Moderate Exercise and Immune Response in Rats: A Model for Cancer Prevention

Exercício Moderado e Resposta Imune em Ratos: Um Modelo para a Prevenção do Câncer

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ABSTRACT
Exercise is believed to cause changes in population and/or activity of immunocompetent cells. Study of this relationship is difficult in humans because of the multifactorial nature of human physiology and concomitant problems in controlling extraneous variables. We
have investigated the effects of 4 weeks of moderate exercise training on percentage of NK cells and Lymphocyte activity in Wistar male rats under tightly controlled laboratory conditions. The animals were randomized into two groups: exercise and control. Exercised rats ran at a treadmill speed of 20 m/min (moderate exercise intensity), for 20 minutes, 6 days/week. Laboratory and handling conditions were matched, and food was controlled for both groups. Twenty-four hours after the exercise training program, lymphocyte response did not change significantly (p>0.05). Exercised rats demonstrated a significant increase in the percentage of circulating NK cell population (p<0.05). Exercise training demonstrated a significantly lower body weight for the exercised rats (p<0.05). With this simple model, this study supports the concept that regular moderate exercise leads to beneficial changes in the immunological response that may prevent cancer.

**Keywords:** Exercise, Wistar rats, Immune response, Cancer.

### 1 INTRODUCTION

Exercise is known to be beneficial to health and a series of studies have reported a protective effect of exercise against metabolic diseases (1), cancer (2) and mental disorders (3), leading some researchers to propose that exercise should be given greater consideration as a mode of therapy (4). Exercise-induced anti-immunosenescence effects might reduce the risk of developing cancer and perhaps benefit patients undergoing cancer therapy (5). Also, exercise may improve immune competency across the lifespan (6).
Exercise is known to cause changes in population of immunological cells and their activity (7) (8). Exercise is thought to have a dual effect on physiological systems depending on its intensity and duration. In addition, the deleterious or beneficial effects of exercise on physiological responses are also associated with a single strenuous exercise event or with training programs. Strenuous exercise is believed to produce an immunosuppressive effect in humans (9) and, in animals (10). On the other hand, regular moderate exercise has been shown to have beneficial effects in animals by enhancing immune function (10). The effects of moderate exercise in humans is shown by improving immune function and increased resistance to infectious diseases (11), prevention and treatment of cancer (12), AIDS (13) and, nowadays exercise has been considered as a tool to help the immune system against Severe Acute Respiratory Syndrome – Coronavirus 2 (SARS-COVID-19) infection (14) (15).

Many interesting findings have been published but interpretation remains conflicting on the immune effects of exercise in human and experimental animal studies. We believe that much of this conflict may be due to the lack of control of the many confounding factors such as exercise type and intensity, lifestyle, diet, stress, environment, the heterogeneity between subjects and species, the complexity of interaction of the physiological systems and, differences in fundamental methodology. All of these may lead to variable outcomes if not controlled in experimental investigations.

There are few reports in the literature that have analyzed the physiological adaptations such as NK cell percentage and lymphocyte response to chronic exercise under conditions of good internal control.

The objective of this work was to analyze immune response, following 4 weeks of regular moderate running exercise in 8 weeks old Wistar male rats, under controlled laboratory conditions. Blood lactate concentrations were first analyzed to give a measure of metabolic intensity of physical activity for male rats.

2 MATERIAL AND METHODS

Animals. A total of sixteen, 8 weeks old SPF (Specific Pathogenic Free) Wistar male rats were used. The animal room environment was controlled at a temperature of 20±2°C, a humidity of 50% and 12 hours light-dark cycle.

Determination of Exercise Intensity. Moderate intensity exercise was determined from blood lactate concentrations measured at different speeds. Only four rats
were randomly selected for determination of exercise intensity. These rats were housed in standard rat cages containing hardwood shavings. After being accustomed to the exercise treadmill (Motor Driven Animal Treadmill, Department of Medical Physics and Clinical Engineering, Royal Hallamshire Hospital, UK), rats were able to run up to 4 different speeds of 15, 20, 25 and 28 m/min at 0% gradient. One blood sample of 100µl was taken by direct venepuncture of tail vein using a disposable syringe and needle at rest (day 1) and immediately after each running speed. Rats ran one single speed/day for a period of 5 min until the incremental speed of 28 min/m (day 5). Blood lactate concentrations were analyzed by enzymatic procedure (YSI Model 23L Lactate Analyzer - Yellow Springs Instruments CO., USA).

**Exercise Program.** An independent group of twelve, 8 weeks old Wistar male rats were randomly divided into 2 groups of 6 rats each: One control (or sedentary) group and one exercise group. Laboratory and handling conditions were matched for both groups of rats. To reduce stress, the exercise group was accustomed to the exercise treadmill in the first week of training, running up to 10 minutes/day at speed of 20 m/min. Rats were then subjected to run at moderate exercise intensity of 20 m/min, 20 min/day, 6 day/week over a period of four weeks. Approximately 24 hours after the last exercise session, rats (exercise and control groups) were sacrificed by brain concussion. Blood samples were taken by direct cardiac puncture for counting the percentage of NK cells population and, spleen was removed for analysis of lymphocyte proliferative response.

**Food Intake and Body Weight.** Exercise and control rats were housed singly in standard cages. Water was given ad libitum. In the first week, food intake was calculated as the weight of food given to the animals minus food spilt (dried) minus food left. Food was then equally restricted for all rats at mean plus 1 standard deviation value. Body weight was measured daily.

**Lymphocyte Preparation** - Lymphocytes were first isolated from spleen for later analysis of mitogenic response. Under sterile conditions, each spleen was teased apart in PBS (Phosphate Buffered Saline). The solution formed was put on the top of lymphoprep (solution for lymphocyte preparation) and the resulting suspension was centrifuged at 2100 rpm for 30 minutes at 18°C. The white cells band at the interface was collected and made up to 100 ml with cold PBS. The mixture was spun at 2100 rpm for 15 minutes at 4°C. The pellet was washed twice with cold cytomedium and centrifuged at 2100 rpm for
15 minutes at 4°C. After the final wash, the cells were suspended in 10 ml of cytomedium. Viable cell counts were determined by trypan blue exclusion.

**Mitogenic-stimulated Cell Proliferation and [³H]-Thymidine Uptake.** The mitogenic response of lymphocytes was determined using three different mitogen doses. One hundred µl of lymphocytes were re suspended in RPMI 1640 at a concentration of 5x10⁴ cells/ml and placed in Costar 96 well round bottomed plates. Fifty µl of cytomedium with PHA (phytohaemagglutinin) in stimulated wells at concentrations of 0 (without PHA = control wells), 2.5, 5 and 10 µg/ml was added in each well and incubated for 72 hours at 37°C in a humidified, 5% CO₂ in air atmosphere. After incubation, 50 µl of 0.5 µCi of [³H]-Thymidine were added in each well and incubated for an additional 4 hours. After this, cells were harvested onto filter mats (Skatron Ltd, UK) using an automated cell harvester (Skatron Ltd) and washed and dried. Incorporation of [³H]-Thymidine was measured by counting the radioactivity on filter mats in 1.5 ml scintillation fluid using a beta spectrophotometer (Camberra Ltd). Counts were for one minute per sample. Each culture was performed in triplicate. Each value given is the mean of these three counts. Lymphocyte response was demonstrated by the proliferation index, which is given by the maximum response in PHA, stimulated wells divided by the control wells.

**NK Cells Count.** NK Cells phenotyping was accomplished by direct immunofluorescence labeling of cell surface antigens with mouse anti-rat monoclonal antibody for NK cells or mouse IgG1 negative control FITC conjugate. Fifty µl of blood was added to 3 µl of monoclonal antibody in centrifuge tubes. The tubes were incubated at room temperature for 15 minutes in the dark. Lyse of the red cells was performed as 2 ml of FACScan lyse solution were added to each tube, mixed and incubated for 10 minutes at room temperature in the dark. The cells were washed twice, firstly spun at 1200 rpm for 15 minutes and secondly spun at 1800 rpm for 3 minutes. The supernatant was discarded and 200 µl of paraformaldehyde was added to each tube and mixed to be ready for reading on the FACScan. Cells were analyzed using a FACScan (Becton Dickinson Ltd) with accessory computer (Hewlett Packard Ltd) and Consort 30 software. Ten thousand cells were included in each measurement. Light scatter gates were set to exclude granulocytes and debris. NK cells were sorted into CD16 and CD56 +ve and -ve populations respectively.
Statistical Analysis. For the immunological analysis the mean and 95% Confidence Interval (95% CI) were calculated. The mean and Standard Error of Mean (SEM) were calculated for food intake and body weight analysis. Comparisons between exercise and control groups were assessed using unpaired t-test. Repeated measures analysis of variance was used for comparisons between blood lactate concentrations at different speeds.

Ethical Issue. This work was undertaken with the permission of the British Home Office.

3 RESULTS

Blood Lactate Concentrations and Running Speeds. Figure 1 illustrates the relationship between blood lactate concentrations at rest and at 4 different running speeds. Repeated measures analysis of variance demonstrated that blood lactate concentrations did not increase significantly at the speeds of 15 and 20 m/min. However, blood lactate concentration increased significantly at the speed of 25 m/min, which corresponds to the lactate concentration of 2.6 mmol/l. The speed of 20 m/min corresponds to an intermediate value of blood lactate concentration of 2.5 mmol/l and therefore this was chosen as a moderate intensity running speed for 8-week-old Wistar male rats.

Figure 1. Blood lactate concentrations at rest and at different running speeds. Values expressed as mean ± 1 semi 95% CI. Significantly different from rest value, * (p<0.05), ** (p<0.01). Each speed = 5 min duration. N=4.

Food Intake and Body Weight. As food was first measured on Week 1, from the second week was offered a daily amount of 25.5 g of food (mean daily food intake of 21.9 ± 3.6 g). A daily increment of 5 g of food was added to each rat to maintain rat’s
normal growth. The relationship between food intake and body weight of exercise and control groups of rats is illustrated in Table 1. There was no significant difference in total food intake between exercise and control group of rats from week 1 to week 4, though exercise rats consumed slightly less food than control ones. At the beginning of the exercise training, no significant difference was found in body weight between exercise and control rats. However, at the end of the training program (week 4) the exercised rats had decreased their body weight significantly when compared to the controls.

Table 1. Food Intake and Body Weight to exercise and control groups of rats. Values expressed as Mean (±SEM). No significant difference between groups ns (p>0.05); significant difference between groups * (p<0.05). N=6.

<table>
<thead>
<tr>
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<th>Week 1</th>
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<tr>
<td></td>
<td>Exercise</td>
<td>Control</td>
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<tr>
<td>Food Intake</td>
<td>152 (4.8)</td>
<td>153 (3.4)</td>
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<tr>
<td>Body Weight</td>
<td>206 (5.1)</td>
<td>214 (2.2)</td>
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**Exercise and Immune Response.** Figure 2 illustrates basal of lymphocyte response and percentage of NK cells circulating in the blood in exercised and control group of rats. Lymphocyte function is demonstrated as a proliferation index of $[^{3}\text{H}]$-Thymidine uptake. Although lymphocyte proliferative activity was slightly increased in the exercised group, it did not support a significant difference when compared to control rats at a PHA concentration of 2.5 µg/ml used. In contrast to the lymphocyte response, there was a significant increase in the percentage of circulating NK cells in the exercise group when compared to the control.

Figure 2. Exercise and immune response. Values expressed as mean ± 1 semi 95% CI. Lymphocyte proliferation index. Exercise group ■; control group □. PHA dose-response relationship, no significant difference between groups ns (p>0.05). N=6. Percentage of NK cells, significant difference between groups * (p<0.05). N=5 (1 sample lost due to processing error).
4 DISCUSSION

In this study we have investigated changes in immune response such as the percentage of NK cell and lymphocyte activity to 4 weeks of moderate intensity treadmill running in 8 weeks old Wistar male rats. Eating habits and body weight were also analysed. Determination of moderate exercise intensity is first illustrated and discussed because it was used as the basis for our study.

**Determination of Moderate Exercise Intensity.** The results clearly demonstrate that, in rats running on the exercise treadmill, blood lactate concentration increased with increasing running speed. Blood lactate concentration was related to exercise intensity in a manner similar to that for humans. It is widely agreed that moderate exercise intensity activity is represented by a lactate level in the range 2.0 to 4.0 mmol/l. Since in this experiment, lactate was sampled in the period immediately following exercise, when the lactate concentration in the blood will have been falling, it was not possible to obtain a precise value for the lactate concentration at the given intensity of exercise. Since the purpose of this experiment was to determine moderate intensity exercise for rats, the speed of 20 m/min was chosen as it corresponded to a lactate concentration of 2.5 mmol/l. This is below the upper "Anaerobic Threshold" of 4 mmol/l stated in humans (16) (17), yet is far enough above the resting lactate level to allow a training effect of the exercise program.

The mean blood lactate concentration at rest was 1.55 mmol/l (95% confidence interval 1.08 - 2.02). This value agrees with those reported by others (18) (19) (20). Above the speed of 15 m/min the lactate concentration increased approximately linearly with increasing running speed. In this experiment, it proved impossible to get the rats to sustain voluntary exercise levels above 28 m/min for periods of 5 minutes. Thus, this treadmill running speed and above was taken to represent high intensity activity for the rat. This observation is in broad agreement with the results reported by Kumagai and Nishizumi (21) and Nakagata et al. (22). We therefore suggest that moderate intensity exercise can be considered as the running speed of 20 m/min for 8 weeks old rats.

**Food Intake and Body Weight.** Food consumption gradually increased in the following weeks of training period for both exercise and control groups of rats. However, it was observed in this study that moderate exercise treadmill for male rats causes a slight but not significant decrease in food intake when compare to sedentary ones over a period of 4 weeks. This finding agrees with that reported by Holloszy (23) who observed that male rats did not increase their food intake to compensate for increased energy
expenditure caused by exercise and, with Foright et al. (24) who reported reduced food intake in 4 weeks of treadmill running in Wistar male rats.

Body weight comparisons between exercise and control animals showed that exercised and control rats weighed the same at the beginning of the exercise program. It was observed however that at the end of the exercise-training period, control sedentary rats weighed significantly more than the exercised rats. As food intake was equally restricted for both groups of rats, it may be suggested that body weight loss in exercised rats was due to the increased energy expenditure caused by treadmill running. This is in agreement with the studies of Boakes (25) and Kim et al. (26) who have demonstrated that voluntary running decreased body weight in food restricted rats.

In this study, four weeks of regular moderate exercise training did not show significant differences in eating habits between exercised and sedentary rats but a significant lower body weight in exercised rat was observed. This may indicate the important role that exercise plays in controlling body weight.

Exercise and the Immune Response. The immunological status of exercised and control rats was determined by the proliferative response of lymphocytes upon stimulation with the mitogen PHA in vitro and, also by the percentage of circulating NK cell population. This was based on the strong relationship between exercise and immune function (27) (28).

Firstly, a dose-response curve, using three different PHA concentrations of 2.5, 5, and 10 µg/ml were used to compare the maximal PHA responses by the lymphocytes. Lymphocyte proliferation index was greater at PHA concentration of 2.5 µg/ml than at concentrations of 5 and 10 µg/ml. As the maximum proliferation index was observed using PHA concentration of 2.5 µg/ml, this was considered to demonstrate lymphocyte activity in our study. Our results demonstrated lymphocyte proliferative response slightly increased in the exercised rats but did not show a significant difference between the groups. Possibly a larger sample size would demonstrate a beneficial trend of the effect of moderate exercise on the basal line of lymphocyte activity in rats.

Other studies have suggested that exhaustive exercise impairs lymphocyte proliferation in animals (29) and in humans (30). However, this concept has changed (31) (32) as intense exercise does not always cause immunosuppression. Regular moderate exercise has been reported to enhance lymphocyte proliferative response (33) (34). We believe this small increase in lymphocyte proliferative response in exercised rats was a beneficial effect of moderate exercise intensity in stimulating immune response since our
measurements were taken at basal levels (i.e. 24 h after training session) and not immediately or one or two hours after exercise, as it is commonly reported.

Data from the literature shows that lymphocyte proliferative response also depends on the mitogens used (PHA, ConA and IL-2) and PWM (Pokeweed Mitogen) (35). This may be one reason for conflicting findings between similar studies. Standardization on lymphocyte stimulation with mitogens and methodology has to be considered. Also, the relationship between exercise and lymphocyte function is yet to be fully understood. The time-course response to post exercise needs more attention for a better understanding of exercise and lymphocyte function.

In terms of NK cells, our results demonstrated that moderate exercise doubled the percentage of circulating NK cell population in exercise group of rats when compared to sedentary controls. This is accordance with the results of Kaufman et al. (36) who reported a 2.5 fold increase in NK cell concentration in moderately trained rats compared to controls after exhaustive exercise, and in fair agreement with Eliakim et al. (37) and Notbohm et al, (38) who reported a significant increase of NK cells number after aerobic exercise in humans.

Moderately exercised rats with doubling of the number of circulating NK cells represents an appreciable enhancement of the first-line defence cells. This is of particular interest as there have been many results that exercise may enhance resistance of experimental animals to tumour growth (39) (40) (41). Recently it has been reported that at least 5 weeks of exercise training are needed to alter the innate immune system, increasing NK cytotoxicity activity (32).

The NK cells are being projected as the main group of cells responsible for immune surveillance with the role of recognizing and destroying the first few neoplastic cells that may have the potential for developing into a malignant cancer. As NK Cells count for approximately 5% to 20% of peripheral blood mononuclear cells usually defined as CD16+ CD56+ CD3- cells (42) (43), it was convenient in this study to investigate lymphocytes activity as a general view of the effects of moderate exercise on immune response. NK Cells take part as a lymphocyte subpopulation with natural cytotoxicity for stressed, viral infected and tumour cells, responding in a non-specific manner. In addition, NK cells kill bacteria, fungi, and parasites (44).

Recently, NK Cells have been shown to be involved in recognizing and destroying SARS-COVID-19 infected cells (14), and to work as a non-pharmacological therapy for cancer patients performing moderate exercise training (45).
These cells are responsive to the physiological alterations of physical exercise. The mechanism of the increased percentage of NK cells induced by exercise is not completely understood. It is suggested that lymphocytes may be redistributed by the increased cardiac output which mobilizes peripheral blood flow and lymphocytes homing at lymphoid organs and, by increased concentration of catecholamines which may also change expression of adhesion molecules (46) and cell mobilization (47). Data available in the literature about the effects of moderate exercise on the hormones associated with immunomodulation are still controversial. This suggests that the relationship between exercise and concentrations of epinephrine, glucocorticoids, serotonin, dopamine, epinephrine (48), cortisol (27) and, other hormones needs further investigation in well-controlled studies.

Therapies have been demonstrating some promising results as NK cells have been shown to fight against tumour (49). A review study reports the evidence for NK cells’ critical role in combating transformed and malignant cells, and how cancer immunotherapies potentiate NK cell responses for therapeutic purposes. This review also describes the challenges that NK cells face that must be overcome by therapeutic modalities to achieve cancer remission (50).

Idorn and Hojman (51) proposed that exercise may be a promising complementary anticancer therapeutic approach based on the effects on NK cell mobilization and activation as well as changes in blood perfusion and body core temperature reinforcing immune cells distribution and transmigrations into tumours. The influence of NK cells in the tumour microenvironment may open new promising avenues for the development of improved anticancer immunotherapeutic strategies (Stojanovic and Cerwenka, 52). On the other hand, NK cell may best be considered as a preventive therapy for cancer, since solid tumours when established may hinder the penetration of immune cells (53). Exercise increases peripheral blood increasing leukocytes extravasation to tumour while in a non-exercised condition, lower blood flow may not allow as many immune cells to enter the tumour microenvironment. This may explain some of the protective function of regular exercise (54).

Considering that cancer is a “wound that does not heal” (55) which is very well related to inflammation at the tumour site, exercise can also help in reducing inflammatory cells and humoral elements. This concept is in agreement with other authors (56) reporting an increasing NK and NKT cell expression of TNF-α, indicating that resistance training may be beneficial in improving the inflammatory profile in breast
cancer survivors and, exercise may be pursued as anticancer treatment through incorporation into standard oncological therapy to benefit cancer patients (12).

Further studies are needed to evaluate post-exercise serum cytokine concentrations of trained individuals after an acute bout of high intensity interval exercise to confirm whether this inflammatory response is exclusively observed in untrained individuals and to support the idea that inflammatory cytokines are required to establish normal angiogenesis (57).

As many modern approaches to cancer immunotherapy rely on cellular cytotoxicity for their effectiveness, unravelling these pathways will be important to further progress these therapeutic strategies (58) and cellular therapy (59). Exercise training have been successfully used as an adjuvant therapy for patients with some types of cancer such as breast (60) (61) (62), prostate cancer (63) (64), colon cancer (64) (65), haematological (66) (67), paediatric solid tumours (68), children and adolescent with leukaemia and solid tumours (69) and, in the prevention and survival of cancer (70).

The therapeutic method for exercise and cancer patients should be evaluated in terms of exercise intensity, duration, frequency, exercise programmes adherence, how long this therapy needs to be carried out related to the health condition of the patient and cancer stage and its side effects (71). Another recent study by Morris et al. (72) has demonstrated the importance of future research across all disciplinary areas of exercise oncology and identified the priority questions to which resources might be directed.

Physical exercise may also reduce the progressive effect of senescence and prevent or protect against some diseases such as cardiovascular disease, Alzheimer's disease, and diabetes in older individuals, certain type of cancers, and autoimmune disease (8). Although there is not yet clear consensus on the association between NK cell number and lymphocyte response at different levels of exercise, it is known that regular exercisers may benefit from a better quality of life and even be more protected against cancer, virus infected diseases and other non-communicable chronic diseases.

Limitations of this Work. One limitation of this study is that NK cells cytotoxicity has not been investigated, but it could provide important information in relation to NK cell numbers as well as to other lymphocyte subpopulations and their activity in further studies. Cytokines and other immunological parameters were also not evaluated, although this controlled animal study demonstrated beneficial influence on the immune system in a small sample size.
5 CONCLUSION

With this well-controlled animal model, it was possible to demonstrate beneficial effects of moderate exercise training on immune response. Moderate exercise training showed an increased, though not significant lymphocyte function demonstrating that moderate exercise did not cause any deleterious effects in lymphocyte response. Four weeks of moderate exercise training had a beneficial effect on circulating NK cells in rats, showing an increased number of these cells. In addition, body weight was slightly reduced by exercise. These are mechanisms whereby moderate intensity exercise confers general health benefit and may reduce the incidence of many common diseases, including cancers.

CONFLICT OF INTEREST

Authors declare that there is no conflict of interest.
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