Excessive physical exercise caused a decrease in CD4+ and Hematopoietic Stem Cell (HSC) levels in Wistar rats

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ABSTRACT

**Background:** Excessive physical exercise can cause injury and disrupt the body’s homeostasis. Excessive physical exercise also causes increasing oxygen demand and reactive oxygen species production (ROS) that induce damage in various cells and body tissue, including HSC and CD4+ cells. This study aims to prove that excessive physical exercise decreases the CD4+ and HSC levels in Wistar rats and evaluates the correlation between HSC and CD4+ cell levels.

**Methods:** An experimental study with a randomized post-test-only control group design was conducted among 44 male Wistar rats in Clinical Pathology Laboratory, Manggusa General Hospital, Bali, from June to September 2022. The samples were divided into two groups; 22 rats received excessive physical exercise (treatment group) and 22 rats as the control group. The CD4+ cells and HSC were examined after 14 days of intervention. Data regarding the means of CD4+ and HSC levels between the two groups were assessed, and the correlation between HSC and CD4+ cell levels was analyzed using SPSS version 21 for Windows.

**Results:** We used 22 rats for each treatment and control group. The mean level of CD4+ and HSC were significantly lower in the treatment group compared to the control group (356.41 vs. 661.41x10^4 cells/µl; p<0.001) and (359.14 vs. 551.09x10^4 cells/µl), respectively. We found a statistically significant difference in CD4+ and HSC levels between the two groups, p<0.001. We also found a positive correlation between HSC and CD4+ cell level with r=0.636; p<0.001. Excessive physical exercise affected the immune function by decreasing CD4+ ad HSC levels in Wistar rats.

**Conclusions:** The impact of excessive physical exercise on immune function showed by decreasing the level of CD4+ and HSC level in Wistar rats. The HSC number also has a positive correlation with CD4+ cell level.

**Keywords:** CD4+ Cell, Excessive Physical Exercise, Hematopoietic Stem Cell, Overtraining.


INTRODUCTION

Physical health can be achieved through balanced physical exercise, which, if done regularly, can benefit health. Physical activity following a healthy lifestyle is only carried out by 9.1% of people in the world, while the rest do a physical activity not following scientific principles. Both lack of and excessive physical exercise will cause an unbalanced hormone secretion that can damage the cell.1,2 Studies showed that regular and balanced physical exercise would reduce mortality rates three times lower than people who do not exercise regularly. Physical exercise can be a stressor that stimulates damage or injury to the muscle, resulting in muscle and connective tissue regeneration. Adaptation to these stressors will increase our functional ability. In contrast, excessive physical exercise will cause injury and disrupt the body’s homeostasis. Excessive physical exercise will cause overtraining syndrome, which is not good for health.3,4

Overtraining is a condition in which an exercise regime’s physiological demand outweighs the body’s ability to adjust to the demand. The consequences of overtraining are widespread, negatively affecting several physiological systems, including the neuroendocrine, immunological, cardiovascular, and musculoskeletal systems. Overtraining could also result in several negative psychological disturbances.4

The incidence of overtraining syndrome is 7-20% of trained athletes. Other data showed that 10-20% of all athletes involved in intensive sports training programs could develop the overtraining syndrome. More than two-thirds of athletes in athletic sports have experienced overtraining syndrome, with the highest risk found in runners, cyclists and swimmers. It is reported that 6% of long-distance runners, 21% of swimmers and more than 50% of Australian football players have been overtrained.4,5

Balanced physical training can increase the number of hematopoietic stem cells (HSC). In contrast, excessive physical training decreases the number of HSC in peripheral blood circulation and viral-specific T-cells lymphocytes. In contrast, excessive physical exercise has caused DNA damage to blood cells,
especially leukocytes and skeletal muscle cells, which induces apoptosis. Several studies also stated that excessive physical exercise could increase proinflammatory cytokine and reactive oxygen species in immunosuppression. Excessive physical exercise causes increasing oxygen demand and reactive oxygen species production (ROS) that induce damage in various cells and body's tissue, including HSC.

Exercise is also linked with the type or intensity-dependent adaptive immune responses, whereas hypoxic stress caused by excessive exercise facilitates the programmed death of CD4 lymphocytes. Weng TP et al., showed a significant decrease in the ratio of T cell leukocyte subtypes, the CD4+ and CD8+ cell levels, after 60 minutes on the treadmill. However, it was found several hours after exercise. The decrease in this ratio lasted for 6 hours after exercise. A decrease in the ratio of CD4+ and CD8+ cell levels indicate immunosuppression. Another study by Natale VM et al., conducted a study on athletes who participated in excessive physical training and found a decrease in the ratio of CD4+ and CD8+ cell levels after exercise at 1 week and 2 weeks. There were many cases of upper respiratory tract infections. Study regarding the impact of excessive physical exercise on HSC and CD4+ cell number is still limited. The study evaluated the correlation between HSC and CD4+ also has not been published yet. Based on those mentioned above, this study aims to determine the correlation between excessive physical exercise with decreasing CD4+ and hematopoietic stem cell numbers in Wistar rats.

METHOD

An experimental study with a randomized post-test-only control group design was conducted to determine the correlation between excessive physical exercise and with decreasing number of CD4+ and HSC in Wistar rats. The study was conducted in the Pharmacology Laboratory, Faculty of Medicine, Universitas Udayana, Bali, for rat observation. At the same time, CD4+ and hematopoietic stem cell counts were examined in Clinical Pathology Laboratory, Mangusada General Hospital, Bali. The study was conducted from June until September 2022.

The sample inclusion criteria were male Wistar rats aged 2.5-3 months and 200 grams weight that were kept in the Pharmacology Laboratory, Faculty of Medicine Udayana University. In contrast, the exclusion criteria were unhealthy rats. Rats that died during the study were categorized as dropout samples. The sample was determined using simple random sampling and divided into control and treatment groups. Sample sizes were calculated using the Federer formula with a total of 44 samples, with 22 Wistar rats as the sample for each group. The control group has not received any intervention, while the treatment group was treated with excessive physical exercise. All rats were adapted for seven days before being treated and placed in a cage with a size of 50x40x15 cm for each of them. On the 8th day, the treatment group received excessive physical exercise, defined as physical exercise in rats by 30 minutes of swimming activity in a bucket with 30 centimeters diameter and 40 centimeters water depth for 14 days regularly. The rat's tail was stimulated manually with determined time to make them keep moving. Signs of fatigue are indicated by the rats almost drowning due to decreased muscle strength, decreased reaction time and frequency of movement and also decreased reflexes. After 14 days of intervention, blood samples were collected intra-orbital from both groups for CD4+ and HSC examination using a vacuum K2-EDTA tube. The number of CD4+ cells was determined by expressing CD4+ and CD3+ using the flow cytometry method in FACS Canto. The number of HSC is defined as the number of endogenous stem cells, such as CD34-marked cells in peripheral blood, using the flow cytometry method on the FACS Canto. The type, age, weight, health condition, environmental condition (temperature, humidity, light), and food and water intake of the rats were controlled in this study. After the intervention, all the rats were euthanized and burned.

Data analysis was performed using the statistical software package SPSS version 21 for Windows. Descriptive analysis was performed to obtain each group's average amount of CD4+ and HSC. The Shapiro-Wilk test was done to determine the normality of data distribution. Levene's test was done to determine the data variance homogeneity. Data were expressed as Means±SD. An Independent sample t-test was done to compare the CD4 and HSC number between the control and treatment groups. To determine the correlation between CD4+ and HSC number Pearson test was used with criteria for significance p<0.05 and 95% Confidence Interval.

RESULTS

We used a total of 44 Wistar rats as a study subject, with every 22 rats for the control and treatment group. The level of CD4+ cells and HSC in both groups were normally distributed based on the normality test using Shapiro-Wilk (p>0.05). Based on Levene's homogeneity test, we found the CD4+ level was homogeneous but not with the HSC level. After 14 days of intervention, all Wistar rats were examined for their CD4+ cell and HSC level.

Based on the descriptive analysis, we found the mean level of CD4+ and HSC in the treatment group was lower than in the control group. The mean CD4+ number in the treatment and control groups were 356.41±157.24 x10⁶ cell/µl and 661.41±184.36x10⁶ cell/µl, respectively. At the same time, the HSC number between the treatment and control groups were 359.14±94.97x10⁶ cell/µl and 551.09±140.13x10⁶ cell/µl, respectively. An independent sample t-test showed a statistically significant difference between CD4+ and HSC levels in treatment and control groups with p <0.001 and <0.001, respectively, as shown in Table 1.

Based on correlation analysis using the Pearson test, we found a statistically significant positive correlation between HSC and CD4+ cell number with p<0.001 with Pearson correlation efficient (r) = 0.636. The positive correlation result means the HSC and CD4+ level was positively correlated, as shown in Table 2. Figure 1 below shows a linear relationship between HSC and CD4+ cell level based on the scatter plot diagram.

DISCUSSION

Our study evaluates the impact of excessive physical exercise in decreasing
Table 1. Mean comparison of CD4+ and HSC level between treatment and control group.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Treatment (Mean±SD)</th>
<th>Control (Mean±SD)</th>
<th>p</th>
<th>95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD4+ (10^6 cell/µl)</td>
<td>356.41±157.24</td>
<td>661.41±184.36</td>
<td>&lt;0.001</td>
<td>200.74-409.25</td>
</tr>
<tr>
<td>HSC (10^6 cell/µl)</td>
<td>359.14±94.97</td>
<td>551.09±140.13</td>
<td>&lt;0.001</td>
<td>119.11-264.79</td>
</tr>
</tbody>
</table>

Table 2. Correlation between HSC and CD4+ levels in Wistar rats.

<table>
<thead>
<tr>
<th>p-value</th>
<th>( r )</th>
</tr>
</thead>
<tbody>
<tr>
<td>HSC and CD4+</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>
been given pre-treatment in the form of caspase 3 and pan-caspase inhibitors. This indicates that oxidative stress triggers apoptosis via an independent pathway to caspase but depends on fluctuations in Bax protein levels.\textsuperscript{13,14,15} Wu G et al, in their study, proved that apoptosis due to excessive physical training involved an inflammation signaling pathway that is marked by increasing levels of TNF-α and NF-Kβ.\textsuperscript{16} Oxidative stress also decreases endogenous antioxidants such as glutathione (GSH), superoxide dismutase (SOD) and catalase. Oxidative stress can induce apoptotic cells that have been given pre-treatment in the form of caspase 3 and pan-caspase inhibitors. This indicates that oxidative stress triggers apoptosis via an independent pathway to caspase but depends on fluctuations in Bax protein levels.\textsuperscript{16} Ishihara Y and Shimamoto N explained that on stress-induced apoptotic events oxidative stress, mitochondria are the target of main damage caused by ROS.\textsuperscript{17} Damage to mitochondria permeability transition (MPT) can cause mitochondrial leakage and apoptotic effectors such as cytochrome C, which exits into the cytoplasm and induces acute apoptosis. In addition, ROS can activate the transcription of proteins involved in increased apoptosis and inhibit cell survival, such as nuclear factor kappa-B (NF-kB), activator protein-1 (AP-1) and p53.\textsuperscript{17}

We also found a positive correlation and linear relationship between the HSC level and CD4+ level. Our study found the power correlation was found to be 0.636 between HCS and CD4+ levels. The positive correlation and linear relationship mean when HCS levels are reduced, CD4 levels are also reduced. According to our results stated by Sellami M et al., the theory states that excessive physical exercise could cause stress to body homeostasis.\textsuperscript{8} The stress causes a decline in HSC and progenitor cell function, which results in increased production of myeloid cell lineage and a decrease in the lymphoid cell lineage. Lymphocytes come from lymphoid cell lineage, and CD4+ is a marker expressed in lymphocytes T cells. Thus, the decreasing number of HSC will affect the level of CD4+ too.\textsuperscript{18}

Excessive physical exercise is very influential to the immune system because it is extremely sensitive to stress, both physiological and psychological condition, and thus potentially could be used as a parameter of stress due to excessive physical exercise.\textsuperscript{19-21} The implication of our study result is that it can be beneficial to regularly do blood examinations in overtrained athletes or athletes who received intensive physical exercise to monitor the impact of excessive physical exercise on their health. The limitation of our study is the relatively limited sample size for each group, and we did not examine the baseline of HSC and CD4+ levels in the treatment group before the intervention.

CONCLUSION

The mean CD4+ and HSC levels in the treatment group were lower than in the control group. We found significantly different CD4+ and HSC levels between the treatment and control groups. We also found a positive correlation between HSC and CD4+ levels; if the HSC level decreases, the cd4 leavel will also decrease. Excessive physical exercise caused a decreasing level of CD4+ and HSC levels in Wistar rats.

CONFLICT OF INTEREST

The authors declare that there is no competing interest regarding the manuscript.

ETHICAL CONSIDERATION

This research was conducted based on the ethical conduct of research from the Ethics Committee of the Medical Faculty, Universitas Udayana, Prof I.G.N.G Ngoerah Hospital Denpasar (Number: 1662/UN14.2.2.VII/LT/2022) and have received permission from the Research and Development Unit (R & D) of Universitas Udayana, Prof I.G.N.G Ngoerah Hospital Denpasar.

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AUTHOR CONTRIBUTION

All of the authors contributed to the study from the conceptual framework, data gathering, and analysis until the study's results were interpreted upon publication.

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