatic

internucleosomal cleavage of DNA is now used by many investigators as a biochemical hallmark of apoptosis. An early indicator of apoptosis in mammalian cells is the loss of the phospholipid membrane asymmetry of the cell (Douglas, Tarshis, Pletcher, Nowell & Moore, 1995). This results in the exposure of phosphotidylserine on the outer surface of the plasma membrane. This change in membrane asymmetry can be analyzed using annexin V (O'Brien, Reutelingsperger, and Holdaway, 1997).

There is a large body of literature showing the effect of gluococorticoids on apoptosis of leukocytes. Weyts et al. (1998) showed the presence of a single class of cortisol-binding sites on peripheral blood leukocytes including NK cells (Weyts, Verburg-van Kemenade & Flik, 1998). It was also shown that elevated levels of plasma corticosterone induced apoptosis in murine bone marrow B-lineage lymphocytes (Garvy, Telford, King & Fraker, 1993) and thymocytes (Gruber, Sgonc, Hu, Beug & Wick, 1994; Telford, King & Fraker, 1991). Weyts et al. (1997) reported increases in plasma cortisol and cortisone level following handling stress and induction of apoptosis in activated PBL including NCCs (nonspecific cytotoxic cells) in fish (Weyts, Verburg-van Kemenade, Flik, Lambert & Wendelaar Bonga, 1997). In addition, apoptosis in mouse thymocytes were observed following acute treadmill exercise (forced running stressor) (Hoffman-Goetz, Zajchowski, S & Aldred, 1999) and restraint stress (Tarcic, Ovadia, Weiss & Weidenfeld, 1998), which was highly correlated with increased plasma corticosterone.

Antidepressant-like Effect of Exercise

It has been previously demonstrated that chronic wheel running has antidepressant-like effects, including increased norepinephrine levels and reduced $-adrenoceptor density in brain cortex, as well as enhanced copulatory performance, in male rats treated neonatally with clomipramine (Yoo, Tackett, Crabbe, Bunnell & Dishman, 2000). Chronic wheel running also blunts the suppression of NK cell activity that is induced by the uncontrollable electric foot shock model of depression (Dishman et al., 1995, 2000). However, it is not known whether

the antidepressant-like effects of wheel running extend to other rat models of endogenous depression, such as the olfactory bulbectomy (OBX) model (Kelly et al., 1997). Although olfactory bulbectomy leads to immunosuppressive effects of reduced numbers and proliferation of lymphocytes (Song & Leonard, 1994), it has not been established whether olfactory bulbectomy suppresses innate functional immunity such as NK cell cytolytic activity. Also, whether chronic wheel running might moderate brain monoaminergic, behavioral, and immunosuppressive features of the OBX model of depression has not been studied.

Exercise/Physical Activity and Immunity

There is a large body of literature describing the relationship between exercise and immune measures, especially NK cell number in circulation and NK functions measured *in vitro*. Both acute exercise and chronic physical training have been shown to affect the numbers and percentages of circulating NK cells as well as those of other leukocytes. However, the findings on the effect of acute exercise on NK cell activity is mixed, and the literature on basal NK cell activity after chronic exercise in humans is limited.

Acute Exercise and NK Cells

Changes in NK cell numbers and functions are typically observed after an acute bout of exercise. However, these responses depend upon the intensity and mode of exercise. In general, acute exercise increases the number of circulating lymphocytes, with a marked increase in NK cell numbers. Moderate-to-high intensity exercise (50 - 85% VO_{2max}) has increased the number of lymphocytes, including T_H, $T_{S/C}$, B, and NK cells, during and immediately after exercise (Fiatarone, Morley, Bloom, Benton, Makinodan & Solomon, 1988; Lewicki, Tchorzewski, Majewska, Nowak & Baj, 1988; Moyna, Acker, Weber, Fulton, Goss, Robertson & Rabin, 1996; Murray, Irwin, Rearden, Ziegler, Motulsky & Maisel, 1992; Rhind, Gannon, Suzui, Shephard & Shek, 1999). The numbers of lymphocytes typically return to the baseline level within 2 (Lewicki et al., 1988) to 24 hours after the exercise (Espersen, Elbaek, Schmidt-Olsen, Ejlersen, Varming & Grunnet, 1996). However, Espersen et al.

(1996) reported lowered numbers of lymphocytes, including NK cells, after 2 hours of exercise, which also returned to the baseline after 24 hours. Rhind et al (1999) showed elevated levels of lymphocyte numbers at 2 and 24 hours after exercise. In other studies, moderate-to-high intensity exercise increased the number of NK cells, but other lymphocytes were unchanged (Palmo Asp, Daugaard, Richter, Klokker & Pedersen, 1995) or decreased (Moyna et al., 1996).

Eccentric exercise using smaller muscle groups were shown to increase (Palmo et al., 1995) or decrease (Malm, Lenkei & Sjodin, 1999) NK cell number in the blood circulation during exercise. In addition, Strasner et al. (1997) showed an increased number of NK cells after a heavy exercise bout (80% VO_{2max}) but not light exercise (40% VO_{2max}).

Mechanisms of Alterations in NK Cell Number and Activity after Acute Exercise

Acute physical stress such as exercise, as well as mental stress, is known to induce leukocytosis. However, the mechanisms underlying lymphocytosis, and a marked increase in NK cell percentage, after exercise are not known. The role of the spleen in leukocyte redistribution during or after acute exercise was investigated in individuals who underwent splenectomy (Baum,Geitner & Liesen, 1996). Marked leukocytosis was shown after exhaustive cycling exercise but did not differ between splenectomized and control individuals. Markedly increased blood pressure and cardiac output during acute exercise might contribute to leukocytosis by propelling cells that are loosely attached to the vessel walls. It was hypothesized that acute exercise affected the adhesion molecules (i.e. shedding of adhesion molecules) on lymphocytes, resulting in a detachment of lymphocytes from the vascular endothelium and a surge into the circulation.

Increased levels of neutrophils and lymphocytes were observed after intense resistance exercise (6 sets of 10-repetition- maximum leg squats) in women, but the percentage of L-selectin expressing T, B, and NK cells in peripheral blood decreased. That finding suggested a shedding of L-selectin (Miles, Leach, Kraemer, Dohi, Bush, & Mastro, 1998). Adrenergic

mechanisms have been hypothesized to influence shedding of adhesion molecules on lymphocytes, including NK cells, which affects migration and homing of lymphocytes to circulation and lymphoid tissues (Benschop et al., 1996, 1997; Carlson, Fox & Abell, 1997). Treadmill running to exhaustion led to increased levels of circulating ICAM-1 and E-selectin which were mitigated after $-adrenergic antagonist treatment (Rehman, Mills, Carter, Chou, Thomas & Maisel, 1997).

Whether these results indicate an exercise-induced decrease in the capacity of NK cells to adhere to adjacent tissues and cells and altered NK cell cytotoxicity during or after acute exercise is not known. However, it is possible that the molecular signaling which results in shedding of the adhesion molecules during exercise might also affect adhesion of NK cells to target cells, which could influence NK cell cytotoxicity.

The effect of acute exercise on NK cell cytotoxic activity also has been investigated. Acute exercise has been shown to augment NK cell activity during or immediately after moderate- to-high intensity exercise, but to decrease NK cell cytotoxicity about 2 hours after exercise (Espersen et al., 1996; Nielsen, Secher, Kappel, & Pedersen, 1998; Rhind et al., 1999). In contrast, there was no change in NK cell activity when NK cell cytotoxicity was appropriately expressed per NK cell (Nieman, Henson, Sampson, Herring, Suttles, Conley, Stone, Butterworth & Davis, 1995; Palmo et al., 1995). Levels of prostaglandins (PGE_2), $-endorphin, and catecholamines have been investigated (Fiatarone et al.1988; Moyna et al., 1996; Murray et al., 1992; Rhind et al., 1999) as possible mechanisms or mediators of altered NK cell activity after acute exercise, but there are too few studies using uniform methods to draw conclusions and further investigation is needed.

Exercise Training and NK Cytotoxicity

There is a large body of literature on the relationship between acute exercise bout or exercise training and immunity. Both acute exercise and exercise training have been shown to affect NK cell cytotoxicity and to alter the number and percentage of circulating NK cells as

well as those of other leukocytes. The literature on the effect of exercise training on NK cell activity is limited and does not support the notion of an immunoenhancing effect of exercise training as NK cell activity was assessed at resting condition.

Baslund et al. (1993) have found that there was no change in NK cell activity as well as in blood mononuclear cell population and proliferative response in 18 rheumatoid arthritis patients after 8 weeks of progressive bicycle training. Also, 12 weeks of walking exercise program (45 min walking at 60-75% HR_{max} , 5d/week) (Nieman, Nehlsen-Cannarella, Henson, Koch, Butterworth, Fagoaga & Utter, 1998), and 8 weeks of a combination of aerobic and resistance training (Scanga, Verde, Paolone, Andersen & Wadden, 1998) did not influence NK cell activity or a circulating level. In addition, it was shown that 6 months of aerobic exercise in elderly participants did not significantly affect NK cell activity nor leukocyte counts (Woods, Ceddia, Wolters, Evans, Lu & McAuley, 1999). Rhind et al. (1994) reported that physically trained individuals (57 ml/kg/min VO_{2max}) showed higher levels of circulating total leukocytes, granulocytes, and NK cells compared to untrained control individuals (39 ml/kg/min VO_{2max}). The minimal effect of exercise training on resting NK cell activity observed in the literature raises questions regarding the nature of the exercise program (i.e. intensity, duration, etc.), possibility of different adaptations/alterations in NK cell activity of different tissues (i.e. peripheral blood, spleen, etc.), and possible mechanisms (i.e. sympathetic innervation of immune tissues and adaptation, activation of HPAC axis and adaptation, etc.).

The effect of exercise training on NK cell activity has been studied more in rats and mice compared to human studies. Ferrandez & De la Fuente (1996) showed that 90 min of moderate intensity of swimming for 20 days resulted in augmentation of NK cell cytotoxicity and of antibody-dependent cell-mediated cytotoxicity (ADCC). Reduced immune functions including NK cell activity in obese Zucker rats were restored following treadmill exercise (5d/wk for 40 wk) (Moriguchi, Kato, Sakai, Yamamoto & Shimizu, 1998). Hoffman-Goetz and colleagues conducted a series of studies on NK cell activity and tumor metastasis following

exercise training in mice. After 9 weeks of wheel running and treadmill training exercise, male C3H mice received an injection of tumor cells and were sacrificed after 3 weeks of tumor development without exercise. Trained mice demonstrated enhanced splenic NK cell activity and lower tumor cell retention (MacNeil & Hoffman-Goetz, 1993). Also, both 8 weeks of forced treadmill exercise and voluntary wheel running resulted in elevated *in vitro* splenic LAK activity in female BALB/c mice received an injection of MMT line 66 tumor cells. But, treadmill group showed a higher tumor multiplicity *in vivo* (Hoffman-Goetz, Arumugam & Sweeny, 1994). 10 weeks of treadmill training enhanced NK cell cytotoxicity in vitro (Simpson & Hoffman-Goetz, 1990) and 9 weeks of both voluntary wheel running and treadmill training increased both *in vivo* and *in vitro* cytotoxicity in mice (MacNeil & Hoffman-Goetz, 1993).

SNS Adaptation and NK Cell Activity after Exercise Training

Whether, or what aspects of, neuroendocrine responses or adaptations are responsible for the altered NK cell activity after acute exercise and chronic exercise training has not been shown. However, there is evidence that the brain noradrenergic and SNS systems in rats adapt to chronic exercise. Increased levels of NE (Dishman, Renner, Youngstedt, Reigle, Bunnell, Burke, Yoo, Mougey, & Meyerhoff, 1997; Dunn, Reigle, Youngstedt, Armstrong & Dishman, 1996), reduced $-adrenoreceptors (Yoo et al., 2000) in brain, and increased turnover of NE in the adrenal gland (Mazzeo, 1991) have been observed in rats that have been exercise trained.

There are studies implicating SNS adaptations in altered NK cell cytotoxicity after chronic physical activity in rats and mice. Jonsdottir and colleagues showed that 4-5 weeks of voluntary wheel running enhanced *in vivo* NK cell cytotoxicity measured as lung clearance of injected YAC-1 cells in spontaneously hypertensive rats. Augmentation of NK cell activity in exercised rats was abolished by the $-adrenergic receptor antagonist, timolol, implying that *in vivo* cytotoxicity of NK cells among exercise trained rats was mediated by the adrenergic

system (Jonsdottir et al., 1997). However, enhanced NK cell activity after chronic exercise was not affected by splenic denervation (Jonsdottir et al., 1996). NK activity in the lung might be enhanced by catecholamines secreted by other sympathetic nerves and the adrenal glands, leading to demarginalization of NK cells and enhanced cell activity despite splenic denervation. Also, the null effect of splenic denervation was limited to basal NK activity.

Modulation of splenic NK cell activity might differ when the SNS is activated by acute stress such as footshock. Dishman et al. (1995) found that 6-weeks of voluntary activity wheel-running among Fischer male rats had a protective effect against suppression of splenic NK cytotoxicity measured *in vitro* after electric footshock without affecting basal NK cell activity. In another study, the splenic NK cell cytolysis after footshock in sympathectomized rats was lower in sedentary rats compared to activity wheel runners and sedentary untrained rats (Dishman, Hong, Soares, Bunnell & Evans 2000). That finding implied that wheel running led to adaptations which offset an altered sympathetic nervous system (SNS) regulation of splenic NK cell activity after sympathectomy, which reduced splenic NE content by about 70%. Thus, chronic physical activity could affect noradrenergic adaptations that add to, or interact with, modulation of splenic NK cell activity by NE.

Cortisol/Corticosterone Response and NK Cell Activity after Exercise Training

The HPAC system is adaptable to chronic stressors, including exercise (Johnson, Kamilaris, Chrousos & Gold, 1992). Whether chronic exercise alters HPAC responses to other stressors, i.e., cross-stressor adaptations, is not established (Sothmann, Buckworth, Claytor, Cox, White-Welkley & Dishman, 1996). Hence, there is a need to better describe the conditions in which responses and adaptations of the HPAC system that occur with exercise. In humans, levels of ACTH, cortisol, and prolactin in circulating blood increase in an intensity- and duration-dependent manner during cycling and running. These responses during exercise appear to partially adapt after chronic exercise training (see Dishman, Bunnell, Youngstedt, Yoo, Mougey & Meyerhoff, 1998 for a review).

Cross-stressor adaptations of the HPAC system following chronic exercise have received very little study in humans or lower animals (Sothmann et al., 1996). Men differing in fitness levels had similar levels of cortisol or ACTH in plasma after the Stroop word-color conflict test under novel or familiar (Blaney, Sothmann, Raff, Hart & Horn, 1990) conditions.

Dishman et al. (1995, 1997) have reported no effects of chronic activity wheel running on plasma levels of ACTH, cortisol, and prolactin after repeated footshock in female Sprague-Dawley and male Fischer rats. Also, There was no moderating effect of voluntary wheel running on plasma corticosterone level following two sessions of footshock separated by 24 hours with cage-switch stress combined (Dishman et al., 1998). However, it can be hypothesized that there might be a cross-stressor adaptation in corticosterone response to a chronic stressor such as olfactory bulbectomy as a result of chronic voluntary wheel running.

Although cross-stressor adaptation at the HPAC axis after chronic exercise, has not been shown consistently in the studies of humans and rodents, there might be a cross-stressor adaptation in glucocorticoid responses among people who suffer chronic stress syndromes such as depression, which has been shown to be immunosuppressing. Dysregulation of HPAC axis is common among patients diagnosed with major depression (Nemeroff, 1998). Elevated basal levels of CRF in cerebrospinal fluid and cortisol in urine, blood, and cerebrospinal fluid, as well as enlargement of adrenal and pituitary glands in some depressed humans are indicative of chronic activation of the HPA axis. Possible cross-stressor adaptation at the HPAC axis after chronic exercise might blunt a suppression of NK cell activity in depressed patients. Manipulation of NK cell activity using animal models of depression (e.g., chronic stress, olfactory bulbectomy, etc.) offer an approach to study those ideas using exercise training.

Although exercise training has been found to augment NK cell activity in rodents, it has not been determined whether exercise training enhances in vitro splenic NK cell cytotoxicity in OBX rats. Also, it is not clear whether or not, or what aspects of neuroendocrine abnormality

of depression induced by OBX can be restored as a result of voluntary wheel running. Further investigations are required to provide better explanations for adaptations of the HPAC axis after chronic exercise as putative mechanisms for a protective effect of chronic physical activity against immunosuppression in depression or after stress.

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THE EFFECT OF EXERCISE ON NATURAL KILLER CELL ACTIVITY: A QUANTITATIVE SYNTHESIS[1]

[1]Hong, S. & Dishman, R. K. (2000). To be submitted to Brain, Behavior, and Immunity.

Abstract

Results from a quantitative synthesis of the literature indicated that there is a moderately large effect of acute exercise on NK cell cytotoxicity in humans. The mean value of effect size d (95% CI) was 0.43 (0.30 to 0.56), 0.57 (0.27 to 0.86), 1.02 (0.65 to 1.39) for acute exercise, exercise training, and cross-sectional studies, respectively, indicating that there were moderate effects of acute exercise and training and a large effect of fitness/training status on NK cell cytotoxicity. Moderator analyses demonstrated a distinct pattern of alteration in NK cell cytotoxicity affected by acute exercise such that NK cell cytotoxicity is markedly increased during and soon after exercise, decreased below the baseline 1-3 hours after termination of exercise, and returned to the baseline value within 24 hours. The effect of exercise was larger in studies that expressed NK cell cytotoxicity in lytic units (4.48 ± 7.73) rather than % specific lysis (1.23 ± 3.02) [$F(1, 71) = 6.562$, $p = 0.013$]. Among various subject characteristics, effects were larger for studies employing sedentary (5.18 ± 7.93) or untrained, but leisurely active (4.32 ± 5.13) participants [$F(4, 71) = 4.645$, $p = 0.002$]. Also, effect sizes obtained from studies that employed a mixed sample of males and females, or female participants only, were larger [$F(3, 71) = 3.96$, $p = 0.012$], compared to studies of male participants. However, effect sizes did not differ by age [$F(3, 71) = 1.42$, $p = 0.245$]. Larger effects were shown when the exercise session was 20 minutes to 2 hours in duration compared to exercise sessions lasting longer than 2 hours [$F(2, 71) = 3.463$, $p = 0.037$]. However, effect sizes did not differ by intensity [$F(3, 71) = 0.446$, $p = 0.721$] or mode of exercise [$F(1, 71) = 2.95$, $p = 0.090$]. Therefore, the present synthesis of the literature on revealed that NK cell activity is influenced by both acute exercise and exercise training, but this effect varied according to characteristics of the exercise and participants.

KEY WORDS: human, innate immunity, fitness, meta-analysis

A large body of literature has described the relationship between exercise and various components of immune system, especially natural killer (NK) cells. The natural killer (NK) cell is a critical component of innate immunity in host defense against viral infections and tumor. Both acute exercise and chronic exercise training have been shown to affect NK cell cytolytic functions measured *in vitro* as well as the circulating numbers and percentages of NK cells. However, the findings on the effect of acute exercise on NK cell activity are mixed, and the literature on basal NK cell activity after chronic exercise in humans is limited in its number (Pedersen & Hoffman-Goetz, 2000).

Acute exercise and NK Cell function

Changes in cytotoxic functions of NK cells are typically observed during and after an acute bout of exercise. In general, NK cell activity is enhanced during or immediately following exercise, but markedly decreased below the resting value during recovery. However, there has been no consensus in human studies. These responses appear to depend upon the characteristics (e.g., fitness level, training status, etc.) of the individuals tested and different exercise stimuli (e.g., mode, intensity, duration of exercise). Acute exercise has been shown to augment NK cell activity during or immediately after moderate- to-high intensity exercise, but to decrease NK cell cytotoxicity about two hours after exercise (Espersen et al., 1996; Nielsen et al., 1998; Rhind et al., 1999). It has generally been shown that NK cell activity was increased when measured during or immediately after exercise, and depended upon the intensity of exercise (Pedersen, Rohde, & Ostrowski, 1998).

Several investigators have speculated that alterations in numbers of circulating lymphocytes, especially a marked increase in NK cell numbers, reported during and immediately after acute exercise (Fiatarone et al., 1988; Lewicki et al., 1988; Moyna et al., 1996; Murray et al., 1992; Pedersen & Hoffman-Goetz, 2000; Rhind et al., 1999) might affect NK cell cytotoxicity measured after exercise. Different methods to express cytolytic function of NK cells have been used in the exercise literature investigating NK cell function including %

specific lysis at various effector to target (E/T) ratios (i.e., percentage of fixed number of target cells lysed by various numbers of NK cells), lytic units (i.e. number of NK cells needed to lyse certain number of target cells), and lysis per NK cell. Investigators have expressed NK cell cytotoxicity per cell by dividing % lysis by the number of effector cells in order to adjust for the difference in percentage of NK cell in isolated leukocytes from peripheral blood. There were no changes found in NK cell activity after acute exercise when cytotoxicity was expressed per NK cell (Nieman et al. 1995; Palmo et al., 1995). However, the validity of this method for expressing NK cell cytolytic function against target cells *in vitro* is not established, and whether different E/T ratios used in experiments affect conclusions about the effects of exercise on NK cell activity is unclear.

NK cells after Chronic Exercise

Compared to the limited number of studies existing in human, the effect of exercise training on NK cell cytotoxicity in rats and mice has received more study. Exercise training has been found to augment NK cell activity in rats and mice (Ferrandez & De la Fuente, 1996; Jonsdottir, Asea, Hoffman, Hellstrand, & Thoren, 1995; Jonsdottir, Johansson, Johannson, Hellstrand, Thoren, & Hoffman, 1997; MacNeil & Hoffman-Goetz, 1993; Moriguchi et al., 1998; Simpson & Hoffman-Goetz, 1990). However, basal NK cell activity did not change after 6 weeks of voluntary wheel running (Dishman, Warren, Youngstedt, Yoo, Bunnell, Mougeyn Meyerhoff, Jaso-Friedmann, & Evans, 1995), 6 weeks (Dishman, Warren, Hong, Bunnell, Mougey, Meyerhoff, Jaso-Friedmann, & Evans, 2000) or 15 weeks of treadmill running (Nasrullah & Mazzeo, 1992) in Fischer 344 rats though suppression of NK cell cytolytic function after footshock was blunted after exercise training (Dishman et al., 1995, 2000). In addition, chronic wheel running or treadmill running have shown to reduce NK cell activity in mice (Blank, Johansson, Pfister, Gallucci, Lee, & Meadows, 1994; Blank, Jones, Lee, Brahler, Gallucci, Fox, & Meadows, 1997).

Human studies examining the effect of exercise training on NK cell activity are limited , and the findings generally have been statistically insignificant. For example, 12 weeks of a walking exercise program (45-min walking at 60-75% HRmax, 5 d/ wk) (Neiman et al., 1998) or 8 weeks of combination of aerobic and resistance training (Scanga et al., 1998) did not significantly influence NK cell activity. Also, 6 months of aerobic exercise in elderly men and women did not significantly affect NK cell activity (Woods et al., 1999).

The insignificant effect of exercise training on resting NK cell activity observed in the human literature raises questions regarding the nature of the exercise program (i.e. intensity, duration, etc.) administered, as it might affect different adaptations in NK cell activity. A quantitative synthesis of literature in exercise training and NK cell activity might find a larger effect of training and influence of characteristics of exercise on changes in NK cell cytotoxicity by conducting moderator analyses.

Thus, we conducted a meta-analytic review to explain various results of the literature that might have resulted for low statistical power of single studies because of small sample size or features of studies that are more clearly described by quantitative analyses than by subjective evaluations alone. The purpose of the study was 1) to examine the changes in NK cell cytotoxicity after single bout of exercise and after exercise training and 2) to investigate possible moderator variables which might influence the magnitude of alterations in cytotoxic activity of NK cells to exercise.

Methods

Forty-three studies published from 1981 through December, 1999, were located by searches of literature published in the English language using *PubMed*, *Current Contents,* and *Biosis* computer searches with exercise, exercise training, physical activity, fitness, cytotoxicity and natural killer cell as key words. Searches were supplemented by bibliographies from the articles retrieved.

Criteria for including a study were: 1) the dependent variable was a measure of NK cell cytotoxicity in peripheral blood from healthy subjects with no immunopathological conditions (e.g., cancer, AIDS, etc.) measured *in vitro* by standard chromium release assay. 2) The independent variable was acute exercise, exercise training, and fitness or training status. 3) The dependent variable was quantified in a way permitting the calculation of effect sizes from percentages, graphs, and F-tests with a single df in studies which did not report means and standard deviations. Nine studies located were excluded from the analyses for the following reasons: Studies that failed to provide a measure of variance (e.g., standard deviation) to calculate effect size or baseline NK activity measure, were excluded (Berk, Neiman, Youngberg, Arabatzis, Simpson-Westerberg, Lee, Tan, & Eby, 1990; Crist, Mackinnon, Thomson, Atterbom, & Egan, 1989; Pedersen, Tvede, Klarlund, Christensen, Hansen, Galbo, Kharazmi, & Halkjaer-Kristensen, 1989; Shek, Sabiston, Buguet, & Radomski, 1995; Tvede, Kappel, Halkjaer-Kristensen, Galbo, & Pedersen, 1993). Cross-sectional studies (Kusaka, Kondou, & Morimoto, 1992; Moyna, Bodnar, Goldberg, Shurin, Robertson, & Rabin, 1999) that failed to use standard measure of fitness or report fitness levels were also excluded. Also, one study was excluded because of an unreliable NK cell cytotoxic assay (i.e. whole blood assay and minimal E/T ratios ranged 2:1 to 0.25:1) (Boas, Joswiak, Nixon, Kurland, O'Connor, Bufalino, Orenstein, & Whiteside, 1996).

A quantitative synthesis of the remaining 5 exercise training, 3 cross-sectional, and 27 studies employing acute exercise was conducted, assisted by Meta 5.3 (Schwarzer, 1991), DSTAT 1.10 (Johnson, 1989) and SPSS windows version 9.0 (SPSS INC., Chicago, IL) statistical software. Effect sizes were calculated by subtracting the mean change for a control group or condition from the mean change for an experimental group or condition and dividing this difference by the initial pooled standard deviation for the studies that employed a control group. Based on guidelines suggested by Cohen (1988), effect sizes of 0.2, 0.5, and 0.8 were considered to be small, moderate, and large effects, respectively. Composite effect sizes (d)

were obtained using the random effects model to aggregate effect sizes because effects were expected to be heterogenous (Hedges & Olkin, 1985).

Homogeneity of each mean effect was tested using a random effects model of variance (Hunter, Schmidt, & Jackson, 1982), and an effect was judged heterogeneous when sampling error was less than 75% of the observed variance. Factors that might moderate the effectiveness of acute exercise or exercise training on NK cell cytotoxicity were considered by comparing effects based on characteristics of participants (i.e., age, gender, fitness level), characteristics of an exercise bout or exercise training program (i.e., intensity, length, duration), and presentations of outcome measure (i.e., % specific lysis or lytic units) (see Table 1).

In order to constrain sampling error, levels of a moderator were collapsed or omitted when the number of effects within a given level was less than 5 (e.g., low intensity of exercise was omitted from moderator analyses). Also, moderator analyses were not performed for exercise training or cross-sectional studies because of the small total number of effects derived. A one-way analysis of variance (ANOVA), followed by Tukey # post hoc tests, was performed for each moderating variable to determine whether there were significant differences among levels of moderators that might account for variability in mean effect size. Moderators with more than two nonordinal levels were dichotomized based on the results of the post hoc contrasts. As a supplementary approach, a multiple linear regression analysis was conducted to determine the independent contributions of the significant moderators for predicting overall effect size.

Seventy-one effects were retrieved from 27 studies employing acute exercise with a total sample size approximating 390 people. Also, 7 and 4 effect sizes were retrieved from 5 exercise training and 4 cross-sectional studies with a total sample sizes of approximating 230 and 110 people, respectively. Analyses were done separately for acute exercise, exercise training and cross-sectional studies. Multiple effects were obtained from studies that reported separate results for gender, included more than one mode of acute exercise or exercise training,

or measured NK cell cytotoxicity at multiple time points after and/or during an acute exercise bout or a training program. Effect size was not correlated with the number of effects per study ($r = -0.18$, $p = 0.142$). When a study reported % lysis at multiple E/T ratios, % lysis at the highest E/T was selected after confirming there were no interactions reported between cytotoxicity and E/T ratios.

Results

A stem-and-leaf display of the 71 effects of acute exercise studies is presented in Figure 1. The distribution of effects was positively skewed ($g1 = 2.77$, $p < .0.001$) and leptokurtic ($g2 = 10.45$, $p < 0.001$). The mean (95% CI) value of d was 0.43 (0.30 to 0.56), 0.57 (0.27 to 0.86), 1.02 (0.65 to 1.39) for acute exercise, exercise training, and cross-sectional studies, respectively. Each moderator is described in Table 1, and effects for each moderator are presented in Table 2. Each study is described in Table 3.

Moderating Variables

Table 2 indicates that effects were larger in studies employing: blood collection for NK cell cytotoxicity measured during or immediately after exercise, data presentation using a conversion of % specific lysis to lytic units, sedentary, or female participants. Effects were smallest in studies that had employed a exercise duration longer than 2 hours and used high level endurance athletes as participants.

Measurement at different time points. Mean effect sizes obtained for different time points of blood drawing indicated that the effect of acute bout of exercise was significantly larger during (4.26 ± 4.55) and immediately after (4.21 ± 6.18) exercise compared to 3-hour (-0.56 ± 1.77) or 24-hour ($-.011 \pm 5.07$) recovery periods [$F(3, 71) = 5.983$, $p = 0.001$].

Expression of cytotoxicity. The effect of exercise was significantly larger when NK cell cytotoxicity was expressed in lytic units (4.48 ± 7.73) compared to % specific lysis (1.23 ± 3.02) [$F(1, 71) = 6.562$, $p = 0.013$].

Participant characteristics. Effects were larger for studies employing sedentary (5.18 ± 7.93) with active, but untrained (4.32 ± 5.13), participants [$F(4, 71) = 4.645$, $p = 0.002$]. Also, effect sizes obtained from studies that employed mixed gender of male and female, and female participants only, were larger [$F(3, 71) = 3.96$, $p = 0.012$] compared to studies with male participants. However, effect sizes did not differ by age [$F(3, 71) = 1.42$, $p = 0.245$].

Features of exercise. Larger effects were shown when exercise session was less than 2 hours compared to exercise sessions that were longer than 2 hours [$F(2, 71) = 3.463$, $p = 0.037$]. However, effect sizes did not differ by intensity [$F(3, 71) = 0.446$, $p = 0.721$] or mode of exercise [$F(1, 71) = 2.95$, $p = 0.090$].

Multiple linear regression analysis

Direct entry of the significant moderating variables into a multiple regression analysis indicated that 2 variables independently accounted for variation in d: fitness of participants (untrained vs. trained) ($= -0.56, t= -1.91, p= 0.06) and expression of NK cell cytotoxicity (% specific lysis vs. lytic units) ($= 0.36, t= 1.89, p= 0.01) at $p < 0.1$, among the moderating variables entered initially: gender (female vs. male) ($= -0.12, t= -0.86, p= 0.4), age (young vs. adult) ($= -0.16, t= -0.99, p= 0.33) of participants, intensity (mild vs. high) ($= 0.16, t= 0.99, p= 0.33), mode (aerobic vs. resistance) ($= -0.21, t= -1.58, p= 0.12), duration (less than 2 hours vs. longer than 2 hours) ($= -0.08, t= -0.2, p= 0.84) of acute exercise. Reentry of fitness of participants and expression of NK cell cytotoxicity into the regression model yielded a multiple R of 0.39, adjusted $R^2 = 0.13$, $F(2,70)= 6.26$, $p < 0.003$. Interactions between fitness level of the participants and duration of exercise did not explain variation in our estimate of effect size d.

Discussion

Results from our quantitative synthesis of the literature indicate that there is a moderate effect of acute exercise on NK cell cytotoxicity in humans. Also, moderator analyses for 'time point of measurement' showed that there is a distinct pattern of alterations in NK cell

cytotoxicity affected by acute exercise such that NK cell cytotoxicity markedly increased during and after exercise, as indicated by large effect sizes, decreased below the baseline 1-3 hours after termination of exercise as indicated by negative effect sizes. The reduction of NK cell cytotoxicity at 2-4 hour postexercise has been also reported in other reviews (Pedersen & Hoffman-Goetz, 2000; Pedersen, Rohde, & Ostrowski, 1998). NK cell activity returned to the baseline value within 24 hours of recovery. These findings are somewhat consistent with previous narrative reviews of the literature. Shephard and colleagues reported that there was an increase in NK cell cytotoxcity soon after moderate exercise and it remained elevated for from 1 hour to, sometimes, 24 hours after termination of exercise. They also reported reductions in NK cell activity found at 2-3 hours postexercise in some studies and remained low up to 7 days after termination of 90 to 120 minutes of moderate exercise (Shephard, , Rhind, & Shek, 1994). This present quantitative synthesis of literature not only confirmed that there is an alterations in NK cell activity during and after acute exercise but also revealed that there is a general pattern of NK cell function changes after exercise across studies.

As shown in figure 1, the effects derived by this analysis were positively skewed to a great extent, and there were six outliers with significantly large effect sizes. Six effects derived from five studies (Brenner, Castellani, Gabaree, Young, Zamecnik, Shephard, & Shek, 1999; Fiatarone, Morley, Bloom, Benton, Solomon, & Makinodan, 1989; Field, Gougeon, & Marliss, 1991; Moyna, Acker, Weber, Fulton, Robertson, Goss, & Rabin, 1996; Strasner, Davis, Kohut, Pate, Ghaffar, & mayer, 1997) were found to be outliers with effect sizes ranged from 11.6 to 28.5. Four of those six effects yielded from three studies (brenner et al., 1999; fiatarone et al., 1989; Moyna et al., 1996) have reported NK cytotoxicity in lytic units indicating that conversion of NK cytotoxic data from % lysis to lytic units might have overestimated the effect of exercise on NK cell activity. Several investigators (e.g., Fiatarone et al., 1989; Shephard & Shek, 1999) have argued that reporting NK cell cytotoxicity in lytic units compared to % specific lysis, is more accurate and has an advantage when different

studies are compared by standardizing the results by estimating number of effector cells to lyse certain number of target cells (e.g., to lyse 20% of 10,000 target cells). However, studies had used inconsistent methods to derive and report lytic units regarding numbers of effector cells or target cells that raises questions of reliability of using lytic units to compare results from different studies. Although, % specific lysis is reported at various E/T ratios across studies, it has an advantage in that measuring and reporting NK cytotoxicity at multiple E/T ratios help readers to evaluate the reliability of the assay. Therefore, integration of studies using different methods of reporting NK cell cytotoxicity need to be further examined.

We have conducted separate analyses for the studies (50 effects) that reported NK cell cytotoxicity in % specific lysis by excluding effects (21 effects) derived from the studies that used lytic units for NK cell function report in order to confirm that expression of NK cell cytotoxicity in lytic units did not affect the effect of exercise on NK cell activity and moderators. Effects were still positively skewed ($g1 = 1.84$, $p < .0.001$) and leptokurtic ($g2 = 5.16$, $p < 0.001$) with studies that used lytic units excluded, but values for skewness and kurtosis were smaller than those when studies with lytic units were included. Mean effect size d was 0.4 (0.24 to 0.55), which was slightly lower than effect (d= 0.43, 0.30-0.56) yielded from studies combined. Additional moderator analysis showed that significant moderators found in original analysis did not differ from the result after exclusion of studies using lytic units though effects were smaller for each level of moderators. Therefore, expression of NK cell cytotoxicity in lytic units did not influence the mean effect or moderating variables significantly though it appeared to yield larger effects. Because number of studies that reported NK cell activity in lytic units was relatively small, it is hard to evaluate the impact of those studies on our quantitative synthesis of the literature. However, the validity of expressing results in lytic units without reporting % specific lysis and combining such results from two different methods of report need to be further addressed.

A recent meta-analysis of the literature conducted by Shephard and Shek (1999) pointed out that the NK cell cytotoxicity results are more accurate when it is expressed in lytic units than it's observed at single E/T ratio. Therefore, they used the lytic unit data when both were reported in some studies. We agree that the reliability of the assay with % specific lysis observed at single or significantly low E/T ratio is questionable. However, when there are multiple E/T ratios that lytic unit is derived from, it is controversial that the conversion to lytic units is a more valid method of reporting NK cell cytotoxicity data. Also, for a quantitative analysis of the literature, it would be more recommended to select prevalent method when it is available. Therefore, we selected % specific lysis rather than lytic units when both were reported for the present analysis. As mentioned above, the number of studies that expressed their results in lytic units is not large enough to affect the effects of exercise on NK cell activity in the literature, but the issue of possible inflation of NK cell cytotoxicity expressed in lytic units needs to be further addressed.

Other reviews reported an augmentation of NK cell activity during and immediately after moderate and intense exercise, but reduction in NK cell activity after intense exercise of long duration (Pedersen et al., 1997). Our meta-analytical review extended their report by investigating the influence of exercise intensity and duration on NK cell activity.

This investigation also found a moderate effect of exercise training on NK cell cytotoxicity, though only 7 effects were derived from 5 studies. Analyses of cross-sectional studies revealed that there is a large effect of fitness level on NK cell activity though comparison of single time-point measure of NK cell cytotoxicity between athletes or trained individuals and age-matched control might be confounded by other factors. Therefore, we grossly concluded from this finding that fitness or training status achieved as a result of chronic physical training might augment NK cell activity in humans.

We found that the duration of acute exercise affected NK cell activity. Exercise persisting longer than 2 hours negatively affected NK cell cytotoxicity. This is in agreement with

previous discussions on immunosuppressive effect of prolonged heavy exertion (Nieman, 1997; Nieman & Pedersen, 1999). Nieman (1994) has argued that there is a relationship between upper respiratory tract infections (URTI) and exercise in a form of "J curve", indicating there is a window of opportunity of infection associated with very mild or intense exercise.

Suppression of NK cell function after prolonged exercise raises questions regarding possible immunosuppressing effect of exercise. Immunomodulation seen after prolonged exercise such as a marathon run included lymphocytopenia, reduced function of lymphocytes, and increased risk for infection. Recent studies also showed that exercise caused DNA damage in leukocytes. Following exhaustive physical exercise, severe mutations at the chromosome level have been shown 24 - 48 hour after exercise (Schiffl, Zieres, & Zankl, 1997). Also, Mars and colleagues showed DNA damage and apoptosis in lymphocytes after ramped treadmill test to exhaustion. (Mars, Govender, Weston, Naicker, & Chuturgoon, 1998). It was argued that apoptosis might contribute to lymphocytopenia and functional modulation of lymphocytes after severe exercise.

Increased levels of glucocorticoids might be the cause of apoptosis of leukocytes after prolonged exercise. Twenty-four hour incubation of thymocytes and splenocytes with concentrations of corticosterone observed during maximal exercise resulted in apoptosis (Hoffman-Goetz et al., 1997). Also, incubation with high dose of corticosteroids reduced the function of NK cells (Pedersen et al., 1994). Therefore, it is speculated that stressful stimuli such as prolonged intense exercise might cause decreased function of NK cells via apoptosis induced by increased glucocorticoid levels. Further investigation is needed examining apoptosis induced during or after prolonged exercise in relation to increased glucocorticoid levels.

Moderator analyses also revealed that the effect of exercise on NK cell activity is influenced by some of the characteristics of individuals. Female participants showed greater augmentation in NK cell cytotoxicity after exercise compared to males. It is not consistent with

what has been reported in the recent review by Pedersen and Hoffman-Goetz (2000) where no effects of fitness level or gender on NK cell activity after exercise was reported. There is no clear explanation for gender-specific responses of NK cells to an acute bout of exercise. It can be speculated that greater augmentation in NK cell function in women might be due to relatively higher intensity of exercise for female compared to male participants in a study, but that is questionable because most of studies relativised exercise intensity to peak oxygen consumption. We did not find different effects according to age of participants, which is in agreement with previous reviews. NK cell function appeared to remain unchanged, though aging has been associated with decreased immune function (Shephard & Shek, 1995). However, only two effects were derived from two studies employing elderly populations for this meta-analysis, and further investigation is needed examining effect of exercise on NK cell cytotoxicity in older people.

It has been argued that alterations in NK cell cytotoxicity during or immediately after exercise is mainly because of changes in numbers of NK cells in peripheral blood during and after exercise (Pedersen & Hoffman-Goetz, 2000). Changes in NK cell numbers and functions are typically observed after an acute bout of exercise though these responses depend upon the intensity and mode of exercise. In general, acute exercise increases the number of circulating lymphocytes, with a marked increase in NK cell numbers in general. Moderate-to-high intensity exercise (50 - 85% VO_{2max}) has increased the number of lymphocytes, including T_H, $T_{S/C}$, B, and NK cells, during and immediately after exercise (Fiatarone et al., 1988; Lewicki et al., 1988; Moyna et al., 1996; Murray et al., 1992; Rhind et al., 1999). The numbers of lymphocytes typically return to the basal level within 2 hours (Lewicki et al., 1988) to 24 hours after the exercise (Espersen et al., 1996). However, Espersen et al. (1996) reported lowered numbers of lymphocytes, including NK cells, after 2 hours of exercise, which also returned to the baseline after 24 hours. Rhind et al (1999) showed elevated levels of lymphocyte numbers at 2 and 24 hours after exercise. In other studies, moderate-to-high intensity exercise

increased the number of NK cells, but other lymphocytes were unchanged (Palmo et al., 1995) or decreased (Moyna et al., 1996). Those reports appear to be a possible explanation of an increase in NK cell activity during or immediately after exercise and decrease in NK cell function 1-3 hours after termination of exercise that returned to a basal value within 24 hours as reported in this review.

In order to adjust for the alterations in the number of NK cells in peripheral blood during or after exercise number of studies calculated cytotoxicity per cell by dividing % specific lysis by number of NK cells. Five studies that were included in this analysis also expressed cytotoxicity per cell basis, and four of those studies showed no alterations in NK cell cytotoxicity during or after exercise when it was adjusted for the number of cells. It indicates that alterations in NK cell number might be the primary explanation of changes in NK cell function. However, the study by Targan et al. (1981) where NK single cell assay was done, showed there was an enhancement in the ability of NK cell to recycle and lyse target cells. It indicates that the estimation of lytic function of single NK cell by a mathematical calculation is different from actual single cell assay, and in order to evaluate true recycle capacity of a NK cell for lysing target cells, single cell assay is more biologically plausible and valid. However, validity of the mathematical calculation of per cell based NK cell cytotoxicity is hard to be judged compared to single cell assay because evidence is limited. Also, a study by Moyna et al. (1996) showed alterations in NK cell number were not accompanied by changes of NK cell cytotoxicity. Therefore, there is evidence that alterations in number of NK cells during and after exercise might not be the sole explanation for the NK cell function changes.

The mechanisms underlying lymphocytosis, and a marked increase in NK cell percentage, after exercise are not clear. The role of the spleen in leukocyte redistribution during or after acute exercise was investigated in individuals who underwent splenectomy (Baum et al., 1996). Marked leukocytosis was shown after exhaustive cycling exercise but did not differ between splenectomized and control individuals. Markedly increased blood pressure

and cardiac output during acute exercise might contribute to leukocytosis by propelling cells that are loosely attached to the vessel walls. It was hypothesized that acute exercise affected the adhesion molecules (i.e. shedding of adhesion molecules) on lymphocytes, resulting in a detachment of lymphocytes from the vascular endothelium and a surge into the circulation.

Increased levels of neutrophils and lymphocytes were observed after intense resistance exercise (6 sets of 10-repetition- maximum leg squats) in women, but the percentage of L-selectin expressing T, B, and NK cells in peripheral blood decreased. That finding suggested a shedding of L-selectin (Miles et al., 1998). Adrenergic mechanisms have been hypothesized to influence shedding of adhesion molecules on lymphocytes, including NK cells, which affects migration and homing of lymphocytes to circulation and lymphoid tissues (Benschop et al., 1996, 1997; Carlson et al., 1997). Treadmill running to exhaustion led to increased levels of circulating ICAM-1 and E-selectin which were mitigated after $-adrenergic antagonist treatment (Rehman et al., 1997).

Whether these results indicate an exercise-induced decrease in the capacity of NK cells to adhere to adjacent tissues and cells and altered NK cell cytotoxicity during or after acute exercise is not known. However, it is possible that the molecular signaling which results in shedding of the adhesion molecules during exercise might also affect adhesion of NK cells to target cells, which could influence NK cell cytotoxicity.

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* denotes included studies

** denotes excluded studies

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Figure 1. Stem-and-Leaf-Display for 71 Effect Sizes (d) from NK cell cytotoxicity during and after acute exercise. The effect sizes d range from -3.10 to 5.5.

Table 1. Moderating variables examined as potential influences on effects (d) of acute exercise.

	TIME-POINT OF MEASUREMENT	EXPRESSION OF CYTOTOXICITY	FITNESS/ TRAINING STATUS	AGE	GENDER
LEVEL	**During:** Blood was drawn during exercise for NK cell cytotoxicity measure Post exercise: Blood was drawn during the last minute of exercise or immediately after exercise **3-hour recovery:** blood was drawn one to three hour after exercise was over. **24-hour recovery:** Blood was drawn 24 hour or longer after exercise was over.	**% Specific Lysis:** Cytotoxicity data was presented in % specific lysis at a single or multiple E/T ratio(s). **Lytic Units:** Cytotoxicity data was presented in lytic units per cells	**No report:** Fitness or training status of subjects was not reported. **Sedentary:** Subjects were not physically active. **Active:** Subjects were leisurely active, but not physically trained. **Trained:** Subjects were physically active and fit, and engaged in physical competition regularly. **Elite Athletes:** Subjects were highly trained athletes.	**No report:** Age of subjects was not reported. **Young:** Subjects were young adults aged up to 25 years. **Adult:** Subjects were adults aged 26-50. **Elderly:** Subjects were aged 65-75.	**Females**: Only females were studied or a separate evaluation of the intervention was made for females. **Males:** Only males were studied or a separate evaluation of the intervention was made for males. **Females & Males:** Evaluation of the intervention was not separated according to sex.

Table 1 continued

	EXERCISE MODE	EXERCISE INTENSITY	EXERCISE LENGTH
LEVEL	**Aerobic:** Subjects were engaged in an aerobic exercise (e.g., running, cycling) **Strength:** Subjects were engaged in resistance exercise	**Low:** Exercise intensity was mild (e.g., lower than 50 % $\dot{V}O_{2max}$). **Moderate:** Exercise intensity was moderate (e.g., 55-65 % $\dot{V}O_{2max}$) **High:** Exercise intensity was high (e.g., 75-85 % $\dot{V}O_{2max}$). **Very high:** Exercise intensity was very high (e.g., triathalon, marathon)	**Less than 0.5 hour:** Length of exercise was shorter than 30 minutes. **Between 0.5 to 2 hours:** Length of exercise was between 30 minutes and 2 hours. **Longer than 2 hours:** Length of exercise was longer than 2 hours

Table 2. Moderators of NK cell cytotoxicity during and after acute exercise effects (d). The number of effects for each level is indicated by N.

	N	d	95% CI
Time Point of Measurement			
During Exercise	7	4.26	5.57,8.46
Post Exercise	33	4.22	2.02,6.41
0.5-3h Recovery	22	- 0.56	-1.35,0.22
24h Recovery	9	- 0.11	-0.75,0.54
Expression of Cytotoxicity			
% Specific Lysis	50	1.23	0.37,2.09
Lytic Units	21	4.48	0.96,8.00
Fitness/Training Status of Subjects			
No Report	12	0.91	-0.32,2.14
Sedentary	13	5.18	0.39,9.97
Active, but Untrained	20	4.32	1.92,6.72
Trained	16	- 0.44	-1.67,0.79
Elite Athletes	10	- 0.21	-1.48,1.05
Age of Subjects			
Young	33	3.38	1.16,5.60
Adult	32	0.92	-0.42,2.26
Gender of Subjects			
Female	12	4.58	1.65,7.51
Male	46	0.80	-0.21,1.81
Female/Male	11	5.40	-0.51,11.31
Mode of Exercise			
Aerobic	64	2.53	1.23,3.82
Strength	7	-0.89	-2.93,1.15
Intensity of Exercise			
Moderate	19	2.04	0.28,3.80
High	46	2.40	0.68,4.13

Table 3. experimental studies examining the relationship between acute exercise or exercise training and NK cell cytotoxicity.

STUDY	PARTICIPANT	RESEARCH DESIGN	EXERCISE	TIME-POINTS OF MEASUREMENT	EXPRESSION OF CYTOTOXICITY
1] Acute Exercise					
Brahmi et al., 1985	male athletes (n=5), untrained males (n=5), and untrained females (n=5) aged 21-37 years	Non-randomized design without control group and repeated measures of NKCA pre- to post-graded exercise test on a cycle ergometer	Graded exercise test on a cycle ergometer with an incremental work load of 15W/min for males and 10W/min for females	Baseline, post-exercise, 2h, and 20h recovery	% specific lysis at 20:1 E/T ratio
Brenner et al., 1999	Healthy males (n=7) aged 20-34 years	Non-randomized design without control group and repeated measures of NKCA pre- to post-exercise across 4 conditions	1-hour cycle ergometer exercise in thermo-neutral water at 55% peak oxygen consumption	Baseline and post-exercise	Lytic units/10^6 cells
Brenner et al., 1996	Healthy and moderately fit males (n=11) aged 23-32 years	Non-randomized design without control group and repeated measures of NKCA pre- to post-exercise across 4 conditions	30-minute cycle ergometer exercise at 50% peak oxygen consumption	Baseline, during, and post-exercise	Lytic units/10^6 cells
Braun et al., 1999	Male distance runner (n=10)	Non-randomized design without control group and repeated measures of NKCA pre- to post-exercise	1-hour treadmill running at 85% peak oxygen consumption	Baseline, post-exercise, and 1.5h recovery	% specific lysis collaped across 2.5, 10, and 20:1 E/T ratios
	Healthy volunteers (n=5)		5-minute running up and down stairs	Baseline and post-exercise	% specific lysis at 10, 20, 40, and 80:1 E/T ratios
Edwards et al., 1984	Helathy females (n=8) aged 24-39 years	Non-randomized design without control group and repeated measures of NKCA pre- to post-exercise	Graded exercise test on a cycle ergometer	Baseline and post-exercise	Lytic units/10^6 cells
Fiatarone et al., 1988		Non-randomized design without control group and repeated measures of NKCA pre- to post-exercise			

Fiatarone et al., 1989	Healthy young (n=8) and old (n=9) females aged 24-35 and 65-79, respectively	Non-randomized design without control group and within subject design with repeated measure	Maximal bicycle ergometer exercise	Baseline and post-exercise	Lytic units/10^6 cells
Field et al., 1991	Healthy males (n=5) with mean age of 26 years	Non-randomized design without control group and within subject design with repeated measure	Maximal bicycle ergometer exercise at 80% of maximal load	Baseline, post-exercise, and 1h recovery	% specific lysis at 20:1 E/T ratio
Gannon et al., 1998	Healthy males (n=10) with mean age of 26 years	Non-randomized design without control group and within subject design with repeated measure	2-hour bicycle ergometer exercise at 65% peak oxygen consumption	Basline, during, 4h, and 24h recovery	% specific lysis at 50:1 E/T ratio
Henson et al., 1999	Male (n=8) and female (n=8) triathletes aged 25-50 years	Non-randomized design without control group and within subject design with repeated measure	2.5-hour treadmill running and cycling exercise at about 75% peak oxygen consumption	Baseline, post-exercise, and 3h recovery	Lytic units/10^7 cells
Kappel et al., 1991	Healthy males (n=8) aged 20-29 years	Non-randomized design without control group and within subject design with repeated measure	1-hour bicycle ergometer exercise at about 75% peak oxygen consumption	Baseline, post-exercicse, and 2h recovery	% specific lysis at 50:1 E/T ratio
Klokker et al., 1995	Healthy males (n=7) aged 20-32 years	Non-randomized design without control group and within subject design with repeated measure	20-minute bicycle ergometer exercise in a recumbent position at about 50% peak oxygen consumption	Baseline and post-exercise	100:1 E/T ratio
Moyna et al., 1996	Healthy males (n=32) and females (n=32)	Randomized design with control group with repeated measure within subject	18-minute incremental bicycle ergometer exercise with 3 stages of 55%, 70%, 85% peak oxygen consumption	Baseline, during, post-exercise, and 2h recovery	Lytic units/10^9 cells
Murray et al., 1992	Healthy males (n=18) and females (n=2) aged 24-43 years	Non-randomized design without control group and within subject design with repeated measure	1-hour treadmill running to exhaustion according to Bruce protocol	Baseline and post-exercise	10, 20, 40, and 80:1 E/T ratios

Nieman et al., 1995	Male marathon runners (n=22) and sedentary males (n=10)	Non-randomized design without control group and within subject design with repeated measure	2.5-hour treadmill running at about 75% peak oxygen consumption	Baseline, post-exercise, 1.5h, and 3h recovery	% specific lysis at 5, 10, 20, and 40:1 E/T ratios
Nieman et al., 1997a	Male (n=9) and female (n=3) marathon runners with mean age of 41 years	Random assignment to vitamin C and placebo groups and blood drawn at multiple time points before and after exercise	2.5-hour treadmill running at 75-80% peak oxygen consumption	Baseline, post-exercise, 1.5h, 3h, and 6h recovery	% specific lysis at 40:1 E/T reported among 40, 20, 10, 5:1 E/T ratios
Nieman et al., 1997b	Male (n=24) and female (n=6) marathon runners aged 25-49 years	random, but uneven assignment to carbohydrate supplement group (n=17) and placebo group (n=13) and blood drawn at multiple time points before and after exercise	2.5-hour treadmill running at about 75% peak oxygen consumption	Baseline, poet-exercise, 1.5h, 3h, and 6h recovery	% specific lysis at 40, 20, 10, and 5:1 E/T ratios
Nieman et al., 1993	Male runners (n=10) aged 17-31 years	One of each pair assigned to one of two intensities (high vs. moderate) and the other assigned to another intensity. Assignment for the pair was reversed at the next visit. blood drawn at multiple time points before and after exercise	45-minute treadmill running and 45-minute treadmill walking at 80 (high intensity) and 50% (moderate intensity) peak oxygen consumption	Baseline, post-exercise, 1h and 2h recovery	Lytic units/10^5 cells
Nieman et al., 1995	Experienced male weight lifters (n=10) with mean age of 25 years	Non-randomized. subjects engaged in two exercise sessions separated by 2 hours blood drawn before and after exercise	sets of 10 repetitions of the parallel leg squat, with a cadence of one repetition every 6 seconds and 3 minutes rest between sets to muscular failure (i.e., inability to continue)	Baseline, post-exercise and 2h recovery	Lytic units/10^4 cells
Pedersen et al., 1988	Healthy untrained male (n=6) aged 23-28 years	Non-randomized assignment to both of aerobic and resistance exercise sessions separated by at least one week and blood drawn at multiple	1-hour bicycle ergometer exercise at 80% peak oxygen consumption and resistance exercise consisting of body lifting in a prone position, leg lifting and pulling back exercises	Baseline, post-exercise, 2h, and 24h recovery	% specific lysis at 100:1 E/T ratio

Rhind et al., 1999	Untrained, but active males (n=10) with mean age of 26 years	Each subject assigned to resting and all of three exercise trials in a randomized and double-blinded manner	Three trials of 2-hour bicycle ergometer exercise at 65% peak oxygen consumption after placebo, indomethacin, or naltrexone administration	Baseline, during exercise, 2h and 24h recovery	% specific lysis at 50:1 E/T ratio
Rohde et al., 1996	Male elite triathletes (n=8) aged 20-45 years	Each subject engaged in an intense endurance exercise session	2500m swimming, 81km bicycling, and 19km running	Baseline, post-each exercise, 2h, 48h, and 72h recovery	% specific lysis at 50:1 E/T ratio and per-cell lysis
Rohde et al., 1998	Healthy males (n=8) with mean age of 27 years	Each subject engaged in 3 exercise sessions with different intensities separated by 2 hours for 2 conditions: glutamine supplementation and placebo separated by 30 days.	1-hour, 45-minute, and 30-minute bicycle exercise separated by 2 hours in between	Baseline, post-exercise sessions, 2h, 24h recovery	% specific lysis at 50:1 E/T ratio
Strasner et al., 1997	Healthy females (n=8) aged 21-33 years	Subjects engaged in three conditions with resting, moderate and high exercise	25-minute bicycle ergometer at 80 % (high-intensity) and 40% (moderate-intensity) separated by at least 5 days	Baseline, post-exercise, 1.5h, and 3h recovery	% specific lysis at 80:1 E/T ratio
Targan et al., 1981	Healthy volunteers (n=5)	Subjects engaged in a moderate exercise session	5-minute bicycle ergometer exercise at 25mph (heart rate exceeding 130 bpm	Baseline and post-exercise	% specific lysis at 20, 10, and 5:1 E/T ratios
Woods et al., 1998	Healthy young males (n=6) and females (n=8) aged 18-27 years, and elderly males (n=14) and females (n=19) aged 58-77 years	Subjects engaged in a maximal exercise graded treadmill test	Maximal exercise graded treadmill test	Baseline and post-exercise	% specific lysis at 50:1 E/T ratio reported among 12.5, 25, and 50:1 E/T ratios

2] Exercise Training

Nieman et al., 1993	Healthy sedentary elderly females (n=32) aged 67-85 years (2 subjects dropped out), conditioned elderly females (n=12) aged 65-84, and young females (n=13) with mean age of 22 years	Subjects randomly assigned to 12-week of aerobic training (n=14) or calisthenic control (n=16) group. Corss-sectional comparison on NKCA was done at baseline between 32 sedentary and 12 conditioned subjects	Five 30-40 minutes of brisk walking sessions per week for 12 weeks	Baseline, after 5 weeks of training, and post-training	Lytic Units/10^7 cells
Nieman et al., 1998	Obese (BMI of 33) females (n=91) aged 25-70 years	Subjects randomly assigned to control, exercise only, exercise and diet,or diet only group	45-minute walking sessions at 60-80% maximum heart rate, five times per week for 12 weeks	Baseline and post-training	Lytic units/10^7 cells
Nieman et al., 1990	Mildly obese (10-40% overweight) females (n=36, 14 dropped out) aged 25-45 years	Subjects randomly assigned to control or exercise training group	45-minute brisk walking session at 60% heart rate reserve, five times per week for 15 weeks	Baseline, after 6 weeks, and post-training	% specific lysis at 50:1 E/T ratio
Watson et al., 1986	Young healthy, but inactive males (n=46 completed out of 52) aged 17-34 years	Subjects randomly assigned to placebo, propranolol, or atenolol administration condition and engaged in exercise training	45-60 minutes of walking sessions at 70-85% peak oxygen consumption, 5 days per week for 15 weeks.	Baseline and post-training	% specific lysis at 25:1 E/T ratio
Woods et al., 1999	Healthy, but sedentary elderly volunteers (n=29 completed out of 33) with mean age of 65	Subjects randomly assigned to exercise training (n=14) or flexibility/toning control (n=15) group	40-minute walking sessions starting at intensity of 50% to 60-65% peak oxygen consumption by the midpoint of the program , 3 days per week for 6 months	Baseline and post-training	% specific lysis at 12.5, 25, and 50:1 E/T ratios

3] Cross-Sectional

Nieman et al., 1994	Male Athletes (n=18) and non-athlete controls (n=11)	NKCA compared at resting between athletes and non-athletes	Baseline	% specific lysis at 5, 10, 20, and 40:1 E/T ratios, Lytic units/10^7, and per NK cell lysis
Nieman et al., 1995	Male marathon runners (n=22) and sedentary controls (n=18) aged 22-57 years	NKCA compared at resting between marathon runners and sedentary control subjects	Baseline	Lytic units/10^7 cells
Pedersen et al., 1989	Male racing cyclists (n=27) aged 19-28 years and untrained controls (n=15) aged 20-29 years	NKCA compared at resting between cyclists and untrained control subjects	Baseline	% specific lysis at 50:1 E/T ratio

THE EFFECT OF VOLUNTARY WHEEL RUNNING ON NATURAL KILLER CELL CYTOTOXICITY AFTER OLFACTORY BULBECTOMY [2]

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[2]Hong, S., Evans, D. L., Holmes, P. V., & Dishman, R. K. To be submitted to Brain, Behavior, and Immunity

Abstract

The effects of olfactory bulbectomy (OBX) on splenic natural killer (NK) cell cytotoxicity *in vitro*, plasma corticosterone level and NK cell apoptosis were examined. Whether voluntary activity wheel running and imipramine would moderate those effects also was examined. A 2 Condition (OBX vs. sham) x 2 Group (activity wheel vs. sedentary) x 2 Treatment (imipramine vs. saline) factorial design was used. Male Long-Evans rats were randomly assigned to groups 24 hours after OBX or sham surgery. After 3 weeks, animals were tested for open-field behavior. Blood and spleens were collected 24-72 hours later for NK cell cytotoxicity using a standard chromium release assay and for plasma corticosterone using radioimmunoassay. NK cell apoptosis was assessed by flow cytometry. Incubation with dexamethasone (100 nM) resulted in apoptosis of NK cells as expected. NK cell activity was not affected by OBX or 3 weeks of voluntary wheel running or imipramine. Also, there was no difference in the percentage of apoptotic cells between OBX (4.58 ± 3.69 %) and sham (4.93 ± 5.09 %) animals. In contrast to expected results, plasma corticosterone level was lower in OBX rats (6.52 ± 6.0 µg/dL) compared to sham surgery animals (10.30 ± 8.70 µg/dL), $p = 0.03$. Though olfactory bulbectomy resulted in hyperactive behavior, it did not result in immunosuppression of splenic NK cell activity through the hypothesized mechanism of glucocorticoid-induced cell apoptosis.

Key words: Animal model of depression, Apoptosis, Corticosterone, Dexamethasone, Exercise, Open field behavior

Depression is associated with immunosuppression. People suffering from major depression have exhibited increased susceptibility to viral infections and higher risk for prolonged chronic illnesses such as cancer (Ballin et al., 1997; LaPerriere, Antoni, Schneiderman, Ironson, Klimas, Caralis & Fletcher, 1990). Clinical depression has been associated with altered numbers or percentages of circulating lymphocytes (e.g., higher T_H :$T_{S/C}$ ratio, leukocytosis, monocytosis, neutrophilia), lower lymphocyte proliferation, lower neutrophil phagocytosis, lower antibody production, and impaired natural killer (NK) cell cytolytic function against tumor cells measured *in vitro* compared to normal individuals (Herbert & Cohen, 1993; Weisse, 1992).

Natural killer (NK) cell activity is a critical component of innate immunity in host defense against viral infections and tumors, and is one of the major functional immune variables studied in the area of psychoneuroimmunology. NK cell cytotoxic function against tumor cells *in vitro* is lower than normal in individuals experiencing psychological stress (Ader & Cohen, 1993; Black, 1995; Kiecolt-Glaser & Glaser, 1987) and in patients diagnosed as having major depression (Bauer, Wadee, Kuschke & O'Neill-Kerr, 1995; Cover & Irwin, 1994). Decreased NK cell activity has been found in clinical populations suffering from chronic illnesses such as cancer (Levy, Herberman, Whiteside, sanzo, Lee, & Kirkwood, 1987; Ballin et al., 1997; Spiegel, 1996) and acquired immunodeficiency syndrome (AIDS) (LaPerriere et al., 1990), who also commonly experience high levels of emotional distress and depressive symptoms. NK cell activity also is significantly lower in women who exhibit depressive symptoms after losing their spouses when compared to age-matched controls (Zisook, Shuchter, Irwin, Darko, Sledge & Resovsky, 1994).

The olfactory bulbectomy (OBX) is an animal model of depression that was developed for the purpose of screening antidepressant drugs (Kelly, Wryne, & Leonard, 1997). Depression-like signs induced by bilateral olfactory bulb ablation including altered brain

monoamine levels (Remond, Kelly, & Leonard, 1999), elevated plasma level of corticosterone (Cairncross, Cox, Forster, & Wren, 1979; Cairncross, 1984), and hyperactivity (e.g., greater distance traveled) in a novel environment measured by the open-field test (O'Connor & Leonard, 1986; Redmond et al., 1999). These signs have been reversed after chronic (i.e., about 14 days) administration of various classes of anti-depressant drugs in rats (Leonard & Tuite, 1981; Richardson, 1991).

A hallmark behavioral feature of OBX rats is greater locomotory activity (i.e., hyperactivity) in a open field compared to sham animals (e.g., Redmond et al., 1999, O'Connor & Leonard, 1986). Higher locomotory activity exhibited by OBX rats is generally regarded as a failure to exhibit a normal behavior in a novel environment (i.e., defensive immobilization) and is thought to resemble "agitated" behavior in human depression.

An elevated serum corticosterone level has been found in bulbectomized rats, consistent with the hypercortisolism found in human depression (Nemeroff, 1998). Levels of plasma corticosterone were elevated after OBX (about 18 µg/dL), with an extreme level (around 70 µg/dL) after footshock, compared to the resting value in sham operated rats (8.1 µg/dL) (Cairncross, Wren, Cox, & Schnieden, 1977). Increased mean level of corticosterone and higher plasma corticosterone morning values were shown in male rats after OBX (Marcilhac, Maurel, Anglade, Ixart, Mekaouche, Hery & Siaud, 1997). Also, elevated plasma corticosterone in OBX rats was reversed after 10 days of antidepressant (e.g., amitrptyline, mianserin, viloxazine) treatment but not after treatment with other psychotropic drugs such as amphetamine (Cairncross, Wren, Forster, Cox, & Schnieden, 1979).

Immunomodulatory effects of OBX in rats have been similar to those found among depressed patients (Kelly, Wrynn, & Leonard, 1997; Song & Leonard, 1995). OBX has induced lower neutrophil phagocytosis (O'Neill et al., 1987; Song & Leonard, 1997), increased leukocyte adhesiveness (Song & Leonard, 1993), lower mitogen-stimulated

lymphocyte proliferation (Song & Leonard, 1992, 1994), and an inhibition of plaque-forming cell count accompanied by thymic involution (Komori, Fujiwara, Nomura & Yokoyama, 1991). Immunomodulatory effects (e.g., decreased neutrophil phagocytosis, lower lymphocyte proliferation, etc.) of OBX have been reversed after chronic (i.e., about 14-20 days) administration of antidepressant drugs such as lithium chloride and fluvoxamine (Song & Leonard, 1995). However, we are unaware of studies that have examined NK cell cytotoxicity after OBX in rats. Also, the possible mechanisms of suppressed NK function in OBX rats are not known.

Hyperresponsiveness and dysregulation of the hypothalmic-pituitary-adrenal cortical (HPAC) system have been thought to contribute to the functional modulation of natural immunity by affecting organs and cells of the immune system that possess receptors for hormones such as CRH, ACTH, and glucocorticoids (Bernton, Bryant & Holaday, 1991; Maier & Watkins, 1998). Reduced surface expression of adhesion and costimulatory molecules on thymic dendritic cells has been found in rats after incubation with dexamethasone *in vitro*, in association with decreased allostimulatory function (Sacedon, Vicente, Varas, Jimenez, Munoz, & Zapata, 1999).

Elevated plasma levels of glucocorticoids can cause apoptotic cell death of leukocytes including NK cells. Apoptosis is a morphological process of programmed cell death that involves chromatin condensation and margination, cell shrinkage, membrane blebbing, and formation of nuclear fragments or apoptotic bodies (Douglas, Tarshis, Pletcher, Howell, & Moore, 1995). Incubation with glucocorticoids has resulted in decreased viability of eosinophils by inducing apoptosis in eosinophils in rats (Nittoh, Fujimori, Kozumi, Ishihara, Mue, & Ohuchi, 1998). Weyts, Verburg-van Kemenade and Flik (1998) showed the presence of a single class of cortisol-binding sites on peripheral blood leukocytes including NK cells. Elevated plasma corticosterone has also induced apoptosis in murine bone marrow B-

lineage lymphocytes (Garvy, Telford, King & Fraker, 1993) and thymocytes (Gruber, Sgonc, Hu, Beug & Wick, 1994; Telford, King & Fraker, 1991). In addition, apoptosis in mouse thymocytes was observed after acute treadmill exercise (i.e., forced running stressor) (Hoffman-Goetz & Zajchowski, 1999) and restraint stress (Tarcic, Ovadia, Weiss & Weidenfeld, 1998), and it was correlated with increased levels of plasma corticosterone.

Hence, it can be hypothesized that an increased level of corticosterone consequent to activation of the HPAC axis after OBX, might modulate activity of NK cells in rats. However, to our knowledge, NK cell cytotoxic function after OBX has not been studied in relation to plasma corticosterone levels.

Both acute exercise and exercise training have been shown to affect NK cell cytotoxicity in rats and mice. NK cell cytotoxicity was enhanced after 40 weeks of treadmill exercise (Moriguchi et al., 1998) and 4-5 weeks of voluntary wheel running (Jonsdottir et al., 1997) in rats. In addition, Hoffman-Goetz and colleagues reported increased NK cell cytotoxicity in vitro after 9 weeks of wheel running or treadmill training (MacNeil & Hoffman-Goetz, 1993a, b), 10 weeks of treadmill training (Simpson & Hoffman-Goetz, 1990), and 8 weeks of either forced treadmill exercise and voluntary wheel running (Hoffman-Goetz et al., 1994a, b) in mice. Also, 90 min of moderate intensity swimming for 20 days resulted in enhanced NK cell activity in mice (Ferrandez & De la Fuente, 1996). Though exercise training has been found to have antidepressant-like effects in rat (Dishman, Renner, Youngstedt, Reigle, Bunnell, Burke, Yoo, Mougey, & Meyerhoff, 1997; Yoo, Tackett, Crabbe, Bunnell & Dishman, 2000), whether exercise training enhances *in vitro* splenic NK cell cytotoxicity in OBX rats has not been examined.

Therefore, the present study was designed to investigate 1) the effect of OBX on splenic NK cell activity, 2) the effect of voluntary wheel running on NK cell activity after OBX manipulation, 3) the effect of 3 weeks of antidepressant (i.e. imipramine hydrochloride)

treatment, as well as the additive effect of drug and wheel running, on NK cell function following OBX, 4) plasma level of corticosterone after OBX and its association with apoptosis of splenic NK cells, and 5) an expected increase in hyperactivity in a novel environment as indicated by open-field behavior in male Long-Evans rats.

The expected results were as follows: Splenic NK cell activity would be lower in the OBX condition, but the voluntary wheel running group and the imipramine treatment were expected to blunt suppression of NK cell activity in OBX rats. Also, an additive effect of imipramine and voluntary wheel running in blunting the NK cell suppression was expected. Lastly, higher plasma levels of corticosterone and percentage of apoptotic NK cells were expected in OBX rats compared to sham conditions, and we expected that those effects of OBX would be blunted by wheel running and imipramine treatment. We also expected that rats in the OBX conditions would exhibit hyperactivity (e.g., greater distance traveled) in an open field compared to the sham conditions, which we expected to be reversed after imipramine treatment.

MATERIALS AND METHODS

Subjects

Long-Evans hooded rats (n = 105, mass ~ 150 g) were obtained from Harlan (Indianapolis, IN) at approximately 45 days of age and allowed to adapt to the vivarium, maintained at 22 ± 2EC, for a week before the surgery. Eighty four animals received experimental manipulations, and 21 rats served as home-cage animals for cytotoxicity assays. Animals were housed individually in a shoebox cage on a 12-h light-dark cycle with a light on at 0700 hours, and food and water were provided ad libitum. Animals were weighed and handled daily throughout the course of the study in accordance with the National Institutes of Health Guide regarding the care and use of animals for experimental procedures. The experiment was approved by the University of Georgia institutional review board.

Experimental Procedures

After a 1-week adaptation to the vivarium, 42 animals were randomly selected to undergo olfactory bulbectomy. The other 42 rats received sham surgery. Twenty-four hours after the surgery, rats were randomly assigned to either activity wheel or sedentary groups. Animals were also randomly selected to receive either imipramine or saline injections i.p. 48 hours after surgery. Imipramine (10mg/kg) was injected intraperitoneally (i.p.) daily, and experimental conditions (voluntary wheel running and imipramine injection) continued for a 3-week period. Twenty-four hours after the final imipramine injection and/or exercise session, animals underwent open-field testing. Each testing lasted 9 minutes for each animal and was conducted in a dimly illuminated, thermoneutral testing room during the light phase. Twenty-four to 72 hours after the behavioral testing, animals were sacrificed by decapitation under halothane inhalation anesthesia. The order of euthanasia was counterbalanced across experimental conditions. Spleens were removed aseptically and were kept in RPMI-1640 containing 10% heat-inactivated FBS at room temperature until one hour later when assays were performed. Trunk blood was collected in a plastic cup containing 0.3 ml of heparin, chilled on crushed ice for approximately 1 hour, and transferred into 5ml collection vials prior to being spun at 3000 g for 15 minutes at 4 EC. The available plasma was pipetted into collection vials and stored at -20EC until the radioimmunoassay.

Olfactory Bulbectomy/ Sham Surgery

Rats were anesthetized with sodium pentobarbital (25mg/kg) and Ketamine (40 mg/kg) administered i.p. (Anpro Pharmaceuticals, Arcadia, CA). The head was shaved, betadine was applied to the scalp, and an incision was made on the scalp midline. Two burr holes (2 mm diameter) were drilled through the skull 6 mm anterior to bregma and 2 mm lateral to midline as described by Kelly et al. (1997). The olfactory bulbs were aspirated using a curved 2 mm diameter plastic pipette tip connected to a suction pump, and the cavity was filled with gel-foam

to control bleeding. Caution was taken to avoid damaging the frontal cortex. Animals in the sham surgery group received the same treatment, but the olfactory bulbs were left intact. The incision was closed with vicryl sutures, and the animals kept warm using towels and heat lamps until they recovered from anesthesia.

Upon termination of the experiment, completion of the bulbectomy was determined by the dry weight of remaining tissue of olfactory bulbs. Seven animals that had remaining tissue which was more than 30% that of sham surgery animals were excluded from the experiment because of incomplete olfactory bulbectomy. In addition, 2 animals were excluded from the experiment because of damage to the cortex and infection in the area after surgery. Rats (n = 9) that were excluded from the study were evenly distributed across all 4 OBX conditions: the wheel plus imipramine (n = 2), sedentary plus imipramine (n = 2), the wheel plus saline (n = 3), and the sedentary plus saline (n = 2) conditions.

Experimental Treatments

Exercise group. Twenty-four hours after surgery, experimental treatments began and continued for three weeks. Half the animals were given free access to an activity wheel (106 cm in diameter) by placing the wheel in the cage. Running distance (m) was determined by multiplying the number of revolution of the wheel recorded by a digital counter by the diameter of the wheel. Rats in the sedentary group were not given access to wheels, but were weighed and handled in the same manner as the activity-wheel group. *Anti-depressant treatment*. Half the rats were randomly assigned to drug treatment. Ten mg/kg of imipramine hydrochloride (Sigma Immunochemicals, St. Louis, MO) was injected i.p. 7 days per week for 3 weeks. The other half of the animals were given saline injection (10 ml/kg).

Open-field Behavior

Behavioral testing began 24 hours after completion of the 3 weeks of experimental manipulation: wheel running and/or imipramine treatment. The open-field apparatus consisted of a four-sided 50 x 50 x 43 cm, plexiglass chamber. The floor was painted black, and the sides were transparent. A video camera was placed 1 meter above the floor of the chamber and a light with a 15 watt bulb was attached to the camera. Rats were transported in a clean shoebox cage to a testing room that was adjacent to the colony room and gently placed in the center of the arena. Distance traveled (i.e., distance that the animal traveled), stereotypic time (i.e., time for repetitive movements including grooming, rearing, etc.), time for ambulating (i.e., time for mobility), and resting time (i.e., time for immobilization) were recorded with an automated video image analyzer (Videomex-V, Columbus Instruments, Columbus, OH). The analyzer was programmed to simultaneously record activity in a central 15 cm^2 area (zone B), the perimeter (zone A), and the entire open-field. Each subject was tested for three, 3-minute periods; 9 minutes in total. The chamber was cleaned with a water soaked sponge between each subject to remove olfactory cues from the previous tested animal.

^{51}Cr-release Assays for NK Cell Cytotoxicity

Single cell suspensions were made by homogenizing spleens in RPMI with 10% FBS. Leukocyte isolation was done by lysing red blood cells in 10 ml of Tris ammonium chloride (1 Tris buffer: 9 ammonium chloride) lysing buffer. Isolated white blood cells were then loaded in nylon wool columns and incubated for an hour at 37 EC in a CO_2 incubator. Nylon wool column-nonadherent cells were collected by passing RPMI (37 EC) through the column for cytotoxic-cell enrichment, which in our laboratory (Dishman, Warren, Youngstedt, Yoo, Bunnell, Mougey, Meyerhoff, Jaso-Friedmann, & Evans, 1995; Dishman, Warren, Hong, Bunnell, Mougey, Meyerhoff, Jaso-Friedmann, & Evans, 2000; Dishman, Hong, Soares, Edwards, Bunnell, Friedmann, & Evans, 2000) contains about 75% NK cells and less than 1%

and 5% contaminating B cells and macrophages, respectively. Effector cells were then washed twice with RPMI and resuspended at desired concentrations.

YAC-1 (mouse T-cell lymphoma cell line) cells were used as target cells. They were labeled with 100 µCi $Na_2{}^{51}CrO_4$ (Amersham, Arlington Heights, IL). After 3 hours of incubation with chromium and two washes with RPMI, target cells were resuspended in RPMI-1640 with 10% FBS at 100,000 cells/ml. Then, 100 µl (10,000 cells) was added to 96-well round-bottom microtiter plates (Costar, Cambridge, MA). Effector cells at desired concentrations were added to the target cells at five different effector to target ratios (12.5, 25, 50, 100, 200:1) in a final volume of 200 µl. Effector and target cells were co-cultured for 4-6 hours at 37 EC in a CO_2 incubator, the plate was centrifuged at 100 g for 5 minutes, and 100 µl of supernatant was then removed from each well. Radioactivity was determined using a Cobra II Auto-Gamma gamma counter (Packard Bioscience, Meriden, CN). The results were expressed as the percentage of specific release (% SR), and determined by using the formula, % SR = [(test release - spontaneous release)/(total release - spontaneous release)] x 100.

There were missing data points (n = 7 experimental animals and 3 home cage animals) because of low target cell incorporation that resulted in minimal total release. Missing data were evenly distributed across experimental groups.

Flow Cytometry

For phenotyping and measurments of apoptosis, flow cytometry was performed on an EPICS XL-MCL flow cytometry system (Coulter Cytometry, Hialeah, FL). A 15-mW argon laser (488 nm) was used for all analyses. Data were stored in list mode and analyzed using SYSTEM II software. The following lenses were used: 488 dichroic, 488 long pass, 560 long pass, 575 band pass, 600 dichroic.

Reagents and Monoclonal Antibodies. Monoclonal antibody (mAb) 5C6 was used for NK cell phenotyping. FITC-conjugated anti-mouse IgM and Propidium iodide (PI) were obtained from Sigma Immunochemicals (St. Louis, MO). Annexin-V-FITC was obtained from Pharmingen (Torreyana, CA), and nuclear isolation media (NIM) was prepared with 0.01% Triton X 100 (Sigma), 0.5% PI, and 0.1% RNAse (Sigma) in PBS. Dexamethasone (Sigma) was prepared in 95 % ethanol and stored at -20EC.

Immunophenotyping. Media control (i.e. cells in RPMI) and conjugate controls (i.e., cells stained with FITC IgM, and PE) were used to examine autofluorescence and background fluorescence. For NK cell phenotyping, NK cells were incubated with anti-5C6 mAb. 5C6-stained cells were than stained with secondary mAb FITC-conjugated anti-mouse IgM (Sigma) for flow cytometric analyses.

Analyses of apoptosis. In order to identify the cells that were undergoing apoptosis, two different methods were used: annexin V staining and NIM (nuclear isolation media) staining. An early indicator of apoptosis in mammalian cells is the loss of the phospholipid membrane asymmetry of the cell (Douglas et al., 1995). This results in the exposure of phosphotidylserine (PS) on the outer surface of the cell membrane and this change in membrane asymmetry can be analyzed using annexin V which binds to PS (O'Brien, Reutelingsperger, and Holdaway, 1997). Apoptotic NK cells were measured by FITC annexin staining and 5C6 mAb and PE annexin double staining. A gate was drawn over PI negative cells, and the FITC annexin-positive population was gated on PI negative cells in order to measure apoptotic cells excluding necrotic cells. Cells were stained with FITC- and PE-conjugated Annexin V (5 µl/100,000 cells) and incubated for 15 minutes in the dark. 0.5 ml of binding buffer (2.5 mM $CaCl_2$) was added prior to the analysis.

In order to examine glucocorticoid induced apoptosis in NK cells, isolated NK cells were incubated with 100 nM dexamethasone for 20 hours as a positive control. Apoptotic cells

were measured by annexin staining as described above. Cell cycle analysis was performed using PI in order to determine the percentage of DNA hypoploidy in isolated rat NK after dexamethasone incubation. PI staining has been used extensively to evaluate apoptosis, where the apoptotic fraction has a reduced DNA content (sub-G_0) due to cleavage and extraction of the DNA (Douglas et al., 1995). NIM was added to the cells, and cells were incubated on ice for 10 minutes in the dark prior to analyses.

Radioimmunoassay (RIA) for Plasma Corticosterone

Commercially available double-antibody RIA kits (ICN biomedicals, Costa Mesa, CA) were used to determine the plasma concentration of corticosterone. The minimum detection of the assay was 12.5 ng/ml with a 50% displacement of 121 ng/ml. The intra- and interassay coefficients of variation were 4.4 to 10.3% and 6.5 to 7.2%, respectively.

Statistical Analyses

The effects of the experimental conditions on each dependent variable were examined using a 2 Condition (OBX vs. sham) x 2 Group (activity wheel vs. sedentary) x 2 Treatment (imipramine vs. saline) factorial design. PC SPSS version 9.0 (SPSS, Chicago, IL) was employed for statistical procedures. The percentage of specific lysis was compared among groups across the five effector-to-target (E/T) ratios by repeated measure ANOVA with E/T ratio as the within-subject factor. Sphericity adjustments were made using Huynh-Feldt ,. The type III error term was used to adjust for unequal cell sizes. The hypotheses that suppression of NK cell cytotoxicity in OBX rats would be blunted after voluntary wheel running and imipramine treatment, were tested by the Condition x Group and Condition x Treatment effects. The hypothesis that voluntary wheel running and imipramine would have additive effects was tested by the Condition x Group x Treatment effect. The main effects for 'Condition' were used to test the effects of OBX on NK cell activity, apoptosis of NK cells, open-field behavior, and plasma corticosterone levels. The effects of chronic voluntary wheel running and

imipramine treatment on the dependent measures were tested by main effects for 'Groups' and 'Treatment' respectively.

Missing observations and data points exceeding the criterion of Grubb and Beck (1972) for outliers were #4% of all observations and were replaced by the cell mean for each variable. Cell size was 7- 10 animals. Sample size was based on an expected effect size of 1.0 standard deviation (SD). Eight animals per cell provided a power of 0.80 at an " of $P < 0.05$. Values are reported as means ± SD in the text and means ± SE in Figs. 1-2. As expected, OBX resulted in hyperactivity in an open field.

Results

NK cell activity was not affected by OBX or activity wheel running. Also, there was no difference in the percentage of apoptotic cells between OBX and sham animals. Contrary to expected results, plasma corticosterone level was lower in OBX rats compared to sham surgery animals, though both values were close to expected control resting values. Incubation with dexamethasone resulted in apoptosis in rat NK cells as expected. OBX animals traveled a greater distance during the first session of the open-field testing indicating that OBX rats exhibited greater locomotor activity in a novel environment compared to sham animals as expected.

NK Cell Cytotoxicity: ^{51}Cr-release Assays

Repeated-measures ANOVA with E/T ratio as the within-subject factor indicated an expected increase in NK activity with increasing E/T [$F(4, 83) = 345.78$, , $= 0.52$, $p < 0.001$] (see Fig. 1). However, there were no E/T x Condition [$F(8, 83) = 0.69$, $p = 0.609$], E/T x Group [$F(4,83) = 0.27$, $p = 0.712$], or E/T x Treatment [$F(4, 83) = 0.67$, $p = 0.481$] effects. The effects of Condition x Group x Treatment for % specific lysis were not significant [$F(8, 83) = 0.86$, $p = 0.3\ 6$)]. Also, interactions between Condition and Group [F (= 0.125, p = 0 .73],

Condition and Treatment [F = 0.19, p = 0.67], and Group and Treatment [F = 0.49, p = 0.49] were not significant. There were no significant main effects for Condition, Group, or Treatment (p > 0.61 for all).

Insert Figure 1A and B

Phenotyping of NK Cells

Cells that were FL 1 positive in a histogram (5C6 mAb positive) were identified as NK cells and backgated on a FS (forward scatter) and SS (side scatter) histogram to examine the distribution of NK cells on a scatter plot (histograms not shown). There were no significant differences in % 5C6 positive cells (72 .0 ± 10.08 %) across 8 experimental groups and 1 home cage group [F(8, 74) = 0.55, p = 0.82].

Corticosterone

OBX rats showed a lower plasma level of corticosterone (6.52 ± 6.0 µg/dL) compared to sham surgery animals (10.30 ± 8.70 µg/dL) [F(1, 72) = 4.853, p = 0.031]. There were no Condition x Group x Treatment [F(1, 72) = 0.23, p = 0.631], Condition x Group [F(1, 72) = 0.59, p = 0.444], Condition x Treatment [F(1, 72) = 0.28, p = 0.602], or Group x Treatment [F(1, 72) = 0.10, p = 0.749] effects. The values for each group and condition are presented in Figure 2.

Insert Figure 2

Apoptosis

Measurement of percent annexin-positive cells indicated that there was no significant difference in percent apoptotic cells between OBX (4.58 ± 3.69 %) and sham groups (4.93 ± 5.09 %) [F(1, 69)= 0.12, p = 0.64]. Percentage of apoptotic cells was minimal and ranged from approximately 3 to 7 % across different groups.

Dexamethasone Incubation

Percent annexin-positive cells indicated that dexamethasone incubation induced apoptosis in NK cells (32.07% of PI negative cells). There was a significant difference in percentage of apoptotic cells between OBX (n = 12, 37.48 ± 9.85 %), sham (n = 9, 24.91 ± 9.85 %), and home cage (n = 2, 31.8 ± 1.56 %) animals [$F(2, 23) = 5.40$, $p = 0.01$] (see figure 3A and B). Higher % apoptosis in OBX rats was not expected because of possible glucocorticoid receptor downregulation after chronic exposure to corticosterone, but the results indicated that NK cells in OBX rats exhibited higher susceptibility to corticosterone. Future receptor-binding study is needed to investigate regulation of glucocorticoid receptor in OBX rats.

Insert Figure 3A and B

NIM staining also showed dexamethasone-induced apoptosis in rat NK cells (32.51 ± 10.32 %). The OBX group showed higher percent apoptosis (34.11 ± 11.26 %) compared to sham animals (30.14 ± 10.64 %), but it was not significant [$F(2, 23) = 0.352$, $p = 0.708$]. Histograms of annexin and NIM staining are shown in figure 4A and B.

Insert Figure 4A and B

Open-field behavior

The results are expressed separately for the three 3-minute sessions for each measurement. Measurements of activity in the open-field apparatus are presented in Table1. The distance traveled (cm) in perimeter area (zone A) during first session was significantly higher in OBX rats compared to sham animals [$F(1, 73) = 5.711$, $p = 0.020$]. However, there were no main effects for Group [$F(1,73) = 0.763$, $p = 0.386$] or Treatment [$F(1,73) = 1.643$, $p = 0.205$]. No Condition x Group [$F(1,73) = 1.825$, $p = 0.181$] or Condition x Treatment [$F(1, 73) = 0.020$, $p = 0.889$] were found for ‘distance traveled’. Resting time (sec.) was

significantly greater in OBX rats [$F(1,73) = 5.490$, $p = 0.022$], and ambulation time (sec.) was greater in sham animals [$F(1, 73) = 4.169$, $p = 0.045$] during the second sessions. Time for stereotypy was larger in sham animals during the first [$F(1, 73) = 15.404$, $p < 0.001$] and third [$F(1, 73) = 3.765$, $p = 0.057$] sessions.

Insert Table 1

Body Mass and Wheel Running

Body mass (g) was significantly different between OBX and sham surgery animals after the surgery beginning from week 1 [$F(1, 72) = 25.77$, $p < 0.001$] to week 2 [$F(1, 72) = 19.71$, $p < 0.001$] and week 3 [$F(1, 72) = 19.40$, $p < 0.001$]. However, mass increased linearly in both OBX and sham groups over the course of 3 weeks of the experimental period from weeks 1 [(236.89 ± 16.69 g), (255.44 ± 14.41 g)], 2 [(270.03 ± 24.78 g), (293.75 ± 20.62 g)], and 3 [(290.86 ± 30.26 g), (320.61 ± 27.32 g)]. There was no significant difference [$F(1, 72) = 0.04$, $p = 0.85$] in body mass between activity wheel (304.84 ± 33.74 g) and sedentary (306.29 ± 31.19 g) animals at the end of the 3 weeks of the experimental treatments.

Running distance increased linearly during the 3 weeks, from week 1 (1706.35 ± 1191.65 m) to weeks 2 (3632.68 ± 2862.35 m) and 3 (5029.48 ± 3039.48 m). The OBX and sham conditions did not differ in running distance during the 3 weeks [$F(1, 37) = 0.34$, 0.37, and 0.17] indicating that olfactory bulbectomy did not affect total activity. **Discussion**

Bilateral ablation of the olfactory bulbs did not affect *in vitro* splenic NK cell cytolytic function in this investigation. OBX has been shown by other investigators to modulate immunity (e.g., O'Neill et al., 1987; Song & Leonard, 1992, 1993a). However, whether OBX influences NK cell activity had not previously been studied. In this investigation, we expected that dysregulation of the HPAC axis, as indicated by an elevated basal plasma corticosterone level, would lead to increased apoptosis in rat NK cells and a subsequent suppression of NK

cell cytolytic function. However, plasma corticosterone levels for bulbectomized animals were in the range of basal levels for home-cage controls (i.e., 4.5 - 9 µg/dL) reported elsewhere (e.g., Cairncross, Wren, Cox, Sheriden, 1977; Dishman, Bunnell, Youngstedt, Yoo, Mougey & Meyerhoff, 1998; Ixart et al., 1977).

Cairncross and colleagues found that OBX resulted in elevated levels of plasma corticosterone (18 µg/dL), which was reversed after antidepressant treatment (Cairncross et al., 1977; 1979). In their experiments, animals were killed at the beginning of the dark cycle (i.e. animal's active period), whereas rats in the present study were killed during the early light cycle (0900-1100 hours). This might explain the different corticosterone levels that were observed between the two studies. Also, Marchilhac and colleagues (1997) reported a phase change in serum corticosterone levels after OBX, showing an elevated value of corticosterone in the early dark cycle. However, in the Cairncross (1977) study, corticosterone levels in control animals were reported to be elevated in the early light cycle. This is perplexing, since other studies (e.g., Ixart et al., 1977; Martin, Wangsness, & Gahagan, 1978) have shown circadian patterns of basal corticosterone levels that were highest in the early dark cycle.

Whether dysregulation of the HPAC system in OBX rats is manifested in an elevated tonic level of corticosterone or shifted circadian pattern of corticosterone raises questions regarding whether the necessary exposure of NK cells to corticosterone was sufficient to affect NK cell function via apoptosis. If OBX rats exhibited elevated tonic levels of corticosterone, total exposure of NK cells to corticosterone might have been greater in OBX rats throughout the day compared to sham animals. However, if a disruption in diurnal rhythm of corticosterone was the main feature in OBX rats, NK cells might have been exposed to a similar total concentration of corticosterone when averaged across the day compared to sham animals.

Also, the relationship between corticosterone release and the time course of the apoptotic process *in vivo* is not clear. Though *in vitro* incubation of splenocytes with

corticosterone with the concentration observed at submaximal exercise for 24 hours induced apoptosis (Hoffman-Goetz & Zajchowski, 1999), apoptotic cells detected using annexin staining were not found immediately or 2 hours after intensive exercise (Hoffman-Goetz, Zajchowski, & Aldred, 1998).

Though some studies have shown that OBX in rats resulted in elevated plasma corticosterone, others have reported no alteration in basal levels of plasma corticosterone after OBX in rats (e.g., Arnold & Meyerson, 1990; Broekkamp, O'Connor, Tonnaer, Rijk, & Van Delft, 1986; Williams, McGinnis, & Lumia, 1992). Similar to the present study, Broekkamp et al. (1986) did not show different corticosterone levels between the OBX and sham animals that were killed during the light cycle. In addition, though plasma corticosterone levels demonstrated circadian variation, they were not different between OBX and sham groups across the different times of the day. This finding of Broekkamp et al. (1986) is contrary to the results reported by Marchillhac et al. (1997), whereby an altered circadian pattern of corticosterone release in OBX rats resulted in abnormally high levels of corticosterone compared to sham animals during the early dark cycle. The basal corticosterone levels reported by Williams et al. (1992) were not different between OBX (11 µg/dL) and sham (8 µg/dL) among Long-Evans rats, and the values were within the range of basal corticosterone levels reported elsewhere (e.g., Cairncross et al., 1977, Dishman et al., 1998) and in the present experiment. Therefore, it is difficult to explain why OBX did not induce an elevated basal corticosterone level in the present experiment.

Exaggerated increases in corticosterone have been shown after an acute stressor, such as footshock (Cairncross et al., 1979). Williams et al. (1992) also reported markedly increased corticosterone levels after restraint stress in OBX rats compared to control animals, though there was no difference in the basal level of plasma corticosterone between the two groups. Corticosterone levels returned to the baseline level 1 hour after the restraint stress in OBX rats, whereas levels in sham animals remained high after 1-hour of recovery. The findings

of Williams et al. suggested that there might have been a negative feedback mechanism that re-regulates the HPAC system, resulting in lowered corticosterone level at rest and faster recovery after stress. Therefore, it is plausible that olfactory bulbectomy might lead to an adaptation in the HPAC system that would result in a hyper-responsiveness to a novel stressor in spite of no change in the tonic resting level of plasma corticosterone. Future investigations should measure plasma corticosterone levels in OBX rats at rest, during, and after an acute stressor at different times of the day. Such studies might provide further explanation of the association between altered corticosterone level and NK cell function in OBX rats.

Contrary to expected results, we did not show apoptosis in OBX or sham animals. The small elevation in plasma corticosterone level likely explains the findings. We demonstrated that dexamethasone induced apoptosis in rat NK cells after 15 hours of incubation. The concentration (100nM) of dexamethasone selected was based on an expected level of corticosterone in OBX rats of about 25μg/dL (e.g., Cairncross, 1984) which was higher than the level found in our experiment. Thus, dexamethasone incubation confirmed glucocorticoid-induced apoptosis in NK cells, but OBX did not result in an elevated plasma corticosterone level.

Voluntary activity wheel running did not alter NK cell activity in our investigation. We have previously shown that 4-6 weeks of chronic wheel running and treadmill training blunted the suppression of NK cell activity induced by uncontrollable electric footshock, but did not enhance basal NK cell activity in Fischer 344 rats (Dishman et al., 1995, 2000). The basal lymphokine activated killer (LAK) cell activity was not altered after 8 weeks of voluntary wheel running (Hoffman-Goetz, Arumugam, & Sweeny, 1994). Also, basal level of NK cell activity did not change after 15 weeks of treadmill running (Nasrullah & Mazzeo, 1992) in Fischer rats. Therefore, the effects of chronic physical activity on basal NK cell activity is unclear. Rats were exposed to 3 weeks of wheel running, which was a shorter period than used in other studies that showed enhanced NK cell activity after training (e.g., Jonsdottir et al., 1997;

Moriguchi et al., 1998). However, the effects of length of exercise training on NK cell activity needs more investigation, as Ferrandez and De la Fuente (1996) showed increased murine NK cell activity after 20 days of moderate swimming.

Hyperactivity of OBX rats in a novel environment has been shown consistently in studies as indicated by higher ambulation (Redmond et al., 1999), rearing and grooming (O'Connor & Leonard, 1986), or numbers of squares crossed in the arena (Broekkamp et al., 1986). As hypothesized, we observed that OBX animals traveled a greater distance during the first session of the open-field testing, which indicated that OBX rats exhibited greater locomotor activity in a novel environment compared to sham animals. Though distance traveled was significantly greater in OBX rats only during the first session among the 3 trials, the first 3 minutes of the testing is thought to be critical as animals are introduced to the novel environment.

There were no effects of wheel running or imipramine treatment on 'distance traveled'. Though previous studies (e.g., Dishman, Dunn, Youngstedt, Davis, Burgess, Wilson, & Wilson, 1996; Frankova, Parizkova, & Milkulecka, 1987; Tharp & Carson, 1975; Weber & Lee, 1968) have reported increased ambulation in the open field after chronic activity wheel running, the method of administering the open-field test in those studies differed from the protocol used in OBX research. No Condition x Treatment effect indicated that there were no effects of imipramine treatment on open-field activity. Our results do not agree with the studies that reported the effects of antidepressant drugs such as mianserine (O'Connor & Leonard, 1986) and Milnacipran (Redmond, et al., 1999) on open-field behavior. However, 2 weeks of mianserine treatment did not affect open-field behaviors such as defaecation and grooming, though it did influence ambulation (O'Connor & Leonard, 1986). In addition, Redmond et al. (1999) reported dose-dependent effects of Milnacipran on open-filed behavior. Two weeks of 30 and 40 mg/kg Milnacipran treatment, but not 20 mg/kg affected open-field behavior. Thus,

the present findings may be explained by the dose-dependent effects of imipramine on open-field behavior.

There are a few methodological issues that need to be addressed in future studies. In order to investigate whether OBX leads to an elevated corticosterone level, measurement of plasma corticosterone levels at different times of the day will help to confirm whether there is a circadian phase shift in corticosterone release. Also, measuring plasma corticosterone levels during and after acute stress in OBX rats would address dynamic rather than tonic regulation and adaptation of the HPAC system and its possible role in modulating NK cell activity.

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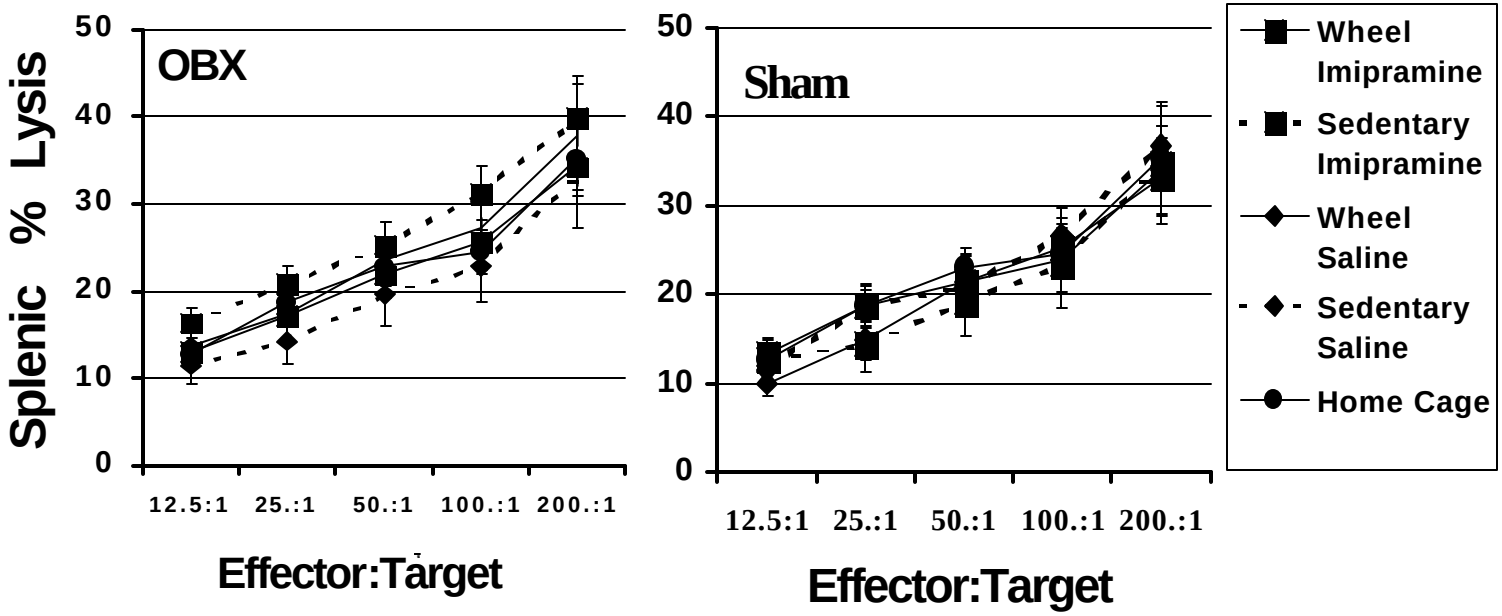

Figure 1A and B. % specific lysis of splenic NK cell in sham (A) and OBX (B) rats. No difference have been found between OBX and sham conditions. Increase in % lysis with increasing E.T was shown.

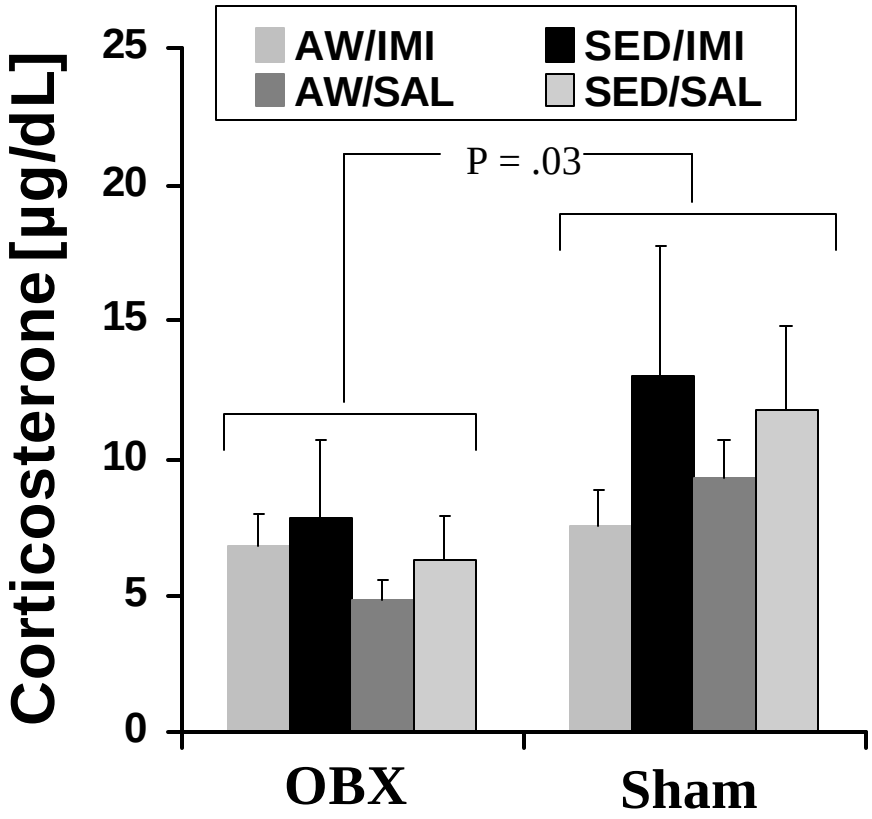

Figure 2. Corticosterone levels (µg/dL) in male Long-Evans rats after OBX and sham surgery. Corticosterone level was higher in sham animals compared to OBX rats. (P = 0.03)

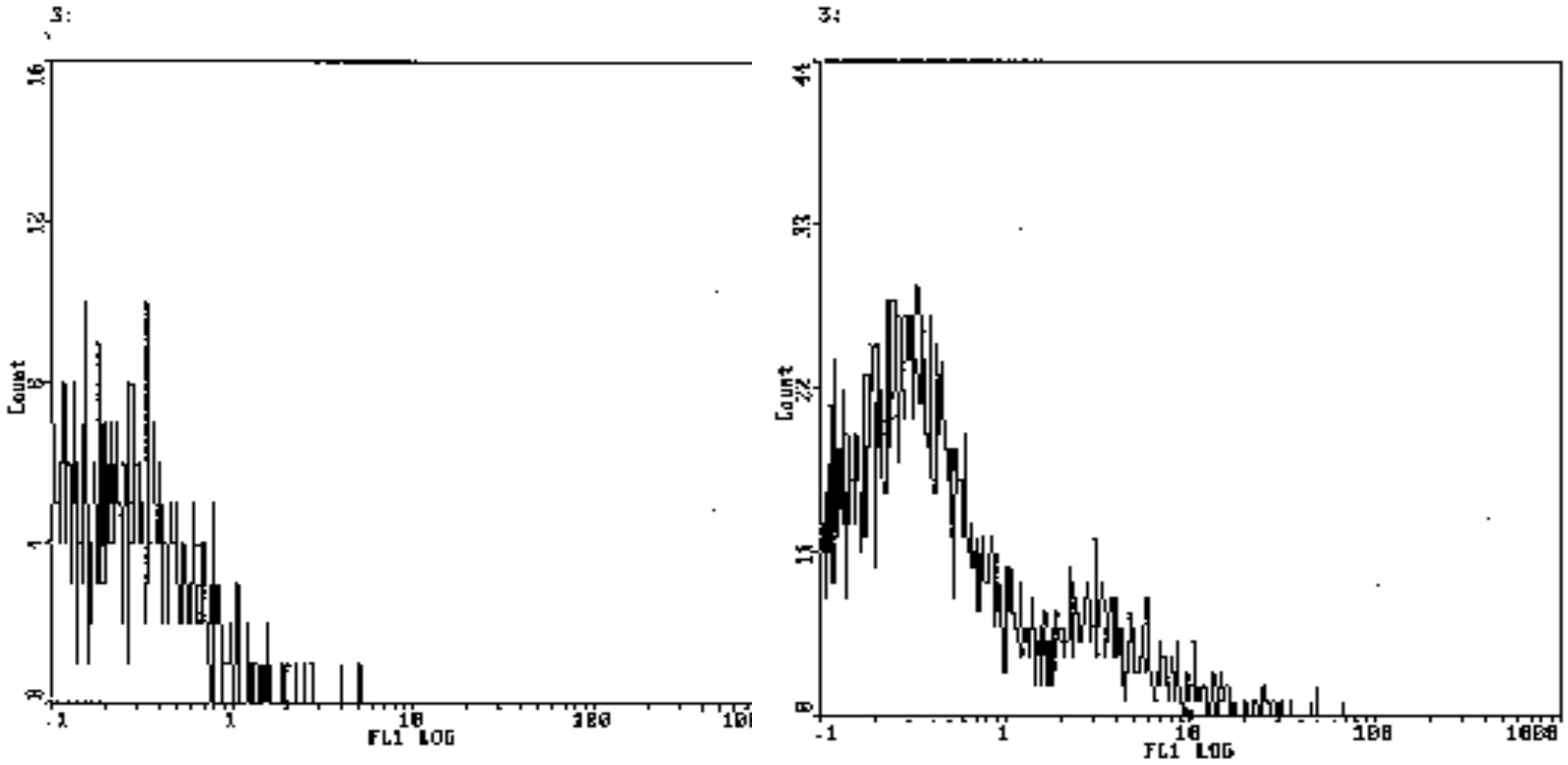

Figure 3A and B. Histograms of rat nylon wool nonadherent cells. Cells were stained with FTIC-conjugated annexin V to detect apoptotic cells. FL1 (FITC positive) histogram was gated on FL2 (PI) negative cells. A) 20 h incubation with media (RPMI with 10% FBS) B) 20 h incubation with dexamethasone (100nM)

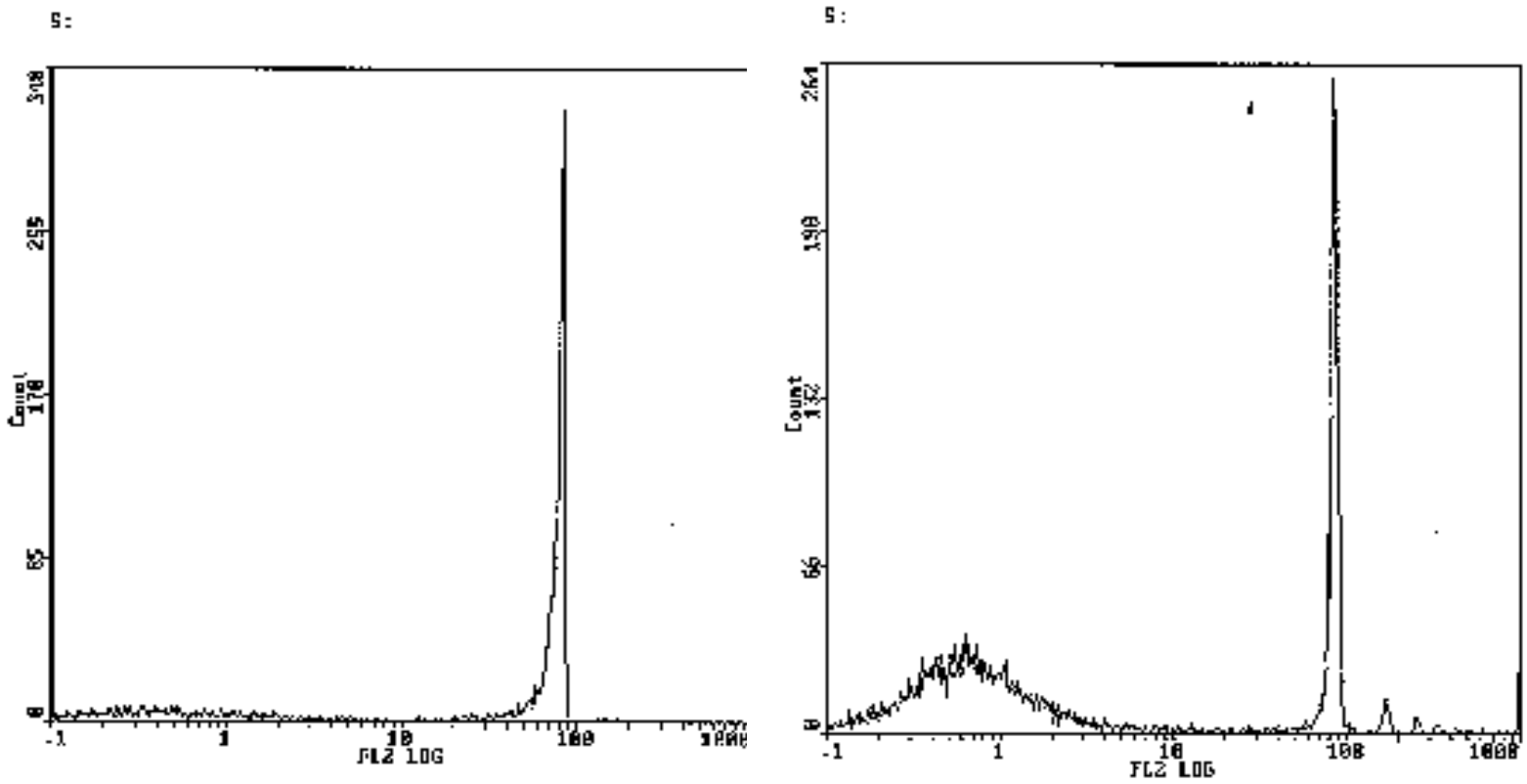

Figure 4. Histograms of rat NWNA cells after NIM staining. A) 20 h incubation with media B) 20 h incubation with dexamethasone

Table 1. Measures of activity in the open field apparatus for bulbectomized and sham rats (means ± standard deviation) are presented. All three 3-minute sessions are presented. Distance traveled in perimeter area (Zone A) during the first session was significantly greater in OBX compared to sham rats. Resting time was greater in OBX rats during the second session. Ambulation time was greater in sham animals during the second session. Stereotypic time was larger in sham animals during the first and third sessions ($P < 0.05^{*}$).

		Distance Traveled in perimeter (cm)	Resting Time Time (sec.)	Ambulation Time (sec.)	Stereotypic Time (sec.)
OBX	session 1	1527.11 ± 649.38*	29 ± 32.97	119.86 ± 33.58	22.72 ± 6.46
	session 2	964.99 ± 622.26	63 ± 49.03*	82.08 ± 42.50	21.44 ± 9.03
	session 3	870.27 ± 674.08	62.47 ± 43.32	74.44 ± 38.69	23.75 ± 10.03
Sham	session 1	1213.79 ± 359.96	23.97 ± 15.51	115.78 ± 20.84	27.65 ± 7.36*
	session 2	1081.66 ± 579.83	41.65 ± 29.35	98.89 ± 28.71*	25.78 ± 7.64
	session 3	836.42 ± 674.08	50.27 ± 27.09	81.65 ± 25.73	28.65 ± 7.98*

Distance Traveled: distance traveled in the perimeter of the open-field; Ambulation Time: duration of ambulation in all areas; Stereotypic Time: total stereotypic (i.e., repetitive behavior including grooming, rearing, etc.) in all areas; Resting time: duration of resting (i.e., immobilization) in all areas

CONCLUSIONS

NK cell activity after olfactory bulbectomy (OBX) has not been previously studied. Bilateral ablation of the olfactory bulbs did not affect *in vitro* splenic NK cell cytolytic function in this investigation, though OBX has been shown by other investigators to modulate other features of immunity (e.g., O'Neill et al., 1987; Song & Leonard, 1992, 1993). As expected, OBX led to hyperacticity in an open-field, consistent with its hallmark depression-like feature of agitation in a novel environment.

In this investigation, we expected that dysregulation of the HPAC axis, indicated by an elevated baseline plasma corticosterone level, would lead to increased apoptosis in rat NK cells and a subsequent suppression of NK cell cytolytic function. However, plasma corticosterone levels for bulbectomized animals were not elevated above resting levels. It is unclear that elevated corticosterone levels of OBX rats in previous studies indicated higher 24-hour absolute levels or a circadian shift of corticosterone secretion.

Whether dysregulation of the HPAC system in OBX rats is manifested in an elevated tonic level of corticosterone or shifted circadian patterns of corticosterone raises questions regarding exposure of NK cells to corticosterone sufficient to affect NK cell function via apoptosis. If OBX rats exhibited elevated tonic levels of corticosterone, total exposure of NK cells to corticosterone would have been greater in OBX rats throughout the day compared to sham animals. However, if a disruption in diurnal rhythm of corticosterone was the main feature in OBX rats, NK cells would likely have been exposed to a similar total concentration of corticosterone averaged across the day compared to sham animals.

Contrary to expected results, we did not show apoptosis in OBX or sham animals. The small elevation in plasma corticosterone level likely explains the findings. We demonstrated that dexamethasone induced apoptosis in rat NK cells after 20 hours of incubation. We can assume that there might be downregulation of glucocorticoid receptors on NK cells if there was a elevated tonic level of corticosterone in OBX rats. Our observation of apoptosis in NK cells after dexamethasone incubation, and previous studies that showed apoptosis of NK cells after incubation with corticosterone or severe exercise, indicate that NK cells undergo apoptosis in the presence of corticosteroids. However, these results all showed acute response of NK cells to glucocorticoids rather than apoptotic process of cells to chronic exposure. Therefore, future study is needed to examine downregulation of glucocorticoid receptors on NK cells after prolonged exposure.

Also, the time course of the apoptotic process of NK cells both in vivo and in vitro after exposure to corticosterone needs to be investigated. Though *in vitro* 24-hour incubation of splenocytes with corticosterone at the concentration observed at submaximal exercise for induced apoptosis (Hoffman-Goetz & Zajchowski, 1999), apoptotic cells detected using annexin staining were not found immediately or 2 hours after intensive exercise (Hoffman-Goetz, Zajchowski, & Aldred, 1998). Therefore, investigation of apoptosis of NK cells at multiple time points after corticosterone exposure might provide further explanations of the association between altered corticosterone level and NK cell function in OBX rats.

It is premature to conclude that the OBX model is not valid to investigate immunosuppression caused by dysregulation of neuroendocrine system. Adaptations that might have occurred at the level of the HPAC axis or higher brain levels, as well as at the cellular level, needs to be considered.

Voluntary activity wheel running did not alter NK cell activity in our investigation. Rats were exposed to 3 weeks of wheel running, which was a shorter period than used in other studies that showed enhanced NK cell activity after treadmill exercise training (e.g., Jonsdottir

et al., 1997; Moriguchi et al., 1998). However, the effects of length of exercise training on NK cell activity needs more investigation, as Ferrandez and De la Fuente (1996) showed increased murine NK cell activity after 20 days of moderate swimming. We have previously shown that 4-6 weeks of chronic wheel running and treadmill training blunted the suppression of NK cell activity induced by uncontrollable electric footshock, but did not enhance basal NK cell activity in Fischer 344 rats (Dishman et al., 1995, 2000). The basal lymphokine activated killer (LAK) cell activity was not altered after 8 weeks of voluntary wheel running (Hoffman-Goetz, Arumugam, & Sweeny, 1994). Also, basal level of NK cell activity did not change after 15 weeks of treadmill running (Nasrullah & Mazzeo, 1992) in Fischer rats. Therefore, the effects of chronic physical activity on basal NK cell activity in rats remains unclear.

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