Nutritional Sciences 5(3), August, 2002, pp. 123-128


# Effects of Acute Exercise on Nitric Oxide Generation from Mouse Macrophages

**Jung-Hee Shin[1§], Jin Kim[2], Hyun-Sook Kim[2] and Nyun-Soo Kwon[3]**

[1]*Dept. of Food and Nutrition, Joongbu University*

[2]*Dept. of Food and Nutrition, Sookmyung Women's University*

[3]*Dept. of Medicine, Chung-ang University*

**Physical activity is a primary cancer control strategy that has received little attention to date. However, an increasing number of epidemiological studies have proposed that physical exercise may be beneficial by enhancing anticancer immune system responses. We investigated the effects of acute exercise on changes in nitric oxide (NO) production and inducible nitric oxide synthase (iNOS) expression. The amounts of NO generated by abdominal macrophages in mice were measured after exercise. Thirty-two mice, which were challenged with thioglycollate broth to activate peritoneal macrophages, were randomly assigned to control, exercise and recovery groups. The mice exercised on a motor-driven treadmill for 3 consecutive days, either moderately (18m/min, 30 min/day, 5% grade) or severely (18-35m/min, 60 min/day, 5% grade). The mice were killed immediately after exercise or after 6 hrs of recovery. Nitric oxide was quantified by the Griess assay. The exercised mice showed higher levels of NO generation than those of the control mice, but the intensity of exercise had no significant effect on NO generation. Mice allowed six hours of recovery after exercise showed higher levels of NO generation than that of animals sacrificed immediately after exercise, but there were no significant differences in NO generation with variations in the intensity of exercise. Increased levels of iNOS were found in the exercised groups, and this was greatest in the groups allowed six hours of recovery compared to those groups sacrificed immediately after exercise. The results of this study suggest that acute exercise may enhance an immune response by inducing macrophage-derived NO generation; these results support the epidemiological findings which support the benefits of exercise in the prevention and control of cancer. Further study is needed to determine the physiological significance of these findings, which could be applied to the use of therapeutic exercises to assist in the prevention and control of cancer.**

**Key words** : exercise, nitric oxide, macrophage

## INTRODUCTION

Recent epidemiological and clinical studies showed that natural immunity is enhanced during moderate exercise, but that the numbers and function of cells mediating cytotoxic activity against virus-infected and tumor target cells are suppressed after intense and long-term exercise.[1-5] In animal studies, exercise delayed the development of cancer induced by chemical changes,[6-10] but exhaustive exercise accelerated cancer development.[11]

Macrophages are a line of defense against microbial invaders[12,13] and malignancies by nature of their phagocytic, cytotoxic, and intracellular killing capacities.[14-16] Macrophages are known to play an important role in host defense mechanisms. Activated macrophages may induce antitumor cytotoxicity, thereby resisting the progression of cancer and preventing metastasis. Immunological stimulation may accelerate the generation of nitric oxide (NO) from macrophages, which in turn produce nitrate and nitrite as oxidation products. Nitric oxide is a very unstable and reactive free radical produced in mammals, and plays significant roles in many physiological functions including antitumor activity when generated by immunological stimulation. The two major groups of nitric oxide synthase (NOS), neuronal and endothelial NOS, are in general constitutively expressed, and NO produced by such isoforms is a key regulator of homeostasis.[17] Moreover, the inducible isoform of NOS (iNOS) plays an important role in the cytotoxic activity of activated macrophages.[18] Stimuli such as the pro-inflammatory cytokine, interferon-γ (IFN-γ), and/or endotoxin and bacterial lipopolysaccharide (LPS), induce iNOS expression; once synthesized, iNOS is responsible for a prolonged and concentrated output of NO.[19] A number of studies on the effect of exercise on immune functions have been reported.[20] According to previous studies, acute exercise enhanced the phagocytic effects and enzymatic activities of human macrophages, and people who maintained physical fitness were healthier and less likely to die from

Accepted : August 6, 2002

§ To whom corresspondence should be addressed.

cancer.[21-22] Some recent research has focused on whether exercise suppresses the generation of tumors, by relating various immune phenomena with exercise. Although the interrelationship between exercise and tumors is not clear, there is increasing evidence that the improvement of a host"s immune functions by exercise can be a key component of cancer prevention.[23-24] In macrophages and lymphocytes, reactive oxygen, nitric oxide and cytokines are known to be important mediators of antitumor and tumoricidal activity.[25] Several empirical studies published between 1994 and 2000 have examined physical exercise and immune system function in cancer survivors. Overall, four out of six studies reported statistically significant improvements in a number of cancer-related immune system components as a result of exercise.[26]

In the present study, we investigated changes in nitric oxide production and iNOS expression by macrophages in mice subjected to various exercise intensities. Woods et al reported that corticosterone, one of the anti-inflammatory hormones, was elevated immediately after exercise and recovered to a standard level after 8h.[27-28] We therefore compared in the present study the levels of NO produced by mouse peritoneal macrophages with the levels of iNOS expression, both immediately after exercise and after 6h of recovery.

## MATERIALS AND METHODS

### 1. Reagents

RPMI medium 1640, lipopolysaccharide (LPS), HEPES, Tris, glycine, sodium hydroxide, gelatin, phenazine methosulphate, nitroblue tetrazolium, and sodium acetate anhydrous were purchased from the Sigma Chemical Co., St. Louis, USA. Sodium deoxycholate, ethylenediaminetetra-acetic acid (EDTA), potassium phosphate monobasic, potassium phosphate dibasic and sodium chloride were obtained from the E. Merk Co. Murine recombinant interferon-γ(IFN-γ) was purchased from the Genzyme Co, and fetal bovine serum (FBS) was purchased from GIBCO BRO (Gaithersburg, MD) or from Hyclone (Logan, UT). Anti- mouse iNOS monoclonal antibody was supplied by Merck Research Laboratories.

### 2. Animals

Four-week-old ICR male mice (n=50) were purchased from the KFDA (Korean Food and Drug Administration) and were kept under standardized animal housing conditions (temperature 22±2 ℃; photoperiod approximately 12 hours of light and 12 hours of dark daily; and relative humidity at 50-60 %). Pelleted food and tap water were available *ad libitum*. Prior to the experiment, two weeks were allowed for the mice to become acclimatized to animal house conditions and daily handling.

### 3. Exercise program

Our exercise protocol consisted of treadmill running. We chose this method of exercise because exercise intensity and duration can be experimentally manipulated and quantified (unlike voluntary wheels or swimming). Fifty mice were subjected to a treadmill exercise (18m/min, 30min/ day, 5% grade) for 6 days, and the final 32 mice were randomly selected. The control mice were exposed to handling, noise, and an environment identical to those of the exercising animals. Mice exercised moderately (18m/ min, 30 min/day, 5% grade) or severely (18-35 m/min, 60 min/day, 5% grade) on a motor-driven treadmill for 3 consecutive days without negative reinforcement by electrical shock. Woods et al reported that plasma corticosterone was significantly elevated (by three times) immediately post-exercise in mice exercised moderately or to exhaustion, compared with control mice, and recovered up to standard levels 8h after exercise.[27-28] Rosa suggested that glucocorticoid had an anti- inflammatory action through inhibition of iNOS induction in vitro.[29] The amounts of NO generated by abdominal macrophages were measured after exercise in mice. Thirty-two mice were inflammatory challenged with thioglycollate broth to activate peritoneal macrophages and were then randomly assigned to control, exercise or recovery groups.

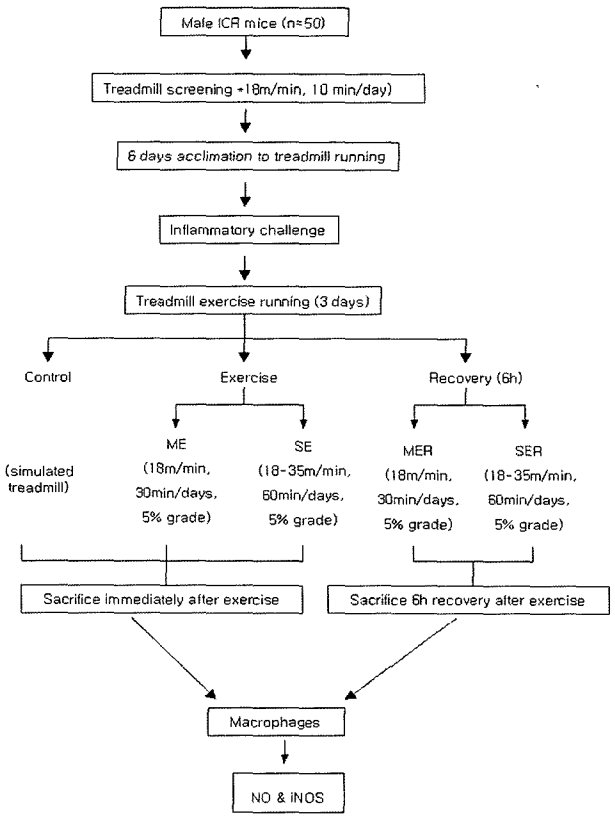


**Fig 1.** Experimental design

### 4. Preparation of peritoneal macrophages and cell cultures

Thioglycollate was intraperitoneally injected into the ICR mice at a rate of 2 ml of 4 % thioglycollate per 30g of body weight. Macrophages were collected from peritoneal lavages of the mice 3 days after injection, washed twice with cold RPMI medium 1640 (L-glutamine, 25 mM HEPES buffer, and sodium bicarbonate), and resuspended in RPMI containing 10 % FBS. Macrophages in the suspension were stained with trypan blue, their numbers counted by using a microscope, and then they were diluted to $1\times10^6$ cells/ml with the same medium. The diluted macrophages were seeded into 96-well culture plates (Corning, USA, 1ml/well) and incubated at 37℃ under 5 % $CO_2$. After pre-incubation for 2h, the medium was replaced with fresh medium to remove the non-adherent cells.

### 5. Cell viability and nitrite assay

Cell viability was assessed using a MTT assay. 10ul MTT solution (5mg/ml of phosphate buffered saline) was added to the cell culture plate and further incubated for 5 h at 37℃. After aspirating the supernatant from the wells, 200ul of SDS (0.1N HCl) was added for the dissolution of formazan crystals. The optical densities (OD) of the samples were monitored by an ELISA reader at a wavelength of 590 nm. MTT (tetrazolium salt) is cleaved only by metabolically active cells and is reduced to a colored formazan, with the color (OD value) reflecting cell viability quantitatively. For the measurement of NO in the culture supernatant, macrophages were plated in a 96-well tissue culture plate in 100ul, and treated with various concentrations of LPS and INF-γ. After incubation for 10h, 50ul of culture supernatant was taken and mixed with an equal volume of Griess reagent (1% sulfanilamide in 5% phosphoric acid + 1% α-naphthylamine in distilled water) at room temperature for 10 min. The absorbance was evaluated with a ELISA reader (Bio-Tek instruments Co.) at 590 nm. The level of nitrite reflects NO synthesis. Nitrite concentration was determined by using sodium nitrite as a standard.

### 6. Western blot analysis of iNOS

Western blot analysis was performed for the estimation of iNOS. Macrophages were plated in 10cm tissue culture plates in 10 ml of 10 FBS RPMI-1640 ($2\times10^6$ cells/ml), and treated with various concentrations of LPS and INF-γ. After incubation for 10h, cells were washed with PBS (pH 7.2), lysed with lysis buffer and briefly sonicated. Cell lysates were centrifuged (10,000 rpm, for 30min, at 4℃), and the supernatants were obtained. 30ug of protein samples from the cell supernatant were separated by 7.5% SDS-PAGE and blotted onto nitrocellulose membranes (Hybond-C super, Amersham, UK). The membrane was blocked for 15 min at 4℃ with TBS of pH 7.5 (25 mM Tris and 150mM NaCl) containing 5% Carnation instant skim milk and 0.05% Tween 20. After the membrane was washed three times in blocking buffer containing 0.2% Tween 20, it was incubated with iNOS antibody, and bands were then revealed using an Enhanced Chemiluminescence (ECL) detection reagent (Amersham, Les Ulis, France) with exposure to Hyperfilm at ambient temperature.

### 7. Statistics

All values are expressed as mean ± SD from 6 observations. The Student's t-tests for unpaired observations between the control and experimental samples were carried out for statistical evaluation of the differences. The level of significance for all statistical tests was set at a probability of 0.05.

## RESULTS AND DISCUSSION

### Effects of exercise on NO production

Nitric oxide is a key mediator in various physiological and pathological processes.[17] We investigated the effect of exercise on the production of NO by IFN-γ/LPS-stimulated mouse peritoneal macrophages. We found that exercise training increased macrophage NO production (as measured by the metabolite nitrite). Nitrite accumulation in the culture medium of mouse peritoneal macrophages was measured at 10h after the stimulation, with a combination of various concentrations of IFN-γ and LPS (Fig 2). The exercised mice (ME, SE) showed significantly higher NO generation than that of the control, but there were no significant differences in NO generation between the groups subjected to various intensities of exercise (Fig 3). It seems that the higher intensity of exercise didn't give any additional stimulus to enhance immune functions in the mice. Woods et al de-

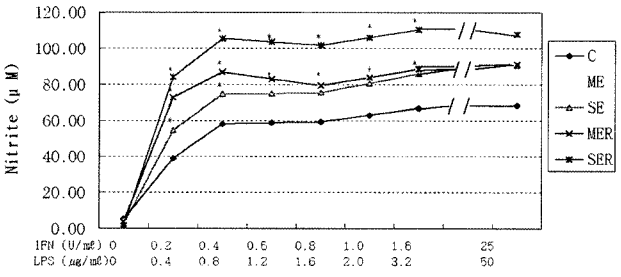


**Fig 2.** Effect of exercise on nitrite generation in peritoneal macrophages activated by various concentrations of LPS and INF-γ

* Denotes both groups are significantly different from control group ($p<0.05$)

C : control, ME : Moderate exercise, SE : Severe exercise,
MER : Moderate exercise and recovery (6h),
SER : Severe exercise and recovery (6h)

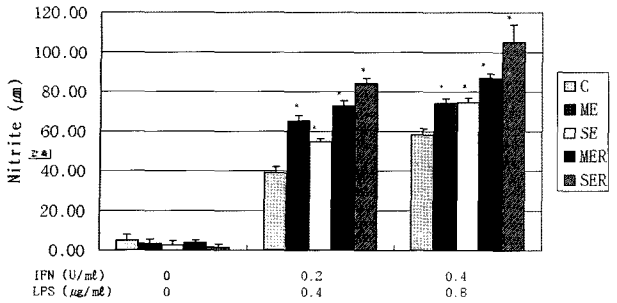


**Fig 3.** Effect of exercise on nitrite generation in peritoneal macrophages

* Denotes values are significantly different from control group ($p<0.05$)

C : control, ME : Moderate exercise, SE : Severe exercise, MER : Moderate exercise and recovery (6h), SER : Severe exercise and recovery (6h)

monstrated that exercise could enhance the ability of inflammatory macrophages to inhibit tumor cell growth *in vitro* (macrohage cytotoxicity), by reporting that NG-monomethyl-L-arginine (NMMA), one of the NO inhibitors, lowered the cytotoxicic effect of exercised mice macrohages.[27] Later, Woods et al demonstrated that moderate exercise may enhance, whereas very heavy exercise or a lack of exercise may attenuate, the immune response. Woods et al also reported that macrophages activated by exercise killed the tumor cells by inhibiting the DNA of the tumor cells, and also the activated macrophages produced more IL-2 and TNF-α than that of the control.[28] These results are similar to the results of our present study which indicate that moderate exercise may enhance the natural immunity by strengthening the cytotoxicity of macrophages. There was a tendency for exercised mice to produce significantly more nitrite when they were stimulated with a low concentration of IFN-γ/LPS (IFN-γ : 0.4U/ml, LPS : 0.8ug/ml) than that of control. However, there were no significant differences when they were stimulated with a high concentration (IFN-γ : 25U/ml, LPS : 50ug/ml) (fig 2).

Groups allowed six hours of recovery after exercise produced more NO than that of groups sacrificed immediately after exercise, but this difference was not significant. It was reported by Woods et al that the plasma corticosterone was significantly elevated immediately post-xercise in moderately or severely exercised mice, but there was no evidence reported for an immuno-uppressive effect of corticosterone on macrophage cytotoxicity, perhaps because of an insensitivity of inflammatory macrophages to glucocorticoid suppression seen in vitro. They concluded that moderate exercise may enhance, whereas very heavyexercise or a lack of exercise may attenuate, the immune response, from the result.[27] In addition, Woods et al reported that moderate exercise can increase the phagocytic capacity of intra-tumoral phagocytic cells, but these changes had no apparent effect on tumor incidence or progression in the study.[28] Rosa suggested that the anti-inflammatory and immunosuppressive actions of glucocorticoids are due to their inhibition of the induction of NOS.[29] Many studies on the cytotoxic effects of glucocorticoids have been performed, but the effects were not consistent. Probably, these inconsistencies are due to several limitations such as the nature of the samples, experimental designs, physical exercise interventions, and immunologic assessments.[26] Cell viability was measured by MTT assay, and more than 80% of the cells survived.

### Effect of exercise on iNOS protein expression

Fig 4 shows the effect of exercise on IFN-γ/LPS-nduced production of iNOS protein as measured by western blotting. Exercised mice peritoneal macrophages were collected and activated with LPS (50 ng/ml) and IFN-γ (25 U/ml) for 10h. The expression of iNOS in the exercised group was increased, and this was very extinctive in six hours of recovery groups. However, there was any significant differences in iNOS protein expression between the groups with differing intensities of exercise. The results of the iNOS protein expression measurements are similar to the measurements of NO production levels.

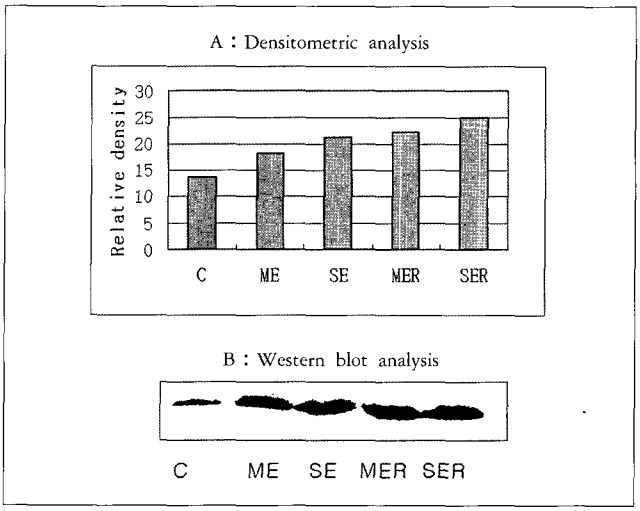


**Fig 4. Effect of exercise on iNOS expression in macrophages**

C : control, ME : Moderate exercise, SE : Severe exercise, MER : Moderate exercise and recovery (6h), SER : Severe exercise and recovery (6h)

## CONCLUSION

With regard to acute exercise effects on the immune response, it has been shown that natural immunity is enhanced during moderate exercise. However, it has been reported that the numbers and functions of cells medi-

ating cytotoxic activity against virus-infected and tumor target cells are suppressed after intense, long-term, exercise.[30-33] In accordance with immune surveillance theory, it is therefore to be expected that moderate exercise protects against malignancy, whereas exhaustive exercise is linked to increased cancer risk. To date there are only limited data to support this theory.[34]

In the present study, exercised mice showed higher NO generation than that of the control, but there were no significant differences in NO generation according to differing intensities of exercise. Mice allowed six hours of recovery after exercise resulted in greater levels of NO generation compared to animals sacrificed immediately after exercise. The expressions of iNOS in the exercised group were increased, and became more distinct, following six hours of recovery. However, there were no significant differences in iNOS expression with variations in the intensity of exercise. The results of this study suggest that acute exercise may enhance the immune response by inducing macrophage-derived NO generation; these results support epidemiological findings of the benefits of exercise in the prevention and control of cancer. Further study is needed to determine the physiological significance of these findings which could be applied in therapeutic exercises for the prevention and control of cancer.

## Literature Cited


1) Blair SN, Kohl H, Paffenbarger RS, Clark DG, Cooper KH and Gibbon LW. Physical fitness and all cause mortality : a prospective study of healthy men and women. *JAMA.* 262 : 2395-2401, 1989
2) Sternfeld B. Cancer and the protective effect of physical activity : the epidemiological evidence. *Med. Sci. Sports Exerc.* 24 : 1195-1209, 1992
3) Albanes D, Blair S and Taylor PR. Physical activity and risk of cancer in the NHANES I population. *Am. J. Public Health.* 79 : 744-750, 1989
4) Frisch RE, Wyshak G, Albright NL, Albright TE and Schiff I. Lower prevalence of non-reproductive system cancers among female former college athletes. *Med. Sci. Sports Exerc.* 21 : 250-253, 1989
5) Severson RK, Nomura AMY and Stemmermann GN. A prospective analysis of physical activity and cancer. *Am. J. Epidemiol.* 130 : 522-529, 1989
6) Cohen LA, Choi K and Wang CX. Influence of dietary fat, caloric restriction, and voluntary exercise on N-nitrosomethylurea-induced mammary turmorigenesis in rats. *Cancer Res.* 48 : 4276-4283, 1988
7) Deuster PA, Morrison SD and Ortega E. Exercise modifies cachexia of tumor growth in rats. *Med. Sci. Sports Exerc.* 17 : 385-392, 1985
8) Hoffman SA, Paschkis DKE, Debias DA, Cantarow A and Williams TL. The influence of exercise on the growth of transplanted rat tumors. *Cancer Res.* 22 : 597-599, 1962
9) Rusch HP and Kline BE. The effect of exercise on the growth of a mouse tumor. *Cancer Res.* 4 : 116-118, 1944
10) Uhlenbruck G and Order U. Can endurance sports stimulate immune mechanisms against cancer and metastases? *Int. J. Sports Med.* 12(suppl.1) : S53-S68, 1991
11) Thompson HJ, Ronan AM, Ritacco KA, Tagliaferro AR and Meeker LD. Effects of exercise on the induction of mammary carcinogenesis. *Cancer Res.* 45:2720-2723, 1988
12) Barlozzari T, Leonhardt J, Wiltrout R, Herberman B and Reynolds CW. Direct evidence for the role of LGL in the inhibition of experimental tumor metastases. *J. Immunol.* 134 : 2783-2789, 1985
13) Herberman RB. Natural killer cells. *Annu. Rev. Med.* 37 : 347-352, 1986
14) Cameron DJ and Stromberg BV. The ability of macrophages from head and neck cancer patients to kill tumer cells. *Cancer* 54 : 2403- 2406, 1984
15) Nakahashi H, Yasumoto K, Nagashima A, Yaita H, Takeo S, Motohiro A, Furukawa T, Inokuchi K and Nomoto K. Antitumor activity of macrophages in lung cancer patients with special reference to location of macrophages. *Cancer Res.* 44 : 5906-5910, 1984
16) Nathan CF. Nitric oxide as a secretory product in mammalian cells. *FASEB J.* 6 : 3051-3064, 1992
17) Porsti I and Paakkari I. Nitric oxide-based possibilities for pharmacotherapy. *Ann Med* 27:407-20, 1995
18) MacMicking J, Xie QW and Nathan C. Nitric oxide and macrophage function. *Annu Rev Immunol* 15:323-50, 1997
19) Maeda H and Akaike T. Nitric oxide and oxygen radicals in infection, inflammation, and cancer. *Biochemistry* (Moscow) 63:854-65, 1998
20) Pedersen BK, Rohde T and Qstrowski K. Recovery of the immune system after exercise. *Acta Physiol Scand* 172, 325-332, 1998
21) Fehr HG, Lotzerich H and Michna H. Human macrophage function and physical exercise : phagocytic and histochemical studies. *Eur. J. Appl. Physiol.* 58 : 613-617, 1989
22) Uhlenbruck G and Order U. Can endurance sports stimulate immune mechanisms against cancer and metastases? *Int. J. Sports Med.* 12 (suppl.1) : S53-S68, 1991
23) Thompson HJ, Wdsterlind KC, Snedden J, Briggs S and Singh M. Exercise intensity dependent inhibition of 1-methyl-1-itrosourea induced mammary carcinogenesis in female F-344 rats. *Carcinogenesis* 17, 1783-1786, 1995
24) Davis JM, Kohut ML, Jackson DA, Colbert LH, Mayer EP and Ghaffar A. Exercise effects on lung tumor metastases and in vitro alveolar macrophage antitumor cytotoxicity. *Am J Physiol.* 274 : 1454-459, 1998
25) Woods JA, Davis JM, Smith JA and Nieman DC. Exercise and cellular innate immune function. *Med Sci Sports Exerc* 31 : 57-66. 1999

26) Fairey AS, Courneya KS, Field CJ and Mackey JR. Physical exercise and immune system function in cancer survivors : a comprehensive review and future directions. *Cancer* 15:94( 2) : 539-51, 2002
27) Woods JA, Davis JM, Mayer EP, Ghaffar A and Pate RR. Exercise increase inflammatory macrophage cytotoxicity. *J.Appl. Physiol.* 75 : 79-866, 1993
28) Woods JA, Davis JM, Kohut ML, Ghaffar A, Mayer EP and Pate RR. Effects of exercise on the immune response to cancer. *Med. Sci. Sports. Exerc.* 26 : 1109-1115, 1994
29) Rosa DM, Radomski M, Carnuccio M and Moncada S. Glucocorticoids inhibit the induction of nitric oxide synthase in macrophages. *Biochem. Biophys. Res. Commun.* 172 : 1246-1252, 1990
30) Bourque SP, Pate RR and Branch JD. Twelve weeks of endurance exercise training does not affect iron status measures in women. *J Am Diet Assoc* 97 : 1116-1121, 1997
31) Fitzgerald L. Exercise and the immune system. *Immunol Today* 9:337-339, 1988
32) Hoffman-Goets L and Pedersen BK. Exercise and the immune system : a model of the stress response? *Immunol Today* 15 : 382-387, 1994
33) Pedersen Bk and Ullum H. NK cell response to physical activity : possible mechanisms of action. *Med Sci Sports Exercise* 27:140-146, 1994
34) Zahm S, Hoffman-Goetz L, Dosemci M, kantor KP and Blair A. Occupational physical activity and Non-Hodgkin"s lymphoma. *Med Sci Sports Exercise* 31:566-571, 1999