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# Physical Condition during Periodization for 3 Months in Female Ekiden Runners

Katsuro YONEDA[1,2], Takashi UMEDA[2], Ippei TAKAHASHI[1], Miyuki KANEKO[1,2], Toshiyuki MASHIKO[3], Manabu TOTSUKA[4], Teruaki KOMURO[1,5], Kazuo SAITO[6], Hiromichi SATO[1], Yuichi UENO[7], Shigeyuki NAKAJI[1]

1 Department of Social Medicine, Hirosaki University Graduate School of Medicine
2 Meijo University
3 National Defense Medical College
4 Department of Health and Physical Education, Faculty of Education, Hirosaki University
5 Kyoto Sangyo University
6 Nippon Sport Science University
7 Ryutsu Keizai University

**Keywords**

1. female ekiden runners
2. periodization
3. muscle fatigue
4. immune function

**Objectives**: In order to assess the appropriates of 3-months periodization for top university female ekiden runner, the changes in muscle fatigue and immune function were investigated in each training phase.

**Methods:** Subjects were 13 female students in the Meijo University ekiden club which won third position in the 28th All Japan Interscholastic Ekiden Competition. There were four monthly assessment points: normal training period; intensive training period; tapering period; and race period. At each assessment, data were collected on the training contents, athletes' body composition, and a variety of immune function markers including immunoglobulins, complements, serum opsonic activity and so on.

**Results:** All myogenic enzymes, complements, immunoglobulins and leukocyte and neutrophil counts tended to be the highest at the intensive training period, and to be the lowest at the race period. Peak height levels of the serum opsonic activity of luminol-dependent chemiluminescence were lowest in the race period out of all assessments. At the race period, helper T, Th1 and B cells showed the lowest numbers out of all assessments.

**Conclusions:** This tapering period was insufficient for full recovery of reduced immune functions, although it was sufficient to allow recovery from muscle fatigue.

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## Introduction

In recent years, a number of female athletes have been delivering outstanding performances in various sports events, including the Olympics. Despite their success, it is a fact that the prevalence of menstruation disorders, eating disorders and osteoporosis are high in female athletes, and are often referred to as the female athletes triad [1]. As well as these sports disorders, many female athletes are also prone to anemia compared to males, and it has been reported to be one of the characteristic health problems for female athletes [2].

According to the study by Nieman et al., immunosuppression was observed in long-distance marathon runners after the race and thus, they were more susceptible to infectious diseases such as upper respiratory tract infection compared to others [3, 4]. This finding is also supported by a number of other studies [5, 6]. Similarly, previous reports from our research group on long-distance runners also observed a reduced neutrophil function after a full-marathon and 30 km marathon [7, 8]. In other words, these results showed that intensive training was carried out in an environment that led to immunosuppression as well as

Table 1. Study subject demographics and the changes in anthropometric parameters during the 4-month study period.

| | Normal training period (3 months before the race) | Intensive training period (2 months before the race) | Tapering period (1 months before the race) | Race period (7 days before the race) |
|---|---|---|---|---|
| Age (Years) | 19.2 ± 0.9 | — | — | — |
| Height (cm) | 159.3 ± 3.8 | — | — | — |
| Weight (kg) | 45.6 ± 3.6 | 45.1 ± 3.1 | 44.6 ± 3.2 ** | 44.7 ± 3.3 ** |
| Relative body fat (%) | 12.9 ± 3.0 | 13.7 ± 2.2 | 13.0 ± 2.5 | 14.1 ± 2.2 ‡ |
| Fat-free mass (kg) | 39.6 ± 2.0 | 38.9 ± 2.0 ** | 38.7 ± 1.9 ** | 38.4 ± 2.1 ** |

Values are shown as the mean ± standard deviation.
**: $p<0.01$, Significant difference from the value of the normal training period.
‡: $p<0.05$, Significant difference from the value of the tapering period.

sports disorders in female long-distance runners. Their daily training involves running long distances, and this makes it extremely difficult for them to get themselves into the best physical conditions for the marathon competition they are aiming to win.

Ekiden, a Japanese term for road relay, is an athletic team sport involving several runners passing on team tasuki (sash) to complete a race. The total number of runners, the distance to be run by each runner, gender of runners and total distance of the race differ in each competition, however, the international standard set by the International Association of Athletics Federations stated that running distance of a full marathon (42.195 km) should be divided between 6 runners (5 km, 10 km, 5 km, 10 km, 5 km and 7.195km) for both male and female races. In Japan, many local and national ekiden races are held between October and January. Many marathon runners thus plan and start their intensive training and tapering between August and September in order to prepare themselves to be in the best physical condition for the competitions. Unfortunately, it has been discovered that some athletes who had inadequate contents of periodization, or when they were not able to follow the adequate schedule for training, suffered from an accumulation of chronic fatigue, inducing the overtraining syndrome and/or the overuse syndrome. As a consequence, they were unable to perform their best in the competition, or in some worst cases, they had to withdraw from the competition [9-11]. However, no medical studies in the past have investigated the association between marathon training and physical conditions in female ekiden runners over a long period of time, or examined the adequacy of periodization that was planned for the athlete with the aim of winning the ekiden competition.

In the present study, in order to assess the appropriates of 3-months periodization for top university female ekiden runner, the changes in muscle fatigue and immune function were investigated in each training phase.

## Subjects and method

Subjects and investigation period

The subjects were 13 female ekiden club members (including 7 reserve runners) at Meijo University. Between 2002 and 2011, the team reached the top 3 of the most prestigious national ekiden competition, the 28th All Japan Interscholastic Ekiden Competition, for 9 consecutive years.

The average age, height, body weight, relative body fat and fat-free mass were 19.2 ± 0.9 years, 159.3 ± 3.8 cm, 45.6 ± 3.6 kg, 12.9 ± 3.0 % and 39.6 ± 2.0 kg, respectively (Table 1).

The investigation was carried out at 4 points between June and October in 2010. On the first investigation day (June 29th), we assessed their health and conditions during the normal training period (3 months before The 28$^{th}$ All Japan Interscholastic Ekiden Competition). The same parameters were assessed for the second and third investigation days on August 25th (2 months before the race immediately after the training camp) during the intensive training period, September 27th (1 month before the race) during the tapering period, and immediately before the race (4$^{th}$ point, 20$^{th}$ October).

Each subject was asked to record the contents of their training regimen over the week before each assessment day including the running distance and training time. According to the information provided, the training time and training distance were calculated.

The study was approved by the Ethics Committee of Hirosaki University School of Medicine. The study protocol and purpose were thoroughly explained and written consent was obtained from all subjects prior to the investigation.

Body composition and blood parameters

After subjects' heights and their body weight were measured, relative body fat and fat-free mass were measured by the impedance method using the multi-frequency body composition meter (MC-190, TANITA Corp, Tokyo, Japan).

Blood biochemical parameters

Blood samples (15 ml) were taken early morning under fasting conditions on each assessment day. Five milliliters of the sample was used to analyze the blood cell components, and the remaining 10 ml was centrifuged at 3,000 rpm for 10 minutes to extract blood serum for further analysis.

For investigation of immune-related functions, leukocyte, neutrophil and lymphocyte counts were determined. Several myogenic enzymes including aspartate aminotransferase (AST), alanine aminotransferase (ALT), lactate dehydrogenase (LDH) and creatine kinase (CK) as well as immunoglobulins (IgG, IgA and IgM) and complements (C3 and C4) were measured. In order to determine the anti-oxidative function, the level of activity of superoxide dismutase (SOD) was also measured.

All blood cell components were measured using an Automated Blood Cell Analyzer (System XE-2100 and SE-9000 Kobe, Japan), and levels of AST, ALT, LDH and CK were measured using the JSCC standardized method. For measurements of immunoglobulins and complements, the turbidimetric immunoassay (TIA) was used. The SOD activity was measured with the NBT reduction method. All measurements of blood biochemical items were consigned to an independent laboratory (Mitsubishi Chemical Medience Corporation).

SOA

SOA was measured with the luminol chemiluminescence assay based on the ROS produced when standard neutrophils phagocytosed opsonized zymosan in serum from the subjects [12, 13]. Luminol (Lm), used as a chemiluminigenic probe, was dissolved with 1 mol/L NaOH and the solution was adjusted to an isotonic state of 12.5 mmol/L at pH 7.4 by adding HCl, ultrapure water and NaCl. The luminol solution was diluted with HBSS to 0.5 mmol/L before use.

Zymosan A (Sigma, USA) was suspended in Hank's balanced salt solution (HBSS) at a concentration of 5 mg/ml and then opsonization was performed by adding the solution to the serum samples (final concentration of 20%) and incubating at 37 °C for 30 min. The particles were then washed twice with HBSS and re-suspended in HBSS at a concentration of 5 mg/ml.

Standard neutrophils were obtained from the peripheral blood of a healthy adult female volunteer, whereby whole blood is modified by centrifugation through Mono-Poly resolving medium. The neutrophils were suspended to $3\times10^6$ cell/ml using an automatic blood cell counter (Coulter MD II, Coulter Co. Ltd., Tokyo, Japan).

Opsonized zymosan (OZ) suspension and chemiluminigenic probes prepared as described above were added to each well of black flat-bottom microplates (Greiner Japan, Tokyo, Japan), and 50 µl of standard neutrophils was added. The plates were automatically measured on the Auto Luminescence Analyzer, Alfa system (Tokken, Funabashi, Japan) [14]. All measurements were performed at 37 °C. The results were evaluated using the maximum light emission (peak height: PH) and the area under the curve (AUC) of chemiluminescence response [14, 15].

Lymphocyte immunophenotyping

Three-color flow cytometry

Immunophenotyping was analyzed with flow cytometry (FACSCantoII ; BD Bioscience). Freshly collected EDTA-anticoagulated whole blood was incubated with the following combinations of conjugated monoclonal antibodies (BD Bioscience): Pacific Blue Mouse Anti-human CD4, Alexa Fluor488 Mouse Anti-Human CD183 (CXCR3), PE Mouse Anti-Human CCR4 and isotype controls at the manufacturer's recommended concentration in the dark at room temperature for 30 minutes. After incubation, the red blood cells were lysed with FACS Lysing Solution (BD Bioscience) for 15 min. The cells were then spun down at 300 g for 5 min and washed with phosphate buffered saline (PBS, pH 7.2).

Six-color flow cytometry

A 50 µl sample of whole blood was stained for analysis on FACS CantoII (BD Biosciences) using 20 µl of CD3 FITC, CD16 PE, CD56 PE, CD4 PE-Cy7, CD19 APC and CD8 APC-Cy7 (BD Multitest 6-color TBNK reagent). After incubation in the dark at room temperature for 30 minutes, the red blood cells were lysed with 2 ml of FACS lysing solution (BD Biosciences) for 15 min.

A total of 10,000 lymphocytes were counted per sample. Lymphocyte subsets were analyzed to determine the percentage of total T (CD3+), total B (CD19+), T helper (CD3+CD4+), T helper 1 (CD4+CD183+), T helper 2 (CD4+CCR4+), T cytotoxic (CD3+CD8+), and natural killer (CD3+CD16+CD56+) cells. Absolute numbers were calculated using the complete blood counts measured with an automated blood cell analyzer (Sysmex XE-2100 SE-9000, Kobe, Japan).

Statistical Analysis

All values were shown as mean ± standard deviation. For statistical analysis between the average values among each investigation day, a one-way ANOVA and the Bonferroni method were used. All values at $p<0.05$ were considered to be statistically significant. SPSS ver.12.0J (SPSS Japan Inc., Tokyo, Japan) was used for analysis of the data.

**Results**

Table 1 shows the changes in body composition

Table 2. Contents of the training sessions during the 4-month study period.

| | Normal training period (3 months before the race) | Intensive training period (2 months before the race) | Tapering period (1 months before the race) | Race period (7 days before the race) |
|---|---|---|---|---|
| Average running distance one week before investigation (km/day)[a] | 19.3 ± 5.1 | 23.8 ± 6.5 ** | 20.7 ± 5.7 | 16.5 ± 3.6 ††, ‡ |
| Average training hours one week before investigation (hours/day)[a] | 2.4 ± 0.7 | 2.8 ± 0.8 | 2.6 ± 0.9 | 2.0 ± 0.6 ††, ‡‡ |

Values are shown as the mean ± standard deviation.
a: These values were calculated from the daily running distance and the training times during the 7 days before each investigation.
**: p<0.01, Significant difference from the value of the normal training period.
††: p<0.01, Significant difference from the value of the intensive period.
‡: p<0.05, ‡‡: p<0.01, Significant difference from the value of the tapering period.

Table 3. Changes in serum myogenic enzymes during the 4-month study period.

| | Normal training period (3 months before the race) | Intensive training period (2 months before the race) | Tapering period (1 months before the race) | Race period (7 days before the race) |
|---|---|---|---|---|
| AST (IU/L) | 44.1 ± 36.6 | 42.4 ± 16.0 | 34.5 ± 8.8 | 29.5 ± 7.9 † |
| ALT (IU/L) | 30.4 ± 18.8 | 29.0 ± 16.6 | 26.0 ± 16.2 | 25.8 ± 11.4 |
| CK (IU/L) | 591.1 ± 877.7 | 592.8 ± 371.1 | 332.9 ± 121.7 | 187.5 ± 82.0 ††, ‡‡ |
| LDH (IU/L) | 319.6 ± 102.4 | 312.2 ± 51.5 | 265.2 ± 28.7 † | 243.3 ± 39.0 *, †† |

Values are shown as the mean ± standard deviation.
*:p<0.05, Significant difference from the value of the normal training period.
†: p<0.05, ††: p<0.01, Significant difference from the value of the intensive period.
‡‡: p<0.01, Significant difference from the value of the tapering period.

Table 4. Changes in blood leukocyte, neutrophil and lymphocyte counts during the 4-month study period.

| | Normal training period (3 months before the race) | Intensive training period (2 months before the race) | Tapering period (1 months before the race) | Race period (7 days before the race) |
|---|---|---|---|---|
| Blood leukocyte count (/μL) | 4346 ± 658 | 5762 ± 1286 ** | 4362 ± 757 † | 4031 ± 750 †† |
| Blood neutrophil count (/μL) | 2243 ± 648 | 3478 ± 1096 ** | 2310 ± 698 † | 1990 ± 562 †† |
| Blood lymphocyte count (/μL) | 1708 ± 295 | 1914 ± 406 | 1703 ± 439 | 1721 ± 437 |

Values are shown as the mean ± standard deviation.
**: p<0.01, Significant difference from the value of the normal training period.
†: p<0.05, ††: p<0.01, Significant difference from the value of the intensive period.

values during the investigation period. Body weight significantly decreased in the tapering and race periods in comparison to the normal training period (both p<0.01). Relative body fat significantly increased in the race periods in comparison to the tapering period (both p<0.05). Fat-free mass significantly decreased in the intensive, tapering and race periods in comparison to the normal training period (each p<0.01).

The training contents are shown in Table 2. The running distance per day or per week in the intensive training period was longer than in the normal training period (both p<0.01). The training hours or running distance per day (or per week) in the race period were significantly shorter compared to the intensive and tapering periods (both p<0.01 against the intensive training period, and p<0.05, p<0.01 against the tapering period, respectively).

Table 3 shows the changes in myogenic enzymes during the investigation period. CK in the race period significantly decreased compared with the intensive training and tapering periods (both p<0.01). LDH in the tapering period significantly decreased compared with the intensive training (p<0.05). LDH in the tapering period significantly decreased compared with the intensive training period (p<0.05).

Table 5. Changes in serum immunoglobulins and complements during the 4-month study period.

| | Normal training period (3 months before the race) | Intensive training period (2 months before the race) | Tapering period (1 months before the race) | Race period (7 days before the race) |
|---|---|---|---|---|
| IgG (mg/dL) | 996 ± 244 | 1032 ± 280 ** | 1012 ± 267 | 1015 ± 232 |
| IgA (mg/dL) | 153.8 ± 62.6 | 166.6 ± 66.1 | 159.3 ± 61.1 | 157.0 ± 59.3 |
| IgM (mg/dL) | 129.2 ± 83.2 | 137.3 ± 76.3 | 131.1 ± 79.8 | 132.0 ± 80.3 |
| C3 (mg/dL) | 93.8 ± 8.7 | 101.4 ± 10.8 | 86.1 ± 11.5 †† | 83.0 ± 9.5 **, †† |
| C4 (mg/dL) | 21.8 ± 6.0 | 24.8 ± 7.3 | 20.6 ± 5.0 | 18.8 ± 3.8 †† |

Values are shown as the mean ± standard deviation.
**: $p<0.01$, Significant difference from the value of the normal training period.
††: $p<0.01$, Significant difference from the value of the intensive period.

Table 6. Changes in neutrophil functions, serum opsonic activity and SOD during the 4-month study period.

| | Normal training period (3 months before the race) | Intensive training period (2 months before the race) | Tapering period (1 months before the race) | Race period (7 days before the race) |
|---|---|---|---|---|
| LgCL・PH (cpm) | 19.6 ± 2.1 | 19.9 ± 2.0 | 19.9 ± 2.1 | 19.5 ± 2.8 |
| LgCL・AUC (cpm*sec) | 550.2 ± 63.0 | 562.1 ± 63.7 | 553.7 ± 58.1 | 552.7 ± 77.6 |
| LmCL・PH (cpm) | 192.9 ± 12.3 | 198.9 ± 14.5 | 193.9 ± 11.4 | 186.1 ± 12.6 † |
| LmCL・AUC (cpm*sec) | 4531 ± 242 | 4615 ± 235 | 4573 ± 276 | 4398 ± 191 |
| SOD (%) | 4.5 ± 1.1 | 3.8 ± 1.1 | 8.1 ± 1.3 **, †† | 5.4 ± 1.5 *, †, ‡‡ |

Values are shown as the mean ± standard deviation.
SOD: Superoxide dismutase
LgCL: Lucigenin-dependent chemiluminescence response
LmCL: Luminol-dependent chemiluminescence response
PH: Peak height
AUC: Area under the curve for 45 min.
*: $p<0.05$, **: $p<0.01$, Significant difference from the value of the normal training period.
†: $p<0.05$, ††: $p<0.01$, Significant difference from the value of the peaking period.
‡‡: $p<0.01$, Significant difference from the value of the tapering period.

Changes in leukocyte and neutrophil counts and the lymphocyte count are shown in Table 4. Leukocyte and neutrophil counts in the intensive training period were significantly higher than in the normal training period (both $p<0.05$). Leukocyte and neutrophil counts in the tapering or race periods were significantly lower than in the intensive training period ($p<0.05$, 0.01, respectively).

Changes in immunoglobulins and complements are shown in Table 5. IgG in the intensive training period significantly increased compared with the normal training period ($p<0.01$). C3 levels in the tapering or race periods were significantly lower than in the intensive training period (both $p<0.01$). C4 in the race period significantly decreased compared with the intensive training period ($p<0.01$).

Table 6 shows the changes in the levels of neutrophil function, SOA and SOD. LmPH in the race period was significantly lower than in the intensive training period ($p<0.05$). SOD in the tapering period was significantly higher than in the normal and intensive training period (both $p<0.01$). SOD in the race period significantly increased compared with the normal or intensive training periods (both $p<0.05$), and decreased than in the tapering period ($p<0.01$).

Table 8 shows the changes in lymphocyte subtypes during the investigation period. The number of helper T cell at the race period was significantly lower than in the intensive training period ($p<0.05$). The number of Th1 cell at the race period significantly decreased compared with the normal or Intensive training period (both $p<0.01$). The number of Th2 cell at the tapering period was significantly higher than in the normal or intensive training period (both $p<0.05$). The number of Th2 cells at the race period was significantly lower than in the tapering period ($p<0.01$). The number of B cells at the race period significantly decreased compared with the normal or intensive training period (both $p<0.01$).

## Discussion

Periodization is a systematic planning of an athlete's physical condition by adjusting the components of the training sessions and having the

Table 7. Changes in subpopulations of lymphocytes during the 4-month study period.

| | Normal training period (3 months before the race) | Intensive training period (2 months before the race) | Tapering period (1 months before the race) | Race period (7 days before the race) |
|---|---|---|---|---|
| T cell (CD3+) [a] | 7049 ± 522 | 7199 ± 629 | 7272 ± 329 | 7057 ± 436 |
| Killer T cell (CD3+CD8+) [a] | 2241 ± 403 | 2253 ± 367 | 2305 ± 365 | 2237 ± 356 |
| Helper T cell (D3+CD4+) [a] | 4229 ± 379 | 4364 ± 579 | 4363 ± 360 | 4102 ± 398 ‡ |
| Th 1 cell (CD4+CD183+) [b] | 948 ± 297 | 980 ± 284 | 874 ± 298 | 790 ± 210 **, †† |
| Th2 cell (CD4+CCR4+) [b] | 655 ± 128 | 667 ± 164 | 1049 ± 251 **, †† | 784 ± 237 ‡‡ |
| B cell (CD19+) [a] | 1194 ± 428 | 1221 ± 407 | 1101 ± 428 | 976 ± 320 **, †† |
| NK cell (CD3+CD16+CD56+) [a] | 1664 ± 521 | 1490 ± 480 | 1454 ± 453 | 1789 ± 488 |

Subjects were 13 female ekiden runners.
Values are shown as the mean ± standard deviation.
a : For phenotypical analyses per 10000 lymphocytes.
b : For phenotypical analyses for 5000 lymphocytes.
**: $p<0.01$, Significant difference from the value of the normal training period.
††: $p<0.01$, Significant difference from the value of the intensive period.
‡: $p<0.05$, ‡‡: $p<0.01$, Significant difference from the value of the tapering period.

athlete complete these components over several training phases such as the intensive training period, tapering period and so on. It is crucial for athletes to follow an adequate training program in order for them to perform their best in the competition, as well as to maintain their physical health in the optimum condition [9, 16, 17]. In terms of periodization carried out by the present subjects, the training period was divided into the normal training period, intensive training period, tapering period and race period, and the amount of training and training elements were adjusted with a set goal for each period. During the athletes' training, the running distance and training duration were the highest during the intensive training period and gradually reduced towards the tapering and race periods.

In the present study, two of the changes observed in athlete body composition were body weight and fat-free mass. They were shown to reduce significantly at the tapering period and the race period compared to the normal training period. The same trend was observed for fat-free mass at the intensive training period. This was suggested to be due to accelerated energy metabolism by the body between the intensive training period and tapering period when running distance and training duration increased, resulting in accelerated fat metabolism and protein catabolism [18, 19]. Also, the period during which the subjects were performing intensive training and tapering were the most humid and hot seasons in Japan. Thus, performing intensive training in such an environment may have increased the acceleration of fat metabolism and protein catabolism [20, 21].

A number of studies have reported that highly intensive physical exercise causes severe contraction of muscles and physical damage, leading to changes in and damage to muscle tissues. Membrane permeability changes and muscle enzymes escape into the vasculature, increasing their concentrations in blood vessels [22-24]. It has also been reported that determination of these levels offers effective indices for examining long-term chronic muscle fatigue [25]. In the present study, the level of CK was the highest at the intensive training period, and lowest at the race period. The levels of AST and LDH were also the lowest at the race period. In other words, these results suggested that muscle fatigue accumulated during the intensive training period and recovered adequately to lower levels by the subsequent tapering.

Many of the past studies have stated that leukocyte levels increased following transient exercise, and the degree of the increase depended on the intensity of exercise being carried out [26]. Levels of lymphocytes and neutrophils have also been reported to increase with physical exercise [26]. These increases were suggested to have strong associations with various inflammatory cytokines and stress hormones such as the catecholamines and cortisol [26-28]. In the

case where athletes developed the overtraining syndrome due to long-term and/or highly intensive training, reduced levels of leukocytes and their functions have also been reported [29]. Moreover, these decreases were suggested to be caused by reduced secretion of catecholamines and increased levels of cortisol, which were induced by the overtraining syndrome [30-32]. Our research group also reported reductions in neutrophil function after intensive training camps for judoists and rugby players [33, 34]. Another study by our research group also proposed that, in a 1-week intensive soccer training camp which involved highly intensive physical exercise to the extent that the overtraining syndrome may have developed, both muscle fatigue and immunosuppression were induced after the training camp. Although both conditions were observed after the training camp, we have concluded that immunosuppression may have developed later than muscle fatigue [35]. Thus, significant increases in the leukocyte and neutrophil counts at the intensive training period in the present study suggested that severe adverse changes in and damage to muscle tissues occurred due to the training involving the longest running time and distance during that period, leading to accelerated stress and inflammatory reactions during the period. In other words, significantly reduced levels of leukocyte and neutrophil counts at the tapering period, with the lowest levels being recorded at the race period, suggested that reductions in the immune function occurred after the peak of muscle fatigue, and lasted for at least 2 months after the training camp. Furthermore, this was suggested to be led by the increased secretion of cortisol and decreased secretion of catecholamines which developed after the tapering period and were caused by the overtraining syndrome, as a result of the highly intensive training carried out in previous period in the periodization program.

In the past, somewhat contradictory studies on the associations between immunoglobulins and complements reported that they either increased, decreased or remained unchanged after isolated and acute bouts of physical exercise [36-38]. In those studies where long-term, highly intensive training was carried out, levels of immunoglobulins were reported to decrease after the training period [39, 40]. Other reports on the levels of complements and long-term training also revealed that athletes who performed highly intensive exercise for a long period of time showed reduced levels of complements compared to members of the general population [36, 41-43]. Additionally, activation of immunoglobulins and complements by physical exercise is related to inflammatory cytokines induced from muscle damage and change, and stress hormones that are de-activated and/or activated by physical exercise [27, 28]. In the present study, the C3 levels were shown to decrease significantly at the tapering period and reached their lowest at the race period. C4 levels also reached their lowest at the race period. These results suggested a reduced volume of complements in the circulating blood (playing a partial role in immune functions) during the tapering and race periods. The highly intensive training carried out during the intensive training period most probably caused the development of the overtraining syndrome after the tapering period, and affected the athletes' general condition for at least 2 months, up until the race period. Moreover, these decreases were suggested to be induced by reduced secretion of catecholamines and increased cortisol, which were brought on by the syndrome.

Neutrophils have the important function in the immune system to destroy foreign substances invading the body by opsonizing the substances to efficiently engulf them for sterilization [44]. When neutrophils attack and sterilize opsonized foreign substances, they do so by engulfing the target and then producing reactive oxygen species (ROS). Although ROS is required for the process, excess production of ROS is known to damage normal cells, leading to oxidative tissue damage [45, 46]. The level of SOA measured in the present study reflected neutrophil ROS productivity after opsonization, and thus SOA levels can be considered as an effective index for determining ROS productivity [12,13]. Also, the level of LgCL determined in the present study reflected the productivity of superoxide ($O_2^-$), which is the primary substance in ROS metabolism and has relatively low toxicity [15, 47, 48]. The level of LmCL on the other hand, reflected the level of highly toxic hypochlorous acid (HOCl/OCl-), which is produced when superoxide is metabolized by myeloperoxidase and thus used as an effective index for determining total ROS productivity [15, 47, 48]. Therefore, SOA levels as determined in the present study were used as the indices for the subjects' ROS productivity and neutrophil functions. As a result, the level of the Lm PH was lowest at the race period, suggesting that the immune function recorded in this study was at its lowest. Such findings supported many of the previous studies on the suppressive effect of long-term, highly intensive training on neutrophil functions [49-51]. The present result also matched the results from our previous report, where reduced neutrophil function was observed during a demanding female judo training camp involving prolonged and repeated highly intensive judo training [34]. Also, reduced neutrophil function was found to appear after muscle fatigue, which was similar to our observations regarding the leukocyte count, neutrophil count and complements [35], and was suggested to be caused by the overtraining syndrome, as it leads to reduced secretion of catecholamines and increased secretion of cortisol [30-32].

It has been reported that available levels of SOD in the body have an important antioxidative role to eliminate ROS that is produced as a result of oxidative stress [52]. A bout of transient physical exercise is also known to accelerate ROS production, which then increases serum SOD activity as an antioxidative reaction [53, 54]. According to the study by Ohishi, ROS productivity is accelerated when highly intensive training is carried out for a long period of time, and the body adapts to the condition by activating SOD[55]. The present result showed the highest level of serum SOD activity at the tapering period, and a higher level at the race period compared to the normal training period or the intensive training period. This suggested that repeated highly-intensive training in the present study produced high levels of ROS, continuously activating serum SOD until the subjects became acclimatized, leading to high serum levels of SOD between the tapering period and the race period.

Lymphocytes are immune cells with the important role of protecting the body against substances that have invaded or occurred within it. As a biological defensive response against foreign bodies, the neutrophils first play their part in the immune which is considered as the innate or natural immunity, and then lymphocytes perform their function as the acquired immunity response [26]. Lymphocytes are classified into T cells, B cells and NK cells, with T cells being further categorized into several subsets. Out of three types of lymphocytes, NK cells is the only one that is considered as part of the innate immune system, recognizing the markers on tumor cell surfaces and attacking cells that are infected by viruses. T cells can be further classified into helper T cells, which play a role in controlling immunity, and killer T cells which remove cells that are infected with viruses. Helper T cells are categorized into Th1, which secrete inflammatory cytokines and Th2, which secrete cytokines that are associated with antibody production. B cells are stimulated by antigens, and they transform, proliferate and mature in order to produce antibodies (immunoglobulins) that correspond to each antigen.

In previous studies on the association between lymphatic functions and transient, highly-intensive and acute physical exercise it was reported that levels of lymphocytes and their subtype increase with exercise, and then decrease to a level lower than the original levels by the end of the exercise [26]. Other studies have reported that long-term, highly intensive training lowered the blood circulation volume of neutrophils, lymphocytes and their subtype [29]. One of the additional reasons why such condition occur is the potential of long-term, highly intensive training to reduce the secretion of catecholamines such as adrenaline and noradrenaline [30, 31]. Other studies reported that training of this type induced the overtraining syndrome, increasing cortisol, one of the stress hormones which is associated with a decreased volume in the circulating blood and reduced functions [32]. In the present study, numbers of helper T cells, Th1 cells and B cells were the lowest during the race period. The number of Th2 cells at first increased at the intensive training period, followed by a decrease at the race period. This result thus suggested that lymphatic functions were the lowest during the race period in this 3-month investigation period. Considering the current results on muscle enzymes and other immune-related indices, reduced lymphatic function was suggested to have developed after muscle fatigue which may have been caused by increased secretion of cortisol and reduced secretion of catecholamine induced by the overtraining syndrome after the training camp. Moreover, the fact that suppression of the lymphatic function appeared after muscle fatigue at the intensive training camp supported the results obtained in our previous studies on muscle fatigue and the immune function of soccer players after a training camp [35].

In the present study, peak muscle fatigue was observed immediately after the intensive training camp, and it gradually recovered during the tapering period. Thus, subjects in this study were considered to have followed the periodization appropriately from the muscle fatigue point of view.

In terms of immune function, its peak was observed not immediately after the intensive training camp, but one month after the training camp in the tapering period, where it was suppressed, and was observed to last for 2 months. Thus, there was a time lag between peak muscle fatigue and immunosuppression, which, as mentioned earlier, supports the results obtained in our previous studies [35]. In the present study, muscle fatigue had recovered adequately at the tapering period, however, the results suggested that 2 months of tapering was insufficient to bring the reduced immune function back to normal, which occurred after the muscle fatigue. This result also suggested that the components and duration of intensive training and tapering performed by the subjects were inadequate from the immunological point of view. Moreover, chronic immunosuppression and increased susceptibility to infection may have developed in subjects during the time between the intensive training period, tapering period and race period as noted in previous studies [56, 57].

It is crucial for athletes to strengthen their skills to reach their goals and perform their best in the competition [9, 16, 17]. The training carried out by the present subjects was conventional training as performed by many other female ekiden runners in Japan and was thus considered to involve training sessions necessary for improving and strengthening

their performance for competitions. Thus, the immunosuppression observed due to the intensive training camp in the present study was considered inevitable. Considering these points and the adequacy of the training carried out during the intensive training period, the training camp should have been held more than 3 months before the race. If athletes are unable to reconsider changing the schedule for their intensive training camp, then the volume of training should be reduced between the tapering period and the race period. Also, the training components should be adjusted to each individual's fatigue conditions, and their physical conditions should be managed by adequate nutritional intake and sufficient rest.

There were several limitations in the present study. It has been reported that female long distance runners are at high risk of sports disorders including menstrual disorders, eating disorders, osteoporosis and anemia [1,2]. Menstrual disorders in particular have been reported to have an association with the immune function as it can be affected by abnormalities in the secretion of female hormones and their functions [58]. Although this should have been considered in the present study, the study involved only a small number of subjects who were at different menstrual conditions in each assessment point, and thus, we were unable to clarify the association between immune function and female hormones or menstrual conditions.

The blood circulation volume and functions of neutrophil and lymphocytes as shown in this study, are adjusted and controlled by various cytokines and stress hormones in the blood [27, 28]. However, they were not measured in the present study. Although we have suggested that the overtraining syndrome developed after the intensive training period caused by activation/deactivation of those cytokines and stress hormones, and led to reduced functions of neutrophils and lymphocytes at the tapering period and the race period, we were unable to confirm the findings by collecting appropriate evidence. Thus, in the future, the study plan should be implemented taking these limitations into consideration.

In conclusion, this tapering period was insufficient for full recovery of reduced immune functions, although it was sufficient to allow recovery from muscle fatigue. Coaches and trainers must therefore pay extra attention in the planning of the tapering period, which should consider not only the muscle fatigue, but also levels of immunosuppression and the time required for recovery.

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Corresponding author: Katsuro Yoneda
Department of Social Medicine, Hirosaki University Graduate School of Medicine
5 Zaifu cho, Hirosaki 036-8562 Japan.
Tel: +81-172-39-5041; Fax: +81-172-39-5038
e-mail: yoneda@ccmfs.meijo-u.ac.jp