ORIGINAL PAPER

Aerobic exercise training enhances cell proliferation and reduces IL-2 and IL-6 cytokine production in spleen of high fat diet-induced obese mice

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ABSTRACT

This study investigated the effect of aerobic exercise training on immune cell proliferation and inflammatory cytokine production in the spleen of high fat diet-induced obese mice. C57BL/6 male mice (4 weeks aged, n=20) were fed a high fat diet (45% fat) for 5 weeks so that obesity was led intentionally. Then, these obese mice were divided into 2 groups; control group (CON, n=10) or exercise group (EXE, n=10). EXE performed treadmill running for 30-60 min/day at 10-22 m/min, 0% grade, five times per week for 8 weeks. After 13 weeks, all the splenocyte was collected and Con A (Concanavalin A 10 µg/ml) was used to stimulate the cell proliferation. MTS and BIOPLEX assay were used for cell proliferation and cytokine production. Independent *t*-test was used and a *p*-value under 0.05 was considered as statistically significant. In the results, body weight, IL-2 and IL-6 production were significantly reduced and Splenocyte proliferation was significantly increased after 8 weeks of exercise training. These findings suggest that aerobic exercise training has a positive effect for improving the obese-induced immune dysfunction.

Keywords: obese, aerobic exercise training, cell proliferation, inflammatory cytokine

INTRODUCTION

Low-level physical activity or a life style including a high fat diet causes energy imbalance induced obesity [1]. Obesity is closely related to atherosclerosis, diabetes, fatty liver, infection, and cardiovascular and immunity diseased, including respiratory track inflammation [2,3]. These physiological phenomena cause chronic low graded inflammation, negatively impacting upon some immunity functions [4,5].

Amar *et al.* [6] reported that the resistant ability of high fat diet-induced obese mice against antigen was significantly lower than that of normal weight mice. Tanaka *et al.* [7], reported that the immune function of high fat diet-induced obese mice was decreased because the sensitivity of immune

cells against antigen had been deteriorated through obesity. Further, a few previous papers reported that the proliferation of B and T lymphocytes was reduced but, the immune function would be changed by exercise type, duration and intensity [8-10].

It was reported that Interleukin-2 (IL-2) produced by the spleen was known as a growth factor of T cells and was reduced by aging and hypokinetic [10]. It was reported that aerobic exercise increased IL-2 expression [11] but another study demonstrated that IL-2 was decreased after aerobic exercise [12]. It seems that IL-2 is closely related to aerobic exercise but the result was not coincidence. Further research will soon be required. It was known that Interleukin-1 beta (IL-1β). Tumor necrosis factor-α (TNF-α) and Interleukin-6

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(IL-6) had a role of signal messenger in the immune system. When these cytokines were excessively produced, it caused the imbalance between pro- and anti-inflammatory cytokines, and as a result, the immune function of the host was decreased [13].

It was known that Interferon gamma (IFN- γ) was a pro-inflammatory cytokine produced by Th1 and a modulator between immunity and inflammation. Also, it was non-specific to host defense at the early stage of immunity and anti-virus activity, and a strong macrophage activator inducing the cell-mediated immune mechanism [14].

As a review of prior studies relating to exercise and inflammatory cytokines, it was known that these immune factors were affected by the increment of sensitivity against infection [15], stress hormone and neuroendocrine [16,17]. However, the majority previous studies measured the changes after using normal mice or a bout of exercise, the results were not coincidence [13,18,19].

Further, it was reported that even though high intensity resistance exercise and high intensity physical activity reduced the proliferation of lymphocytes [8,9], the studies about cell proliferation including various exercise intensity and diet intervention were still insufficient.

It was reported that the combination of a low fat diet and moderate aerobic exercise reduced inflammatory cytokines expression in high fat diet-induced obese mice [20]. This suggests that the combination treatment might improve the deteriorative immune function caused by obesity. However, this result did not show the effect of exercise alone but the combination treatment. Thus, it is needed to understand the effect of aerobic exercise on immune dysfunction, immune cell proliferation, and cytokine production as a regulator of immune function caused by obesity. Hence, the purpose of this study was to investigate the effect of aerobic exercise on splenocyte proliferation and inflammatory cytokines in high fat diet-induced obese mice.

METHODS AND MATERIALS

Animals and diet

4-week-old male C57BL/6 (Central Experiment Animal, Korea, n=20) mice were housed in a cage in a standard experimental laboratory, illuminated from 06.00 to 18.00, at temperature $22 \pm 2^\circ\text{C}$, with $60 \pm 5\%$ of humidity. The protocols were approved by the Animal Experimentation Committee of Chungnam National University (number 09-3-29), Korea. The mice had free access to tap water during

Table 1. Formulas of rodent feed

Product #	High fat Diet	
	g%	kcal%
Protein	24	20
Carbohydrate	41	35
Fat	24	45
Total		100
kcal/gm	4.73	

the experiment. After a one-week acclimatization period the mice were fed high a fat diet (45% of energy from fat, Orient Bio Inc., # D12451) *ad libitum* for 5 weeks. Then the mice were divided into 2 groups for 8 weeks: (1) CON group (n = 10) received a high-fat diet (45% of energy from fat, Orient Bio Inc., # D12451) *ad libitum* for 8 weeks; (2) EXE group (n = 10) received a high-fat diet (45% of energy from fat, Orient Bio Inc., # D12451) *ad libitum* and performed moderate aerobic exercise training for 8 weeks.

The food consumption was monitored daily and body weight was measured once per week by using a standard table scale. The diet composition is shown in Table 1 below.

Exercise protocol

In the early stage of moderate exercise, all the mice of EXE ran at 8-10 m/min for 10 minutes on the automatically operated rodent treadmill.

After that, the speed and time of exercise were gradually increased up to 10-22 m/min for 30-60 minutes. The volume of this protocol represents 60-75% of the maximum oxygen consumption of the rodents as moderate intensity exercise [21]. The exercise group was subjected to this training for 8 consecutive days within each week, over the eight week period. During training of the exercise group (maximum 60 min day⁻¹), the control group was exposed to environmental stress as well as vibration and noise from the treadmill, and their food and water supply were removed.

Preparation of splenocytes

All procedures were conducted under aseptic conditions. For this part of the experiment, ten mice were used from each group. Mice were sacrificed by cervical dislocation 24h after the last exercise and the single cell suspension was prepared by pressing the spleen between two slide glasses.

The cell suspensions were passed through a 70um Nylon Cell Strainer (BD Falcon, USA) and then let to stand to remove tissue fragments. Contaminating red blood cells were lysed by suspending cells in a Red Blood cell Lysing Buffer

(Sigma, UK). The cell suspensions were centrifuged and then resuspended gently in FBS-RPMI 1640.

These cells were counted, checked for viability in trypan blue, and used within *in vitro* assays for the production of inflammation cytokines.

Measurement of splenocytes proliferation

The 1mL of spleen cell suspension at a concentration of 1×10^6 cells/mL were incubated at 37°C with 5% CO₂ in humidified air for 48h with ConA (10 ug/mL). The proliferation of spleen cells were assayed using Cell Titer 96 A Queous assay (Promega, USA). Then, optical density at 490 nm was measured by the microtiter plate reader (Bio rad, USA). Proliferation was calculated using the following equation. Proliferation Index = Sample OD/Control OD

Measurement of cytokine

The 1mL of spleen cell suspension at a concentration of 1×10^6 cells/mL were incubated at 37°C with 5% CO₂ in humidified air for 48 h with Con A (10 ug/mL) [22].

The IL-2, IL-1 β , IL-6, TNF- α , IFN- γ production in culture supernatants were determined by using the Bioplex array reader.

Briefly, PBS 100 μ l (with BSA 1%) to each well, add standard, sample, reference and blank 100 μ l, incubate 30min at room temperature with shaking. Washing PBS 150 μ l (1% BSA+ 0.05% Tween 20) three times. 50 μ l of Biotinylated Abs to each well, incubate at room temperature for 30min. Streptavidin-PE to each well, incubate 10 min after filter washing three times. Washing PBS 120 μ l (1% BSA + 0.05% Tween 20). Reading the plate by Bio-Plex array reader (Bio-Rad, Hercules, CA).

Statistical methods

The data were analyzed by using SPSS (version 17.0). All values were expressed as means $\pm$ SE. When comparing groups, statistical significance was determined by using independent *t*-test. The differences were considered statistically significant at $p < 0.05$.

RESULTS

Body weight change

After 8 weeks of aerobic exercise, the weight of EXE was

Table 2. The results of independent t-test on body weight (g)

	Groups	N	M $\pm$ SD	t	P
PRE	CON	10	26.74 $\pm$ 1.59	.104	.932
	EXE	10	27.23 $\pm$ 1.94		
POST	CON	10	40.81 $\pm$ 4.75	3.31	.005**
	EXE	10	34.57 $\pm$ 2.40

Fig. 1. Effects of 8wks aerobic exercise on body weight in high fat diet induced obese mice. ** $p < .01$. Data is presented as mean $\pm$ SD

significantly lower than that of CON ($p = .005$, Fig. 1, Table 2). The weight of EXE was approximately 18% lower than that of CON.

Proliferation of splenocytes by mitogen concanavalin A stimulation

After 8 weeks of aerobic exercise, the splenocyte proliferation of EXE was significantly increased ($p = .021$, Fig. 2, Table 3). The splenocyte proliferation of EXE was about 16% higher than that of CON.

Table 3. The results of independent t-test on proliferation index

Groups	N	M $\pm$ SD	t	P
CON	10	0.92 $\pm$ 0.09	-2.86	.021*
EXE	10	1.07 $\pm$ 0.07

Fig. 2. Effects of 8wks habitual exercise on proliferation index in high fat diet induced obese mice. * $p < .05$. Data is presented as mean $\pm$ SD

Table 4. The results of independent t-test on IL-2 production (pg/ml)

Groups	N	M $\pm$ SD	t	P
CON	10	111.64 $\pm$ 45.55	4.507	.001**
EXE	10	36.52 $\pm$ 4.58

Fig. 3. Effects of 8wks habitual exercise on IL-2 production in high fat diet induced obese mice. ** $p < .01$. Data is presented as mean $\pm$ SD**Table 5.** The results of independent t-test on IL-1 β production (pg/ml)

Groups	N	M $\pm$ SD	t	P
CON	10	4.40 $\pm$ 1.01	.717	.497
EXE	10	3.73 $\pm$ 1.88

Fig. 4. Effects of 8wks habitual exercise on IL-1 β production in high fat diet induced obese mice. Data is presented as mean $\pm$ SD

IL-2 production

After 8 weeks of aerobic exercise, IL-2 production of EXE was significantly decreased. ($p = .001$, Fig. 3, Table 4). IL-2 production of EXE was about 68% lower than that of CON.

IL-1 β production

After 8 weeks of aerobic exercise, there was no difference between CON and EXE in IL-1 β production (Fig. 4, Table 5).

TNF- α production

After 8 weeks of aerobic exercise, there was no difference between CON and EXE in TNF- α production (Fig. 5, Table 6).

Table 6. The results of independent t-test on TNF- α production (pg/ml)

Groups	N	M $\pm$ SD	t	P
CON	10	58.81 $\pm$ 37.74	.123	.908
EXE	10	54.88 $\pm$ 40.10

Fig. 5. Effects of 8wks habitual exercise on TNF- α production in high fat diet induced obese mice. Data is presented as mean $\pm$ SD**Table 7.** The results of independent t-test on IL-6 production (pg/ml)

Groups	N	M $\pm$ SD	t	P
CON	10	15.67 $\pm$ 5.43	2.89	.013*
EXE	10	7.58 $\pm$ 5.38

Fig. 6. Effects of 8wks habitual exercise on IL-6 production in high fat diet induced obese mice. * $p < .05$. Data is presented as mean $\pm$ SD

IL-6 production

After 8 weeks of aerobic exercise, IL-6 production of EXE was significantly decreased ($p = .013$, Fig. 6, Table 7). IL-6 production of EXE was about 52% lower than that of CON.

IFN- γ production

After 8 weeks of aerobic exercise, there was no difference between CON and EXE in IFN- γ production (Fig. 7, Table 8).

Table 8. The results of independent t-test on IFN- γ production (pg/ml)

Groups	N	M $\pm$ SD	t	P
CON	10	4.41 $\pm$ 1.88	-1.12	.297
EXE	10	6.02 $\pm$ 2.61

Fig. 7. Effects of 8wks habitual exercise on IFN- γ production in high fat diet induced obese mice. Data is presented as mean $\pm$ SD

DISCUSSION

In the previous studies, high fat diets increased obesity, inflammation of fat tissue, blood glucose and insulin resistance. Also, it causes immune dysfunction [23-26].

We investigated the effect of aerobic exercise as an effective way to improve the immune dysfunction of high fat diet-induced obese mice [27], on splenocyte proliferation stimulated by Con A and cytokines production relating to immunity. In the present study, after 8 weeks of aerobic exercise training, the weight of the exercise group (EXE) was significantly lower than that of the control group (CON). The result coincided with previous studies [23,28,29]. Hence, it was proved that long-term moderate aerobic exercise was effective to weight loss because the exercise program increased the energy expenditure of the exercise group even though the mice were fed a high fat diet during the exercise training period. The spleen is a special organ against antigen for immunity so that the splenocyte proliferation was affected by various factors such as IL-1 or IL-2, and essential immune function to evaluate the function of immune cells [30]. It was reported that moderate aerobic exercise improved lymphocyte proliferation [22,31]. However, other studies reported that after high intensity resistance exercise, the lymphocyte proliferation was decreased and if the exercise intensity was higher than 40-60% of maximal capacity, the lymphocyte proliferation was decreased [9,32]. The results were not coincident. One of the possible reasons might be the difference of exercise intensity. In this present study, after 8 weeks of moderate aerobic exercise (60-75% maximal capacity) the splenocyte proliferation was increased. It seems that the exercise program of this study did not excessively

produce epinephrine. This is why the DNA of lymphocyte was not damaged. This result was the same as previous studies [33,34]. Thus, the ability of cell proliferation was not negatively affected but rather positively affected by 8 weeks of aerobic exercise even though the mice were fed a high fat diet.

It was reported that IL-2 production of the spleen stimulated by mitogen was decreased after 15 weeks of treadmill exercise in young mice but increased in old mice [12]. The result showed that the effect of exercise is directly related to the capacity of metabolic function. Thus, it is thought that the metabolic function would be further decreased if a high fat diet were fed continuously. The important finding of the present study was that aerobic exercise significantly decreased the IL-2 production of EXE. It means that aerobic exercise overcomes the high fat diet-induced metabolic deterioration.

However, we did not find the mechanism pathway between IL-2 and cell proliferation. Thus, further research is required to find the reason.

In general, the inflammatory cytokines are decreased when the weight is reduced in obese mice [20]. IL-1 β plays a key role to activate the T and NK cell and a signal messenger to protect the host while the tissue is damaged. However, it is known that over expression IL-1 β causes the imbalance between anti-inflammatory and pro-inflammatory cytokines and results in destroying the immune system of the host [13].

This inflammatory cytokine is related to exercise, and it is especially increased after high intensity exercise [35,36]. TNF- α is a key factor to be increased following weight gain and is increased in obese mice [37]. It was reported that moderate exercise decreased TNF- α production [38,39].

However, it was reported that high intensity exercise or a low-fat diet with exercise increased TNF- α production. This coincident result seems that TNF- α production of immune cells is affected by the difference of exercise intensity and diet treatment. In the present study, 8 weeks of aerobic exercise with a high fat diet did not affect IL-1 β and TNF- α production. However, our previous result showed that moderate aerobic exercise with a low fat diet decreased IL-1 β and TNF- α production [20]. It seems that IL-1 β and TNF- α production are highly related by both exercise and diet treatment. In our previous study, C57BL/6 mice were fed a high fat diet for 5 weeks first then, switched to a low fat diet for 8 weeks. While the mice were fed a low fat diet, the exercise group performed the treadmill exercise exactly as in this present study. In this case, IL-1 β and TNF- α production of the EXE group was significantly decreased [20]. Thus, diet treatment should be considered to ameliorate obese-induced inflammatory cytokine production especially

IL-1 β and TNF- α .

It is known that IL-6 has both a pro- and anti-inflammatory function. Thus, IL-6 production was different depending on various conditions such as exercise type, intensity and time. It was reported that there was no difference between the control and exercise group on IL-6 production of splenocytes stimulated by Con A in obese mice [40]. Another study reported that moderate exercise with a high fat diet significantly increased IL-6 production [41]. In the present study, IL-6 production of EXE was significantly lower than that of CON. The result was the same as Barbara *et al.* [42], whereby weight loss by exercise interacts with anti-inflammation. It means that aerobic exercise improves low grade inflammation induced by obesity prior to the facilitation of energy metabolism [43].

IFN- γ is produced by NK cells and, CD4+ and CD8+ T cells. Also, it has a strong inhibitor action to the humoral immune pathway and induces NK cell activity [14]. It was reported that IFN- γ production was increased after voluntary wheel exercise in splenocytes stimulated by Con A [40]. Another study reported that IFN- γ production was significantly increased after exercise in the macrophage of high fat diet-induced obese mice [44]. In the present study, there was a trend that IFN- γ production was increased but it was not significant. This result shows that a high fat diet during exercise training diminished the positive effect of exercise training on IFN- γ production. It seems that IFN- γ production is also increased by both exercise and diet treatment.

Taken together, it seems that moderate aerobic exercise with a high fat diet has a positive effect to ameliorate the metabolic disturbance caused by high fat diet-induced obesity by promoting immune cell proliferation while inhibiting the production of inflammatory cytokines.

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