ORIGINAL ARTICLE

ASSOCIATION OF SEX STEROID HORMONES WITH NEUTROPHIL FUNCTION IN THE GENERAL POPULATION

Satoshi Sato[1], Ippei Takahashi[1], Hitomi Komame[1], Naoki Akimoto[1],
Kaori Sawada[1], Rina Tanaka[1,2], Osamu Munakata[1], Hiroshi Iizuka[1],
Nobuyuki Shibata[1], and Shigeyuki Nakaji[1]

Abstract   There are certain sex differences in the prevalence and severity of immunity-related diseases. Previous studies have shown the influence of sex steroid hormones, such as estrogen and testosterone, on the immune system. The aim of this study was to investigate the association of sex steroid hormones with neutrophil function among normal healthy subjects in the general population. Subjects included 540 residents (358 males and 182 females), who participated in the Iwaki Health Promotion Project in 2014. We examined the association of estradiol and testosterone with neutrophil ROS production and phagocytic activity. As a result, estradiol and testosterone negatively correlated with basal ROS production in males and luteal phase females (>15th day of menstrual cycle), although such trend was not observed in follicular phase females (<14th day of menstrual cycle) and postmenopausal females. In contrast, the association of testosterone with stimulated ROS production and phagocytic activity was observed only in males. In conclusion, both estradiol and testosterone were found to have an influence on neutrophil function. Furthermore, the association of sex steroid hormones with neutrophil function was found to vary according to the phase of the menstrual cycle in addition to the sex difference.

Introduction

Sex differences exist in the prevalence and severity of immunity-related diseases. For example, males are more prone to infections and have a higher potential to be severely infected than females[1]. Furthermore, the response to vaccines against measles, hepatitis virus, and yellow fever is less in males compared with that in females[1,2]. In contrast, autoimmune diseases, such as rheumatoid arthritis and systemic lupus erythematosus, are overwhelmingly more common in females than in males[3,4]. Moreover, there are sex differences in the prevalence of malignant tumors depending on its types.

The influence of sex steroid hormones, such as estrogen and testosterone, on the immune system has been pointed out as one of the factors for sex differences[5,6], and estrogen replacement therapy has been reported to prevent the decrease in immune function[7].

The immune system can be broadly divided into innate and adaptive immune responses. Neutrophils and macrophages are responsible for innate responses, whereas lymphocytes play a major role in adaptive responses. The innate immune system responds against infections at an early stage, whereas the adaptive immune system requires several days to become activated and gives stronger immune strength. Many

---

[1] Department of Social Medicine, Hirosaki University Graduate School of Medicine
[2] Department of Cancer Epidemiology and Community Health, Hirosaki University Graduate School of Medicine

studies have investigated the association of sex steroid hormones with the adaptive immune system. For example, estrogen activates the adaptive immune system by increasing the antibody production by lymphocytes[8-10]. In contrast, androgen suppresses the adaptive immune system by decreasing antibody production and promoting the apoptosis of lymphocytes[11, 12]. However, the association of sex steroid hormones with innate immune system has rarely been studied in the past.

Neutrophils play an important role in the innate immune system. It comprises the highest proportion of immune cells in blood and plays an important role as a defense against foreign substances, including microorganisms. Neutrophils engulf microorganisms [phagocytic activity (PA)] and produce reactive oxygen species (stimulated ROS production)[13, 14]. ROS is also produced continuously by neutrophils under non-stimulated conditions (basal ROS production), which can become one of the causes of oxidative stress in the body[15]. Therefore, reduction in the activity of neutrophils to remove foreign substances reflects susceptibility against infections[15]. In contrast, overproduction of basal ROS causes chronic oxidative stress[16]. However, the association of sex steroid hormones with basal ROS production has rarely been studied.

Several reports have studied the association of sex steroid hormones with neutrophil function and have found that females have a higher neutrophil function than males because of the influence of female steroid hormones[17]. Furthermore, both estrogen and androgen are reported to decrease the oxidative stress of neutrophils[18-21].

Various studies have reported that sex steroid hormones increase the activity of neutrophils to remove foreign substances[23], whereas others have reported the opposite[21, 23] or no change[24] in the removal activity of neutrophils. These contradictory results may

be explained by studies concentrating solely of the role of neutrophils and not measured their function in the whole blood, overlooking the influence of various cytokines on neutrophils[25, 26]. Furthermore, most previous studies have targeted stimulated ROS production, whereas the basal ROS production in relation to oxidative stress has rarely been acknowledged. Thus, we used the neutrophils obtained from whole blood and measured both basal and stimulated ROS production simultaneously.

Previous studies have used a small number of subjects and targeted young males or females only. Furthermore, in premenopausal females, the average length of the menstrual cycle is 28 days and female steroid hormones, such as estrogen and progesterone levels change periodically during menstruation. In particular, female steroid hormone levels are greatly different before and after the ovulation day, i.e., the 14th day of menstruation. In contrast, after menopause, female steroid hormone levels rapidly decline. For these reasons, epidemiological studies considering the menstrual cycle and menopause are considered necessary.

Immunity also changes during the menstrual cycle. For example, excessive inflammatory reaction occurs during the menstrual phase, whereas the inflammatory reaction is suppressed after ovulation in the luteal phase[28]

Although previous studies have reported that neutrophil counts increase or decrease during the menstrual cycle[29-31], the association of menstrual cycle with neutrophil function has not been studied.

The secretions of sex steroid hormones are controlled by gonadotropin. Luteinizing hormone (LH) and follicle stimulating hormone (FSH) are the main gonadotropin hormones, and both LH and FSH are reported to increase the reactive oxygen of neutrophils[27]. In other word, gonadotropin may influence to the association of

sex steroid hormones with neutrophil function as confounding factor.

Even in healthy people, individual difference exists in the immune function under the influence of age, physique and lifestyle habit. For the purpose of clarifying the effect of sex steroid hormones at immune function in various factors that influence to the immune function, the epidemiologic study for a greater number of general inhabitants is useful method to consider these factors.

In this study, we investigated the association of estradiol and testosterone with neutrophil function (basal ROS production, stimulated ROS production and PA) among healthy subjects from the general population.

Subjects and Methods

1. Participants

A total of 1167 adults of both sex aged >20 years living in the Iwaki region of Hirosaki City in Aomori Prefecture, northern Japan participated in this study. All participants attended the 2014 Iwaki Health Promotion Project. The purpose of this project is to maintain and promote the health of local people in the community to prevent lifestyle-related diseases and prolong their lifespans. Approval for the study was obtained from the ethics committee of Hirosaki University Graduate School of Medicine, and all subjects gave written informed consent prior to the research project.

Participants with malignant tumors, immune disorders, gynecological diseases, or diabetes mellitus; those taking steroid, anti-inflammatory drugs (NSAIDs), or sex steroid hormones; pregnant and lactating females, or premenopausal females with irregular menstruation; and participants with a lower detection limit, which varied for sex steroid hormones, and missing data were excluded from the study. A total of 627 participants (85

males, 123 premenopausal females, and 419 postmenopausal females) were excluded. Finally, a total of 540 participants (358 males, 139 premenopausal females, and 43 postmenopausal females) were finally enrolled into the study.

2. Lifestyle habits and physical measurements

Self-reported questionnaires were sent to subjects prior to the investigation day, and these were collected after reviewing the answers during personal interviews on the day of the study. In the questionnaire, subjects were asked about their age, sex, current illnesses, past illnesses, medication histories, smoking habits (daily number of cigarettes and years of smoking), alcohol use (daily alcohol volume), and exercise habits (frequencies of weekly exercise). Pack-years and the quantity of pure alcohol consumed per day were then calculated. Subjects who exercised more than once per week were defined as the exercise habit group. Furthermore, females were also asked about menstruation and menopause. Body mass index [BMI, weight (kg)/height (cm)^2] was calculated as an index of obesity.

3. Blood parameters

Blood samples were collected on the day of investigation when the subjects were under fasting conditions. Neutrophil counts were measured using an automated blood cell analyzer. Measurements of estradiol, testosterone, luteinizing hormone (LH), and follicle stimulating hormone (FSH) levels were handled by the LSI Medience Corporation after serum was separated from the whole blood by centrifugation. Estradiol, testosterone, luteinizing hormone (LH), and follicle stimulating hormone (FSH) levels were measured using chemiluminescent immunoassay.

ROS generation and PA in peripheral blood neutrophils

Basal ROS production is considered to be a reflection of oxidative stress[16]. In contrast, stimulated ROS production and PA reflects the activity of neutrophils to remove foreign substances, such as bacteria[15].

Neutrophil function (basal ROS production, stimulated ROS production and PA) were measured using the flow cytometry (Becton Dickinson, San Jose, CA, USA) using the two-color method. ROS production was measured using the ROS-reacting fluorescent agent hydroethidine (HE; Polyscience Inc., Warrington, PA, USA). HE, a redox-sensitive probe, has been widely used to detect intracellular superoxide anion[32]. Phagocytic capacity was measured using fluorescein isothiocyanate (FITC)-labeled opsonized zymosan (OZ) (Sigma Chemical Co., St Louis, MO, USA)[32].

In brief, 44 μL of 8 μM HE (Polyscience) was added to 200-μL aliquots of heparinized whole blood and then incubated at 37°C for 5 min. A 100-μL aliquot of the mixture was added to 25 μL (5 mg/mL) of FITC-OZ and incubated at 37°C for 35 min to measure neutrophilic phagocytosis of FITC-OZ. Basal ROS production was measured in non-stimulated neutrophils that were not treated with FITC-OZ, and stimulated ROS production was measured in FITC-OZ-treated neutrophils.

After incubation, 1 mL of a hemolytic agent was added to each sample and mixed well. After confirming hemolysis of the red blood cells, 250 μL of the fixative (Polyscience) was added to the samples, and they were allowed to stand for 5 min. The samples were washed twice in phosphate-buffered saline containing sodium azide, and 50 μL of 5 % paraformaldehyde was then added. To measure the phagocytic activity, 30 μL (0.25 mg/mL) of trypan blue was added to the samples just before measuring fluorescence intensity (FI) to exclude FITC-OZ, which was not incorporated into the neutrophils or adherent to their surfaces.

In flow cytometry, neutrophils were irradiated with a 488-nm laser beam generated from a 15-mW argon laser with forward- and side-scattering emission, which was simultaneously recorded. Green fluorescence generated from FITC was detected through a 530-nm filter, and orange fluorescence generated from HE was detected through a 585-nm filter. FI was measured as the value of neutrophils per 10,000 screened from the forward- and side-scattering emission for each sample. Cumulative FI, i.e., the sum of the values of FI multiplied by the percentage of positive cells, was used as a quantitative index.

In this study, the amount of superoxide production was used as an index of neutrophilic ROS production. Superoxide is the upstream substance of ROS metabolism, and all of the ROS are metabolites of superoxide. Accordingly, the amount of superoxide production is considered to be the reflection of the entire production of ROS. Therefore, superoxide might be an indicator of the approximate amount of ROS production that causes oxidative stress.

4. Statistical analyses

Statistical analyses were performed after participants were divided into four groups. First, participants were divided into two groups on the basis of sex. Second, females were divided into pre- and postmenopausal groups. For further analysis, premenopausal females were divided into two groups around ovulation day. We defined the menstruation cycle from 1 to 14 days groups as "follicular phase group" and the menstruation cycle 15 days later groups as "luteal phase group" for convenience.

One-way analysis of variance with Tukey's test and chi-squared tests were used to determine differences in the characteristics of participants. Bivariate (Spearman) correlation coefficients were used to analyze the association of neutrophil function (basal ROS production, stimulated ROS production, and PA) with age,

Table 1. Characteristics of the participants

	Males (n=358)	Females		
		Follicular phase (n=63)	Luteal phase (n=76)	Postmenopausal (n=43)
Age（year）	51.0 ± 15.0	36.9 ± 7.4**	38.1 ± 7.3**	62.9 ± 13.1** ††‡‡
BMI（kg/m²）	23.6 ± 3.2	20.5 ± 2.5**	21.5 ± 3.9**	23.8 ± 4††‡‡
Exercise habits（%）	32.7	19.0	18.4	32.6♯
Pack-Years	14.9 ± 20.3	3.2 ± 5.9**	3.2 ± 7.0**	2.5 ± 6.8**
Alcohol use（g/day）	23.3 ± 24.0	7.8 ± 14.5**	3.8 ± 9.5**	2.3 ± 7.3**
Estradiol（pg/mL）	22.0 ± 7.1	106.4 ± 120.2**	129.6 ± 76.8**†	17.7 ± 19.2††‡
Testosterone（ng/mL）	5.7 ± 1.7	0.3 ± 0.1**	0.3 ± 0.1**	0.3 ± 0.1**
LH（mIU/mL）	3.5 ± 3.0	6.6 ± 8.2**	5.3 ± 6.9	19.8 ± 10.0** ††‡‡
FSH（mIU/mL）	7.4 ± 7.4	6.4 ± 3.1	5.7 ± 9.4	53.4 ± 24.8** ††‡‡
Leukocyte cell counts（×10³/μL）	5.6 ± 1.5	5.0 ± 1.5*	5.1 ± 1.3*	5.1 ± 1.2
Neutrophils cell counts（×10³/μL）	3.1 ± 1.1	2.8 ± 1.1	2.8 ± 1.0	2.8 ± 1.0
Basal ROS production, ×10³, CFI	4.9 ± 7.4	5.4 ± 6.6	4.2 ± 3.6	6.5 ± 17.8
Stimulated ROS production, ×10³, CFI	532.5 ± 176.8	539.0 ± 151.4	525.3 ± 165.6	527.5 ± 185.8
Phagocytic activity, ×10³, CFI	1370.0 ± 493.9	1417.0 ± 462.4	1390.4 ± 462.5	1328.9 ± 411.2
Menstrual cycle（days）		28.3 ± 3.0	28.4 ± 2.4	

Data are presented as mean ± SD or number（%）

BMI: body mass index, CFI: cumulative fluorescence intensity

*p＜0.05 **p＜0.01, vs Male

†p＜0.05 ††p＜0.01, vs Follicular phase females

‡p＜0.05 ‡‡p＜0.01, vs Luteal phase females

♯p＜0.05, among groups

BMI, pack-year, and alcohol use. And the Mann-Whitney U test was used to compare neutrophil function（basal ROS production, stimulated ROS production, and PA as dependent valuables）between the exercise and non-exercise habit groups. Multiple regression analysis was used to analyze the association of gonadotropin（LH and FSH as independent valuables）and sex steroid hormones（estradiol and testosterone as independent valuables）with neutrophil functions（basal ROS production, stimulated ROS production and PA as dependent valuables）. Furthermore, after adjusted at LH and FSH, multiple regression analysis was used to analyze the association of sex steroid hormones with neutrophil functions. At all multiple regression analyses, age, BMI, exercise frequency, and pack-year served as adjusted parameters.

Although the main variables were in skewed distribution, multiple regression analysis was a robust analysis method and was suitable for multivariate analysis for the objective variable of neutrophil function.

Data analyses were performed using the Statistical Package for the Social Sciences（SPSS）version 12.0 J statistical software（SPSS Inc., Chicago, IL, USA）. The differences were considered to be statistically significant at p < 0.05, and the differences were considered to have a statistical trend at p < 0.1.

Result

1. Physical characteristics and blood biochemical values of participants and their lifestyle habits

The characteristics of the participants are listed in Table 1. The average age was higher in postmenopausal females than in other groups. BMI was higher in males and postmenopausal females than in premenopausal females. For

S. Sato, *et al.*

Table 2. Bivariate correlation of Basal ROS production, CFI with selected variables

| | Males | Females | | |
| | | Follicular phase | Luteal phase | Postmenopausal |
	P	ρ	ρ	ρ
Age	0.075	0.062	−0.049	−0.047
BMI	0.046	−0.053	−0.051	−0.300
Pack-years	−0.095	−0.105	−0.119	0.162
Alcohol use	−0.067	−0.042	−0.027	0.160

ρ : Spearman's rank correlation coefficient

Table 3. Bivariate correlation of Stimulated ROS production, CFI with selected variables

| | Males | Females | | |
| | | Follicular phase | Luteal phase | Postmenopausal |
	ρ	ρ	ρ	ρ
Age	0.059	−0.224	−0.134	−0.263
BMI	−0.055	−0.134	0.005	−0.263
Pack-years	0.131*	0.006	0.023	0.227
Alcohol use	−0.012	−0.049	−0.221	0.177

ρ : Spearman's rank correlation coefficient
*p<0.05

Table 4. Bivariate correlation of Phagocytic activity, CFI with selected variables

| | Males | Females | | |
| | | Follicular phase | Luteal phase | Postmenopausal |
	ρ	ρ	ρ	ρ
Age	0.095	0.066	0.106	−0.447**
BMI	0.027	0.050	−0.051	−0.400**
Pack-years	−0.081	0.103	−0.089	−0.139
Alcohol use	−0.034	−0.148	0.107	0.109

ρ : Spearman's rank correlation coefficient
**p<0.01

Table 5. Comparison of Basal ROS production, $\times 10^3$, CFI between exercise and non-exercise habit groups

	Exercise	Non-exercise
Males	5.2 ± 8.2	4.8 ± 7.0
Follicular phase	4.6 ± 2.8	5.6 ± 7.2
Luteal phase	5.6 ± 4.4*	3.9 ± 3.4
Postmenopausal	3.9 ± 2.3	7.8 ± 21.7

Values are mean±SD
*p<0.05

lifestyle habits, exercise frequencies per week were different among groups. Pack-year and alcohol use were higher in males than in females. For blood biochemical values, estradiol was higher in premenopausal females than in males and postmenopausal females. Furthermore, estradiol was higher in luteal phase females than in follicular phase females. Testosterone was higher in males than in females. LH and FSH were higher in postmenopausal females

Table 6. Comparison of Stimulated ROS production, $\times 10^3$, CFI between exercise and non-exercise habit groups

	Exercise	Non-exercise
Males	509.7 ± 160.7	543.6 ± 183.4
Follicular phase	562.7 ± 155.9	533.4 ± 151.3
Luteal phase	564.6 ± 160.2	516.4 ± 166.7
Postmenopausal	525.7 ± 191.2	528.3 ± 186.5

Values are mean±SD

Table 7. Comparison of Phagocytic activity, $\times 10^3$, CFI between exercise and non-exercise habit groups

	Exercise	Non-exercise
Males	1330.8 ± 513.6	1389.0 ± 484.0
Follicular phase	1356.4 ± 567.7	1431.3 ± 439.4
Luteal phase	1495.0 ± 377.4	1366.8 ± 479.1
Postmenopausal	1171.1 ± 292.1	1405.0 ± 442.2

Values are mean±SD

Table 8. Multiple regression analysis with gonadotropin（Males）

Dependent variable	Independent variable	β	p	R^2
Basal ROS production, CFI	LH	−0.061	0.301	0.015
	FSH	−0.057	0.365	0.014
Stimulated ROS production, CFI	LH	−0.089	0.135	0.018
	FSH	−0.105	0.091	0.020
Phagocytic activity, CFI	LH	−0.084	0.154	0.026
	FSH	−0.094	0.130	0.026

Values are adjusted for age, body mass index, exercise habit and pack-year.
β: standardized partial regression coefficient, R^2: coefficient of determination
CFI: cumulative fluorescence intensity,   ROS: reactive oxygen species

than other groups. Leukocyte counts was higher in males than in premenopausal females. In premenopausal females, the average length of the menstrual cycle was 28 days.

2. Association of neutrophil function with age, BMI, and lifestyle habits

Bivariate correlations of neutrophil function with age, BMI, pack-years, and alcohol use are listed in Table 2–4. In males, stimulated ROS production positively correlated with pack-years. In postmenopausal females, phagocytic activity negatively correlated with age and BMI. Comparisons of neutrophil function between the exercise and non-exercise habit groups are listed in Table 5–7. In premenopausal females after menstruation, basal ROS production was higher in the exercise habit group than in the non-exercise habit group.

3. Association of LH and FSH with neutrophil function

In males, the FSH level negatively correlated with stimulated ROS production（table 8）. Although we could not detect any significant correlations in follicular phase females, both LH and FSH levels positively correlated with basal ROS production in luteal phase females （table 9, 10）. In contrast, we could not detect any significant correlations in postmenopausal

S. Sato, *et al.*

Table 9. Multiple regression analysis with gonadotropin（Premenopausal females in follicular phase）

Dependent variable	Independent variable	β	p	R^2
Basal ROS production, CFI	LH	0.112	0.405	0.032
	FSH	−0.013	0.926	0.020
Stimulated ROS production, CFI	LH	−0.100	0.440	0.085
	FSH	0.052	0.694	0.078
Phagocytic activity, CFI	LH	−0.147	0.275	0.032
	FSH	−0.128	0.347	0.026

Values are adjusted for age, body mass index, exercise habit and pack-year.
β: standardized partial regression coefficient,   R^2: coefficient of determination
CFI: cumulative fluorescence intensity,   ROS: reactive oxygen species

Table 10. Multiple regression analysis with gonadotropin（Premenopausal females in luteal phase）

Dependent variable	Independent variable	β	p	R^2
Basal ROS production, CFI	LH	0.361	0.002	0.173
	FSH	0.591	<0.001	0.356
Stimulated ROS production, CFI	LH	0.110	0.362	0.045
	FSH	0.022	0.863	0.034
Phagocytic activity, CFI	LH	0.135	0.259	0.056
	FSH	0.199	0.109	0.073

Values are adjusted for age, body mass index, exercise habit and pack-year.
β: standardized partial regression coefficient,   R^2: coefficient of determination
CFI: cumulative fluorescence intensity,   ROS: reactive oxygen species

Table 11. Multiple regression analysis with gonadotropin (Postmenopausal females)

Dependent variable	Independent variable	β	p	R^2
Basal ROS production, CFI	Estradiol	0.012	0.944	0.046
	Testosterone	−0.005	0.978	0.045
Stimulated ROS production, CFI	Estradiol	−0.103	0.535	0.136
	Testosterone	−0.080	0.670	0.132
Phagocytic activity, CFI	Estradiol	−0.129	0.364	0.368
	Testosterone	−0.002	0.992	0.354

Values are adjusted for age, body mass index, exercise habit and pack-year.
β: standardized partial regression coefficient,   R^2: coefficient of determination
CFI: cumulative fluorescence intensity,   ROS: reactive oxygen species

females（table 11）.

4. Association of estradiol and testosterone with neutrophil function

In males, the estradiol level negatively correlated with basal ROS production, and the testosterone level positively correlated with stimulated ROS production and PA（table 12）. Although we could not detect any significant correlations in follicular phase females, both estradiol and testosterone levels negatively correlated with basal ROS production in luteal phase females（table 13, 14）. In contrast, we could not detect any significant correlations in

Table 12. Multiple regression analysis with sex hormones（Males）

Dependent variable	Independent variable	β	p	R^2
Basal ROS production, CFI	Estradiol	−0.126	0.019	0.028
	Testosterone	−0.056	0.323	0.015
Stimulated ROS production, CFI	Estradiol	−0.025	0.640	0.012
	Testosterone	0.117	0.038	0.024
Phagocytic activity, CFI	Estradiol	−0.087	0.104	0.027
	Testosterone	0.135	0.016	0.036

Values are adjusted for age, body mass index, exercise habit and pack-year.
β: standardized partial regression coefficient, R^2: coefficient of determination
CFI: cumulative fluorescence intensity, ROS: reactive oxygen species

Table 13. Multiple regression analysis with sex hormones
（Premenopausal females in follicular phase）

Dependent variable	Independent variable	β	p	R^2
Basal ROS production, CFI	Estradiol	0.054	0.698	0.024
	Testosterone	0.046	0.759	0.023
Stimulated ROSproduction, CFI	Estradiol	−0.121	0.372	0.088
	Testosterone	0.073	0.621	0.079
Phagocytic activity, CFI	Estradiol	−0.059	0.675	0.014
	Testosterone	0.138	0.361	0.026

Values are adjusted for age, body mass index, exercise habit and pack-year.
β: standardized partial regression coefficient, R^2: coefficient of determination
CFI: cumulative fluorescence intensity, ROS: reactive oxygen species

Table 14. Multiple regression analysis with sex hormones（Premenopausal females in luteal phase）

Dependent variable	Independent variable	β	p	R^2
Basal ROS production, CFI	Estradiol	−0.320	0.007	0.145
	Testosterone	−0.348	0.004	0.155
Stimulated ROS production, CFI	Estradiol	0.188	0.120	0.000
	Testosterone	0.132	0.293	0.049
Phagocytic activity, CFI	Estradiol	−0.040	0.744	0.040
	Testosterone	−0.075	0.551	0.043

Values are adjusted for age, body mass index, exercise habit and pack-year.
β: standardized partial regression coefficient, R^2: coefficient of determination
CFI: cumulative fluorescence intensity, ROS: reactive oxygen species

postmenopausal females（table 15）.

5. Association of estradiol and testosterone with neutrophil function after adjusted at LH and FSH

In males, the estradiol level negatively correlated with basal ROS production, and the testosterone level positively correlated with stimulated ROS production and PA（table 16）. Although we could not detect any significant correlations in follicular phase females, both estradiol and testosterone levels negatively correlated with basal ROS production in luteal

S. Sato, *et al.*

Table 15. Multiple regression analysis with sex hormones (Postmenopausal females)

Dependent variable	Independent variable	β	p	R^2
Basal ROS production, CFI	Estradiol	−0.015	0.934	0.046
	Testosterone	0.044	0.802	0.047
Stimulated ROSproduction, CFI	Estradiol	0.200	0.234	0.160
	Testosterone	0.182	0.275	0.155
Phagocytic activity, CFI	Estradiol	0.015	0.918	0.019
	Testosterone	0.098	0.497	0.362

Values are adjusted for age, body mass index, exercise habit and pack-year.
β: standardized partial regression coefficient,　R^2: coefficient of determination
CFI: cumulative fluorescence intensity,　ROS: reactive oxygen species

Table 16. Multiple regression analysis with sex hormones after adjusted at LH and FSH (Males)

Dependent variable	Independent variable	β	p	R^2
Basal ROS production, CFI	Estradiol	−0.123	0.023	0.030
	Testosterone	−0.052	0.365	0.017
Stimulated ROS production, CFI	Estradiol	−0.021	0.704	0.020
	Testosterone	0.123	0.030	0.033
Phagocytic activity, CFI	Estradiol	−0.084	0.124	0.033
	Testosterone	0.142	0.012	0.044

Values are adjusted for LH, FSH, age, body mass index, exercise habit and pack-year.
β: standardized partial regression coefficient,　R^2: coefficient of determination
CFI: cumulative fluorescence intensity,　ROS: reactive oxygen species

Table 17. Multiple regression analysis with sex hormones after adjusted at LH and FSH (Premenopausal females in follicular phase)

Dependent variable	Independent variable	β	p	R^2
Basal ROS production, CFI	Estradiol	−0.088	0.645	0.043
	Testosterone	<0.001	0.999	0.039
Stimulated ROS production, CFI	Estradiol	−0.015	0.934	0.100
	Testosterone	0.136	0.375	0.113
Phagocytic activity, CFI	Estradiol	−0.016	0.933	0.035
	Testosterone	0.169	0.286	0.055

Values are adjusted for LH, FSH, age, body mass index, exercise habit and pack-year.
β: standardized partial regression coefficient,　R^2: coefficient of determination
CFI: cumulative fluorescence intensity,　ROS: reactive oxygen species

phase females (table 17, 18). In contrast, we could not detect any significant correlations in postmenopausal females (table 19).

Discussion

To the best of our knowledge, this is the first study that looked at the influence of sex, menstrual cycle, and menopause on the association of sex steroid hormones with

Table 18. Multiple regression analysis with sex hormones after adjusted at LH and FSH
（Premenopausal females in luteal phase）

Dependent variable	Independent variable	β	p	R^2
Basal ROS production, CFI	Estradiol	−0.185	0.078	0.401
	Testosterone	−0.289	0.008	0.436
Stimulated ROS production, CFI	Estradiol	0.171	0.185	0.080
	Testosterone	0.089	0.511	0.061
Phagocytic activity, CFI	Estradiol	0.009	0.945	0.074
	Testosterone	−0.057	0.672	0.076

Values are adjusted for LH, FSH, age, body mass index, exercise habit and pack-year.
β: standardized partial regression coefficient,　　R^2: coefficient of determination
CFI: cumulative fluorescence intensity,　　ROS: reactive oxygen species

Table 19. Multiple regression analysis with sex hormones after adjusted at LH and FSH
（Postmenopausal females）

Dependent variable	Independent variable	β	p	R^2
Basal ROS production, CFI	Estradiol	−0.039	0.852	0.047
	Testosterone	0.054	0.772	0.049
Stimulated ROS production, CFI	Estradiol	0.310	0.108	0.200
	Testosterone	0.188	0.286	0.166
Phagocytic activity, CFI	Estradiol	0.174	0.280	0.429
	Testosterone	0.054	0.712	0.411

Values are adjusted for LH, FSH, age, body mass index, exercise habit and pack-year.
β: standardized partial regression coefficient,　　R^2: coefficient of determination
CFI: cumulative fluorescence intensity,　　ROS: reactive oxygen species

neutrophil function（basal ROS production, stimulated ROS production and PA）in a general population. We considered that gonadotropin influenced to the association of sex steroid hormones with neutrophil functions as confounding factors. Although we investigated the association of estradiol and testosterone with neutrophil function before and after adjusted at LH and FSH, the statistically significant or trend were observed.

Although we could not detect any significant association of sex steroid hormones with neutrophil function in follicular phase females and postmenopausal females in this study, we were able to detect a significant association in males and luteal phase females. Our results suggest that sex differences exist in the association of sex steroid hormones with neutrophil function and are influenced by factors relating to the menstrual cycle.

In luteal phase of premenopausal females, the corpus luteum secretes high level of progesterone. Progesterone maintains endometrial receptivity for the implantation of the fertilized eggs and raises the basal body temperature. Furthermore, progesterone are considered to have anti-inflammatory effects by attenuating TNF-α, nitric oxide and prostaglandin E_2[33]. Although we did not measure progesterone, the possibility that the significant association of sex steroid hormones with neutrophil function was observed because of progesterone in the luteal phase females was considered.

In our study, the association of sex steroid

hormones with neutrophil function was observed in males. The testosterone level is very higher in males than females. Testosterone suppresses pro-inflammatory cytokines such as TNF-α, Interleukin-1β and interleukin-6 and increases anti-inflammatory effect such as interleukin-10[34, 35]. Furthermore, testosterone suppresses the adaptive immune system by decreasing antibody production and promoting the apoptosis of lymphocytes[11, 12]. We considered the possibility that because of anti-inflammatory effects of testosterone, the significant association of sex steroid hormones with neutrophil function was observed in males.

In our study, both estradiol and testosterone levels negatively correlated with basal ROS production. Therefore, as a mechanism to decrease the oxidative stress of neutrophils, sex steroid hormones might have suppressed the basal ROS production of neutrophils. We were able to detect a significant association of both estradiol and testosterone with basal ROS production in females. In contrast, the significant association of testosterone with basal ROS production was not observed in males.

Marin et al. have reported that the decreasing influence of testosterone on the oxidative stress of neutrophils attenuate in high testosterone concentrations[20]. Furthermore, Posma et al. have reported that the levels of inflammatory cytokines, such as IL-12 and IL-1β from monocytes, increased when testosterone levels were not in high concentrations[36].

These phenomena suggest that the influence of testosterone on oxidative stress attenuates or disappears at high testosterone concentrations. Because testosterone levels are much higher in males than in females, the association of testosterone with basal ROS production might not be observed. Furthermore, neutrophil function can be influenced by various cytokines[25, 26], and the influence of cytokines on neutrophil function might contribute to our

result as confounding factors.

Stimulated ROS production and PA reflect the activity to remove foreign substances[15]. Although various studies have investigated the association of sex steroid hormones with the neutrophils' activity to remove foreign substances, consensus has not been obtained[20-24, 37]. In our study, no significant association of sex steroid hormones with stimulated ROS production and PA was observed in females; however, we observed a significant association in males. In this reason, the high testosterone concentration or cytokines which are modulated by testosterone might influence the association of sex steroid hormones with neutrophil's activity to remove foreign substances. And, in females, the small influence of sex steroid hormones on neutrophil's activity to remove foreign substances.

In our study, testosterone level positively correlated with stimulated ROS production and PA. There are several differences between previous studies and our study with regards to experimental methods. For instance, previous studies have used isolated neutrophils, although neutrophils can be influenced by various cytokines[25, 26]. Therefore, we used the whole blood to measure neutrophil function. Furthermore, the number of subjects in previous studies has ranged from several to dozens, and they were solely young males or females. In the current study, we simultaneously investigated a greater number of subjects (545 adults in total) of both sex and in a wide range of age groups. Furthermore, confounding factors such as gonadotropin, age, physique and lifestyle habits were adjusted in our study. These differences in experimental methods might have caused the differences in results.

Although some studies have stated the decreasing influence of sex steroid hormones on oxidative stress of neutrophils, they differ in results showing an association of sex steroid

hormones with neutrophils' activity to remove foreign substances. Therefore, this latter association between sex steroid hormones and neutrophils' foreign substances removal activity is considered to be weaker than the oxidative stress of neutrophils.

However, our study has some limitations. First, we only used interviews to determine the menstrual cycles of participants. To divide premenopausal females into two groups around ovulation day, we interviewed premenopausal females regarding the number of days after menstruation and whether that menstruation was regular, and then divided them into two groups, i.e., before and after the 14th day of menstrual cycle. However, there are significant differences in menstrual cycles even in females whose menstrual cycles are regular. Second, although we did not measure progesterone. Therefore, the measurements of basal body temperature, progesterone levels are necessary to evaluate the menstrual cycle more accurately. Third, this study used multivariate analysis to investigate the association of sex steroid hormones with neutrophil function. Although we adjusted LH, FSH, age, BMI, pack-years, and exercise habit that the statistically significances were observed in between neutrophil function as confounding factors, the association of sex steroid hormones with neutrophil function can be influenced by other various factors, such as various cytokines and steroid hormones. So, these factors that we did not measure might tend to influence our results.

Conclusion

In our study, we investigated the association of estradiol and testosterone with neutrophil function（basal ROS production, stimulated ROS production and PA）among normal healthy adults in the general population. Both estradiol and testosterone were found to influence neutrophil function. Furthermore, the association of sex steroid hormones with neutrophil function was found to vary according to the menstrual cycle in addition to sex difference.

Reference

1）Klein SL. The effects of hormones on sex differences in infection: from genes to behavior. Neurosci Biobehav Rev 2000;24:627-38.

2）Klein SL, Poland GA. Personalized vaccinology: one size and dose might not fit both sexes. Vaccine 2013;31:2599-600.

3）Ngo ST, Steyn FJ, McCombe PA. Gender differences in autoimmune disease. Front Neuroendocrinol 2014;35:347-69.

4）Rhonda Voskuhl. Sex differences in autoimmune diseases. Biol Sex Differ 2011;2:1.

5）Dorak MT, Karpuzoglu E. Gender Differences in Cancer Susceptibility: An Inadequately Addressed Issue. Front Genet 2012;3:268.

6）Bouman A, Heineman MJ, Faas MM. Sex hormones and the immune response in humans. Hum Reprod Update 2005;11:411-23.

7）Porter VR, Greendale GA, Schocken M, Zhu X, Effros RB. Immune effects of hormone replacement therapy in post-menopausal women. Exp Gerontol 2001;36:311-26.

8）Weetman AP, McGregor AM, Smith BR, Hall R. Sex hormones enhance immunoglobulin synthesis by human peripheral blood lymphocytes. Immunol Lett 1981;3:343-6.

9）Kanda N, Tamaki K. Estrogen enhances immunoglobulin production by human PBMCs. J Allergy Clin Immunol 1999;103:282-8.

10）Olsen NJ, Kovacs WJ. Gonadal steroids and immunity. Endocr Rev 1996;17:369-84.

11）Kanda N, Tsuchida T, Tamaki K. Testosterone inhibits immunoglobulin production by human peripheral blood mononuclear cells. Clin Exp Immunol 1996;106:410-5.

12）McMurray RW, Suwannaroj S, Ndebele K, Jenkins

JK. Differential effects of sex steroids on T and B cells: modulation of cell cycle phase distribution, apoptosis and bcl-2 protein levels. Pathobiology 2001;69:44-58.

13) Benestad HB, Laerum OD. The neutrophilic granulocyte. Curr Top Pathol 1989;79:7-36.

14) Sawyer DW, Donowitz GR, Mandell GL. Polymorphonuclear neutrophils: an effective antimicrobial force. Rev Infect Dis 1989;11:S1532-44.

15) Smith JA. Neutrophils, host defense, and inflammation: a double-edged sword. J Leukoc Biol. 1994;56:672-86.

16) Finkel T. Oxidant signals and oxidative stress. Curr Opin Cell Biol 2003;15:247-54.

17) Skafar DF, Xu R, Morales J, Ram J, Sowers JR. Clinical review 91: Female sex hormones and cardiovascular disease in women. J Clin Endocrinol Metab 1997;82:3913-8.

18) Lefèvre G, Beljean-Leymarie M, Beyerle F, Bonnefont-Rousselot D, Cristol JP, Thérond P, Torreilles J. Evaluation of lipid peroxidation by measuring thiobarbituric acid reactive substances. Ann Biol Clin (Paris) 1998;56:305-19.

19) Wakatsuki A, Ikenoue N, Sagara Y. Effects of estrogen on susceptibility to oxidation of low-density and high-density lipoprotein in postmenopausal women. Maturitas 1998;28:229-34.

20) Marin DP, Bolin AP, dos Santos Rde C, Curi R, Otton R. Testosterone suppresses oxidative stress in human neutrophils. Cell Biochem Funct 2010;28:394-402.

21) Békési G1, Kakucs R, Várbíró S, Rácz K, Sprintz D, Fehér J, Székács B. In vitro effects of different steroid hormones on superoxide anion production of human neutrophil granulocytes. Steroids 2000;65:889-94.

22) Molloy EJ, O'Neill AJ, Grantham JJ, Sheridan-Pereira M, Fitzpatrick JM, Webb DW, Watson RW. Sex-specific alterations in neutrophil apoptosis: the role of estradiol and progesterone. Blood 2003;102:2653-9.

23) Miyagi M, Aoyama H, Morishita M, Iwamoto Y. Effects of sex hormones on chemotaxis of human peripheral polymorphonuclear leukocytes and monocytes. J Periodontol 1992;63:28-32.

24) Cassidy RA. Influence of steroids on oxidant generation in activated human granulocytes and mononuclear leukocytes. Shock 2003;20:85-90.

25) Bokoch GM. Chemoattractant signaling and leukocyte activation. Blood 1995;86:1649-60.

26) Yoshie O, Imai T, Nomiyama H. Chemokines in immunity. Adv Immunol 2001;78:57-110.

27) Shirai F, Kawaguchi M, Yutsudo M, Dohi Y. Human peripheral blood polymorphonuclear leukocytes at the ovulatory period are in an activated state. Mol Cell Endocrinol 2002;196:21-8.

28) Maybin JA, Critchley HO. Menstrual physiology: implications for endometrial pathology and beyond. Hum Reprod Update 2015;21:748-61.

29) Tikare SN, Das KK, Dhundasi SA. Blood leukocyte profile in different phases of menstrual cycle. Indian J Physiol Pharmacol 2008;52:201-4.

30) Bouman A, Moes H, Heineman MJ, de Leij LF, Faas MM. The immune response during the luteal phase of the ovarian cycle: increasing sensitivity of human monocytes to endotoxin. Fertil Steril 2001;76:555-9.

31) Rajnee A, Binawara B.K, Choudhary S, Chawla V.K, Choudhary R. Haematological and electrocardiographic variations during menstrual cycle. Pak J Physiol 2010;6:18-21.

32) Takahashi I, Umeda T, Mashiko T, Chinda D, Oyama T, Sugawara K, Nakaji S. Effects of rugby sevens matches on human neutrophil-related non-specific immunity. Br J Sports Med 2007;41:13-8.

33) Lei B, Mace B, Dawson HN, Warner DS, Laskowitz DT, James ML. Anti-Inflammatory Effects of Progesterone in Lipopolysaccharide-Stimulated BV-2 Microglia. PLoS One 2014; 9:e103969.

34) Bobjer J, Katrinaki M, Tsatsanis C, Lundberg Giwercman Y, Giwercman A. Negative association between testosterone concentration and inflammatory markers in young men: a nested cross-sectional study. PLoS One 2013;8:e61466.

35) Maggio M, Basaria S, Ceda GP, Ble A, Ling SM, Bandinelli S, Valenti G, Ferrucci L. The relationship

between testosterone and molecular markers of inflammation in older men. J Endocrinol Invest. 2005;28:116-9.

36）Posma E, Moes H, Heineman MJ, Faas MM. The effect of testosterone on cytokine production in the specific and non-specific immune response. Am J Reprod Immunol. 2004;52:237-43.

37）Lagranha CJ, Alba-Loureiro TC, da Silva AI, de Lima DDB, Pedroza AA, Ferreira DS, Pithon-Curi TC. Differences in age modulates neutrophils function. Advances in Biological Chemistry 2014;4:51-58.