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Article

Usefulness of Skin Autofluorescence as a Biomarker of Oxidative Stress in Young Japanese Long-distance Runners: A Cross-Sectional Study

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Abstract: Chronic oxidative stress in long-distance runners adversely affects conditioning. Therefore, it is important to objectively assess and monitor oxidative stress but measuring oxidative stress can be invasive or require skill to measure. Therefore, this study aimed to verify whether skin autofluorescence (SAF), a noninvasive, rapid, and easily calculable metric for calculating advanced glycation end products (AGEs), is useful as an oxidative stress biomarker. The subjects were 50 young Japanese male long-distance runners (aged 20.2 ± 1.2 years); 35 average male university students (aged 19.8 ± 1.1 years) served as controls. The interactions and relationships between SAF and plasma pentosidine and oxidative stress markers (reactive oxygen metabolite-derived compounds [d-ROMs], biological antioxidant potential [BAP], and the BAP/d-ROMs ratio) in runners were examined, and SAF in the runners and controls was compared. The results suggest that plasma pentosidine in runners is associated with oxidative stress markers and that plasma pentosidine can assess oxidative stress. However, SAF was not validated as an oxidative stress marker because it was not associated with oxidative stress marker. In future, clarifying the factors affecting SAF may clarify the relationship between SAF, plasma pentosidine, and oxidative stress markers.

Keywords: advanced glycation end products; skin autofluorescence; pentosidine; d-ROMs; BAP; BAP/d-ROMs ratio; athletes; runner

1. Introduction

Long-distance runners' prolonged and intense exercise induces oxidative stress when the body's defence mechanisms are overwhelmed by the formation of reactive oxygen species (ROS), a collective term for oxygen molecules derived from highly reactive oxygen molecules (O_2) and others [1]. Oxidative stress has been shown to adversely affect skeletal muscle fatigue and immune function [2,3]. Their chronicity in long-distance runners may lead to adversely affects conditioning. Therefore, quantification and objective assessment and monitoring of oxidative stress are important to minimise these risks. Although several biomarkers exist to assess exercise-induced oxidative stress in athletes, measurement of these markers is invasive because it requires blood sampling or, because of the unstable nature of ROS, the several markers require specialized technology to measure [4]. Assessment of oxidative stress should be noninvasive and simple for applications, including the early detection of the risk of sports injuries and overtraining.

High oxidative stress in vivo promotes the formation of advanced glycation end products (AGEs) [5]. Pentosidine, a major AGE, is formed by non-enzymatic reactions and oxidation processes of arginine residues and lysine groups, and unlike the unstable ROS, it is a product with a stable structure and exhibits fluorescence and cross-linking [6]. Because of its properties, pentosidine is useful as a marker to assess and predict the onset and progression of diseases associated with oxidative stress [7,8]. AGEs with fluorescent

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functions, including pentosidine, can be estimated noninvasively, quickly, and simply as skin autofluorescence (SAF) using the AGE Reader [9]. SAF has been reported to be associated with a number of diseases affected by chronic glycation and oxidative stress. SAF is a useful predictive biomarker for the evaluation of peripheral artery disease (PAD) and cardiovascular disease mortality in patients with renal failure and diabetes [10-14].

In summary, SAF has the potential to be used to noninvasively assess chronic oxidative stress, but to our knowledge, no reports have been investigated in athletes with a focus on this metric. Therefore, this study was designed to examine the interactions and relationships between SAF and plasma pentosidine and oxidative stress-related markers to test whether SAF is a valid assessment index to estimate oxidative stress in long-distance runners. Study 1 will use controls as a point of comparison to obtain basic data on SAF in long-distance runners. Study 2 will examine the correlation between SAF, plasma pentosidine, and oxidation-related markers. Study 3 will examine whether SAF evaluates plasma pentosidine in long-distance runners. The hypothesis of this study is as follows: SAF in runners reflects plasma pentosidine concentrations and is associated with oxidative stress biomarkers.

2. Materials and Methods

2.1. Subjects

The study design was a cross-sectional. Participation in the study was by invitation. All surveys were conducted in August-September 2020. The participants in this study consisted of 52 long-distance runners from the distance division of the men's track and field department at the University of A as the runners' group and 39 male college students as the control group. All study participants were free of chronic diseases (e.g., diabetes, atopy, and other skin diseases). A reliable measure of SAF is possible in subjects with skin reflectance greater than 6% from I to IV of the Fitzpatrick skin typing [15,16], and no subjects with type V or higher in were included of this study.

The final analysis included 50 and 35 participants in the runners and control groups, respectively. Two persons who abstained from blood collection were excluded from the analysis of runners. In the case of the control group, three individuals in whom SAF was unable to be measured, as well as one smoker, were excluded. Smoking was used as an exclusion criterion because tobacco is a factor that increases the concentration of AGEs in the body [17].

The runners' athletic status was at a comparatively elite level of competitiveness among universities, practicing twice a day, six times a week. The controls confirmed prior to conducting the study that they were not athletes and were not involved in any club activities that involved physical activity. Runners' bodies and SAF levels were measured at the subjects' dormitory (Tokyo, Japan), and blood samples were taken at a general hospital (Tokyo, Japan). The controls' bodies and SAF were measured in a classroom on the university campus (Shizuoka, Japan).

This study was approved by the Ethics Review Committee of the College of International Relations of Nihon University (2020-001) on August 6, 2020, and all procedures were performed in compliance with the Declaration of Helsinki. All subjects were informed orally and in writing about the purpose and methods of this study in advance, and their written consent to participate in the study was obtained after they fully understood the content of the study.

2.2. Anthropometry

Height was measured using a height meter with an accuracy of 0.1 cm. Runners' weight were measured using InBody720 (Inbody Japan Co., Tokyo, Japan). Controls' body weights were measured using a multi-frequency body composition analyser MC-190 (Tanita Corp., Tokyo, Japan). Body mass index (BMI) was calculated by dividing weight (kg) by height squared (m^2).

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2.3. SAF

SAF was measured using the AGE Reader (AGE Reader, Diagnoptics Technologies B.V., Groningen, The Netherlands, Japanese import agent Serista Corporation). The instrument irradiates the skin with an excitation wavelength (300–420 nm), measures the fluorescence wavelength (420–600 nm) emitted from the skin, and automatically expresses the integral value of the fluorescence intensity as a SAF value (unit: AU). Higher values indicate higher amounts of AGEs. Measurements were taken in a sitting position, and the measurement site was the inner forearm (5–10 cm below the elbow). Previous studies have established the validity and reliability of this measurement site for measuring SAF values in Japanese people [18]. A location free of scars, moles, or bruises was selected, and adjacent areas were measured three times, shifting the location slightly, and the average value was used as the representative value. Since products such as skin lotions and sunscreens affect the measurement results [19], we ensured that the subjects did not use them. As a precaution, the measurement site was disinfected with an alcohol wipe and dried well before measurement.

2.4. Biochemical measurements

Blood samples were taken only for runners. Blood samples were drawn from the elbow vein on the same day as body composition and SAF measurements. In terms of AGE concentration, plasma pentosidine levels (FSK PEN ELISA kit; Fushimi Pharmaceutical Co., Marugame, Japan) were tested using enzyme-linked immunosorbent assay (ELISA) kits. The FSK PEN ELISA kit has been found to correlate well with values measured by high-performance liquid chromatography (r = 0.936) [20]. In addition, creatine-kinase (CK) and lactate-dehydrogenase (LDH), indicators of muscle damage, were included in the survey as secondary outcomes. Their analysis was outsourced to a laboratory (SRL, Inc., Tokyo, Japan).

Since the oxidative stress of a living body is determined by the equilibrium between oxidation and anti-oxidation, it is necessary to evaluate the phenomenon as oxidative balance by measuring oxidation alongside multiple other biomarkers. Reactive oxygen metabolite-derived compounds (d-ROMs) and biological antioxidant potential (BAP) can measure and evaluate oxidative stress and antioxidant capacity, respectively [21-23]. By combining them, the ratio of BAP/d-ROMs can be calculated to evaluate which indicates the equilibrium and balance between oxidative stress and antioxidant capacity. The collected blood was immediately centrifuged at 3000 rpm for 10 minutes and stored in a freezer until assay. Each serum sample was analysed using a free radical analyser (FREE Carrio Duo, Diacron International SRL, Grosseto, Italy). The free radical analyser is an instrument equipped with a photometer, which automatically calculates d-ROMs and BAP values after inserting a cuvette into the measuring cell. The d-ROMs test can comprehensively evaluate oxidative stress in vivo by measuring the concentration of plasma hydroxyperoxide, produced by reactive oxygen species and free radicals in the body, using a colour reaction. Its validity has been proven by its correlation with free radical values using the electron spin resonance method [23].

For the assay procedure, 20 μL of serum sample is placed in a cuvette containing pH 4.8 buffer and gently inverted and stirred. Next, 20 μL of colourless chromogen (N, N-diethyl-paraphenylenediamine) was added to the cuvette, gently inverted, and stirred again, and the absorbance at 505 nm was measured. The principle of this measurement is that when blood is diluted with an acidic buffer solution of pH 4.8, Fe^{2+} and Fe^{3+} are separated from blood proteins and then act as catalysts to decompose hydroperoxide in the blood into alkoxyl radical and peroxy radical. The d-ROMs test value is calculated by calculating the change in absorbance of the coloured radical cations when adding a colourless colouring solution. The unit of measure is U.CARR, where 1 U.CARR corresponds to 0.08 mg/dL of hydrogen peroxide. The BAP test can evaluate antioxidant capacity by measuring the ability of an oxidant to give electrons to reactive oxygen species/free radicals and stop oxidative reactions.

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For the BAP test procedure, each serum sample (10 μL) was placed in a cuvette containing BAP colouring solution (50 μL) and inverted and stirred. The cuvettes were then placed in a thermostat for 5 minutes and then set in a photometer to measure absorbance at 505 nm. The principle of this measurement is that the trivalent iron salt $FeCl_3$ in the BAP colouring solution turns red as a function of trivalent iron Fe^{3+} ions when dissolved in a colourless solution containing a certain thiocyanate derivative. However, when added, plasma is reduced to divalent iron Fe^{2+} ions by the action of antioxidants in the plasma and decolourized. The BAP test value is calculated by calculating the amount of change in its colour. The coefficients of variation in previous studies ranged from 0.2 to 2.1% for d-ROM and from 0.1 to 1.1% for BAP [24].

2.5. Self-administered questionnaire survey

For the survey on mileage in runners, respondents were asked to answer, "Distance in km" to the question "Please tell us your weekly mileage (from the previous day to the past week).

The survey on exercise frequency given to the controls asked the question, "Do you currently exercise regularly?" and asked them to respond to the question, "Type of activity, time/day, frequency/week." If they did not exercise, they were asked to answer "none".

2.6. Statistical Analysis

Each statistical method was analysed using SPSS version 28 (IBM, Japan Inc., Tokyo, Japan). The significance level was set at 5% with a two-tailed test. The normality of each variable was evaluated using the Shapiro-Wilk test. Results are expressed as means ± standard deviations or the median values (25–75% CI). All analyses used normal or non-normal based test methods. Comparisons between two independent groups were made using either the unpaired t-test or the Mann-Whitney's U test. Spearman's rank correlation coefficient was used for correlation.

3. Results

3.1 Subjects' characteristics (Study 1)

The characteristics of the subjects (runners, n=50, controls, n=35) are shown in Table 1. The mean age of the runners was 20.3±1.2 years, and BMI was 19.1±1.1 kg/m^2. SAF, an indicator of AGEs, was 1.20 (1.0-1.3) AU, and plasma pentosidine was 0.0473±0.0103μg/mL. The d-ROMs test, a measure of exercise-related fatigue, was 274 ± 42 U.CARR. BAP test was 2143(2034-2291) μmol/L. The BAP/d-ROMs ratio was 8.2(7.4-9.9). CK, an indicator of muscle fatigue, was 406.3±310.5 U/L, and LDH was 213.5±39.6 U/L. BMI in the controls was within the range of the average BMI of Japanese 15-29-year-olds (21.1±3.6-22.9±4.1 kg/m^2) [25], confirming that the controls were indeed a group of average-sized Japanese men.

A comparison of the survey items between runners and controls showed that runners had significantly lower weight, BMI, and SAF than controls, while age and height were not significantly different (all $p>0.05$).

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Table 1. Subjects' characteristics. Values are mean ± SD, medians [IQRs]. Between-group comparisons were performed using the Student's t-test or Mann–Whitney U test. d-ROMs: diacron-reactive oxygen metabolites, BAP: biological antioxidant potential, CK: creatine kinase, LDH: lactate dehydrogenase.

Characteristics	Runners(n=50)	Controls(n=35)	*p Value*
Age, year (SD)	20.2 ±1 .2	19.8 ± 1.1	0.061
Height, cm (SD)	169.8 ± 5.0	170.3 ± 6.4	0.510
Weight, kg (SD)	55.3 ± 4.0	65.4 ± 3.1	＜0.001
Body mass index, kg/m² (SD)	19.1 ± 1.1	22.6 ± 1.0	＜0.001
Skin autofluorescence, AU (IQR)	1.20 (1.0-1.3)	1.30 (1.2-1.4)	0.011
Pentosidine, μg/L (SD)	0.0473 ± 0.0103	_	_
d-ROMs, U.CARR (SD)	274 ± 42	_	_
BAP, μmol/L (IQR)	2143 (2034-2291)	_	_
BAP/d-ROMs ratio (IQR)	8.2 (7.4-9.9)	_	
CK , U/L (SD)	406.3 ± 310.5	_	_
LDH, U/L (SD)	213.5 ± 39.6	_	_
Mileage, km/week (SD)	102.4 ± 52.0	_	_
Frequency of Exercise, times/week (SD)	_	0.8 ± 1.4	_
Exercise time, min/week (SD)	_	32.6 ± 63.6	_

3.2. Correlation between SAF and anthropometric values (Study 1)

Correlations between SAF and anthropometric values in runners and controls were examined (Table 2). The results showed that SAF in runners and controls were not associated with anthropometric values (age, height, weight, and BMI) (all p>0.05).

Table 2. Correlation between SAF and plasma pentosidine and anthropometric values.

	Skin autofluorescence (AU)			
	Runners(n=50)		Controls(n=35)	
	r	*p*	*r*	*p*
Age, yer	0.008	0.955	0.195	0.229
Height, cm	-0.128	0.387	-0.252	0.062
Weight, kg	-0.126	0.394	0.008	0.962
Body mass index, kg/m²	-0.025	0.868	0.121	0.471

Correlation coefficients (r) and P are calculated using the Spearman correlation analysis.

3.3. Correlation between SAF and plasma pentosidine and markers of oxidative stress and muscle damage (Study 2)

Correlations between SAF and plasma pentosidine and oxidation-related markers in runners were examined (Table 3). The results showed that plasma pentosidine was positively correlated with d-ROMs (r=0.299, p=0.035) and negatively correlated with the BAP/d-ROMs ratio (r=-0.321, p=0.023). SAF was not associated with any markers of oxidative stress and muscle damage (all p>0.05).

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Table 3. Correlation between SAF and plasma pentosidine and markers of oxidative stress and muscle damage.

	Skin autofluorescence (AU)	Pentosidine (μg/mL)		
	r	*r*	*r*	*p*
d-ROMs , U.CARR	0.124	0.391	0.299	0.035
BAP , μmol/L	-0.108	0.457	-0.134	0.352
BAP/d-ROMs ratio	-0.187	0.192	-0.321	0.023
CK , U/L	0.179	0.215	0.037	0.799
LDH, U/L	0.190	0.187	-0.084	0.562

Correlation coefficients (r) and P are calculated using the Spearman correlation analysis. d-ROMs: diacron-reactive oxygen metabolites, BAP: biological antioxidant potential, CK: creatine kinase, LDH: lactate dehydrogenase.

3.4. Correlation between SAF and plasma pentosidine in runners (Study 3)

When the correlation between SAF and plasma pentosidine in runners was examined, there was no significant difference (r=-0.023, p=0.872; Figure 1).

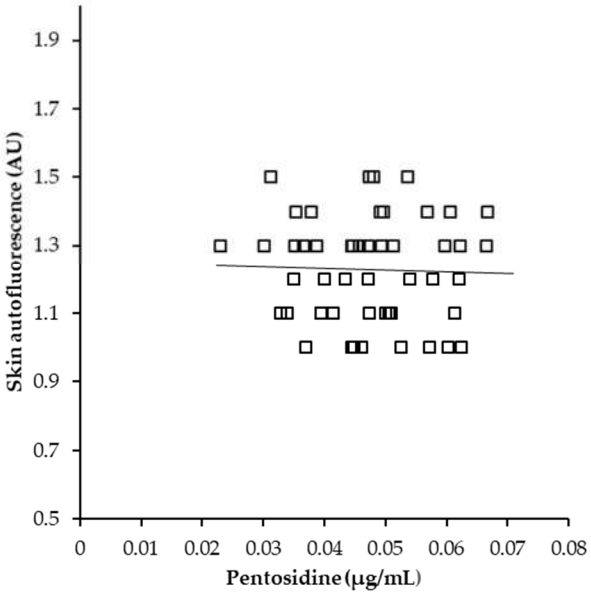

Figure 1. Correlation between SAF and plasma pentosidine in runners (n= 50). Correlation between SAF and plasma pentosidine in runner were performed using the Spearman correlation analysis. SAF and plasma pentosidine in runner were no significant difference (r=-0.023, p=0.872) .

4. Discussion

This study aimed to test whether SAF is a valid assessment index to estimate chronic oxidative stress in young Japanese long-distance runners and reported the interrelationship between SAF and plasma pentosidine and oxidative stress-related markers. This is the first report of its kind. Plasma pentosidine was positively correlated with d-ROMs and negatively correlated with BAP/ d-ROMs ratio, suggesting that plasma pentosidine concentration in long-distance runners is valuable in evaluating oxidative stress and oxidative stress balance. However, SAF was not associated with plasma pentosidine and oxidative stress-related markers, and the usefulness of SAF as an indicator for assessing oxidative stress was not confirmed in this study.

The plasma pentosidine concentration of runners in this study was 0.0473±0.0103μg/mL (Table 1), and this is higher than the reference value. The reference

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value, according to the manufacturer of the ELISA-based pentosidine test kit used in this study, is 0.00915–0.0431μg/mL [26]. Although regular exercise is believed to reduce AGE concentrations because it suppresses the formation of reactive oxygen species [27], acute and high-intensity exercise may cause an overproduction of reactive oxygen species and a progressive increase in blood AGEs concentrations. This paradox cannot be solved through only this study, it is desirable to accumulate research reports focusing on the effects of oxidative stress due to high-intensity exercise on AGEs accumulation.

Study 2 showed that plasma pentosidine concentrations were not only positively correlated with d-ROMs, the total oxidant capacity (r=0.299, p=0.035), but also negatively correlated with BAP/d-ROMs ratio, an index of antioxidant potential (r=-0.321, p=0.023; Table 3). Under oxidative stress, the production of sugar-derived carbonyl compounds, precursors of pentosidine, is enhanced, and pentosidine levels increase [28]. In addition, AGEs bind ligands to RAGE and activate NAPDH oxidase, which enhances ROS formation [29,30]. These relationships suggest that plasma pentosidine level is a marker that can assess oxidative stress status in long-distance runners.

On the other hand, SAF was not associated with oxidative stress markers and plasma pentosidine (Table 3; Figure 1). This is consistent with previous studies reporting that SAF failed to detect certain blood AGEs and was not consistent with variations in oxidative stress markers [31,32]. This may be due to the difference in metabolic turnover rates of AGEs in tissue and blood. The metabolic turnover rate of most proteins in the blood may be higher than long-lived proteins in tissues, such as collagen. Hoonhorst et al. also consider that this may be influenced by the presence of fluorescent substances in the skin [32]. Since SAF is calculated from the ratio of skin fluorescence to reflectance using light, it is influenced by several factors in the skin, including skin reflectance [33], and the presence of fluorescent substances in the skin [34,35]. In addition, melanin is a fluorescent substance and absorbs light in the ultraviolet and visible regions, and there has been concern that elevated melanin levels due to sun exposure may be a confounding factor for SAF [36]. Since the forearm is a site that does not tan throughout the year, risk factors due to sun exposure did not need to be considered in non-Athletes. However, because athletes training outdoors routinely have longer UV exposure times, the skin of athletes may be more affected by sunburn than non-Athletes [37]. The SAF for this study was measured in late August-early September. The Global Solar UV Index (UVI), an indicator of the level of solar ultraviolet radiation that comprehensively evaluates the degree of impact on the human body [38], was 7.4 in August [39], which was the highest value in Japan compared to other seasons, and it was a time when there was an impact of ultraviolet radiation compared to other seasons. This is the highest value in Japan compared to other seasons. Although all subjects in this study had no subjects above V in the Fitzpatrick skin types, it is possible that the runners had lower skin reflectance due to the effects of pigmentation from extreme sun exposure. Therefore, it is suggested that SAF in the runners in this study may have been affected by sunburn, and no association with plasma pentosidine could be found.

Study 1 also showed that SAF in runners was significantly lower than in controls (p=0.011). The results may also be influenced by the SAF-related factors mentioned above. Runners had lower SAF values than controls, probably due to skin pigmentation caused by sunburn and the absorption of excitation light necessary to excite fluorescent AGEs by the fluorescent substances (melanin, etc.) in the skin, which were elevated by sunburn.

Another reason could be that the beneficial effects of exercise may have worked to reduce glycation, and the amount of AGEs in the body was lower than in controls of the same age group. The exercise frequency for the controls in this study was 0.8±1.4 times/week, and the exercise duration was 32.6±63.6 minutes/week. These did not meet the WHO recommended of at least twice a week, 150-300 minutes of moderate-intensity exercise, or 75–300 minutes of high-intensity exercise [40], both of which are recommended by the WHO, so the controls in this study were a relatively inactive population. Regarding the relationship between SAF and the amount of physical activity, several

studies have reported that an increase in physical activity leads to a decrease in SAF [41,42]. However, there exist reports of no association between physical activity and SAF [43].

Furthermore, a previous study examining SAF in runners reported that SAF in runners and untrained controls was not significantly different between the two groups [41,42]. Thus, the average of SAF in runners and the effect of exercise on SAF remain controversial. Based on the above, we believe that a longitudinal examination of SAF, taking into account factors that influence SAF, will clarify basic data on SAF in runners and the relationship between SAF and plasma AGEs concentration.

This study has several limitations. First, because this is a cross-sectional, it is not possible to discuss the causal relationship between SAF and changes in oxidative stress markers, nor SAF and plasma pentosidine. Therefore, it is necessary to examine these longitudinally. In addition, this study did not validate the controls' oxidative stress markers and plasma pentosidine. It is possible that verifying these values in the controls could also clarify the nature of oxidative and glycative stress in runners and the relationship between them and SAF. Finally, this study did not examine tanning, skin colour, or other factors that affect SAF. Future studies may clarify the relationship between this investigation and oxidative stress by simultaneously examining factors that may affect SAF.

Despite these limitations, the present study suggests that plasma pentosidine in long-distance runners is associated with oxidative stress markers and that plasma pentosidine can assess oxidative stress. However, SAF was not associated with oxidative stress and plasma pentosidine and was not validated as an oxidative stress marker. In the future, clarifying the factors affecting SAF may clarify the relationship between SAF, plasma pentosidine, and oxidative stress.

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Institutional Review Board Statement: This study was approved by the Ethics Review Committee of the College of International Relations of Nihon University (2020-001) on August 6, 2020, and all procedures were performed in compliance with the Declaration of Helsinki.

Data Availability Statement: The data presented in this study are available on request from the corresponding author. The data are not publicly available due to privacy reasons.

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Conflicts of Interest: The authors declare no conflict of interest.

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