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Research article

Reference urinary biopyrrin level and physiological variation in healthy young adults: relation of stress by learning

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ABSTRACT

Background: Biopyrrins are end products of oxidation reactions of bilirubin with reactive oxygen, and urinary biopyrrin (UBP) levels might increase under oxidative stress. The authors examined the reference UBP level for healthy adults and its physiological variation in 40 healthy volunteers recruited from among students of our university (20 students each from third-year and fourth-year), and compared the results with data on 8-hydroxy-2-deoxyguanosine (8OHdG).

Methods: UBP and 8OHdG levels could be considered as oxidative stress markers. The UBP levels were measured with a competitive ELISA kit using biopyrrin antibody 24G7, according to the manufacturer's protocol. 8OHdG levels were measured with a Highly Sensitive 8-OHdG Check kit. UBP and 8OHdG measurements were performed in triplicate and means values calculated. For both parameters, creatinine (Cr) correction was performed using urinary creatinine levels measured by an enzymatic method.

Results: A comparison of the UBP levels between different grades revealed that the third-year students under high stress from clinical training and other course work tended to have a higher UBP level than fourth-year students. Therefore, we compared the current UBP levels in fourth-year students (samples collected in 2018) with their UBP level when they were in the third-year (samples collected in 2017) to examine the annual change. We found that the UBP level in 2017 samples was significantly higher than that in 2018 samples ($P < 0.05$). No difference in the 8OHdG level. Additionally, no effect of menstrual stress on the UBP level was observed.

Conclusions: These results suggest that the UBP levels may be related to school-related stress and menstruation has no effects on urinalysis results.

1. Introduction

The development of new technology in the field of clinical laboratory have been remarkable [1, 2]. The authors have developed measurements of cholesterol and have reported that elevated cholesterol causes oxidative stress in the human body [3, 4]. The present period is referred as the age of stress, and changes in the social structure, which are mentally and materially intertwined in a complex manner, produce a variety of stress [5]. Recently, saliva has attracted attention as an easily accessible type of biological sample, and substances in saliva, such as cortisol, α-amylase, chromogranin A, and secretory immunoglobulin A, have been identified to reflect stress [6]. These are controlled by the hypothalamic–pituitary–adrenocortical axis and the sympathetic–adrenomedullary axis, and have many problems such as diurnal variations and age-dependent differences [7, 8]. Meanwhile, bilirubin, one of the bile pigments that are heme metabolites, is a potent low-molecular-weight antioxidant in vivo [9]. Unlike antioxidative nutrients, the bilirubin level in blood is constant and does not fluctuate owing to variable intake. In addition, as bilirubin has a unique molecular structure that forms an intramolecular hydrogen bond, it is interconvertible between the hydrophobic and hydrophilic forms, and passes freely through the cell membrane. This low-molecular-weight antioxidant was found not only in the blood circulation system but also has been ubiquitously present in tissues and cells [10, 11].

Biopyrrins are end products of the oxidation reaction of the suicide antioxidant bilirubin with reactive oxygen species (ROS) (Figure 1) [12]. An increase in bilirubin consumption owing to oxidative stress is reflected through an increased level of urinary biopyrrins (UBP). Owing to these

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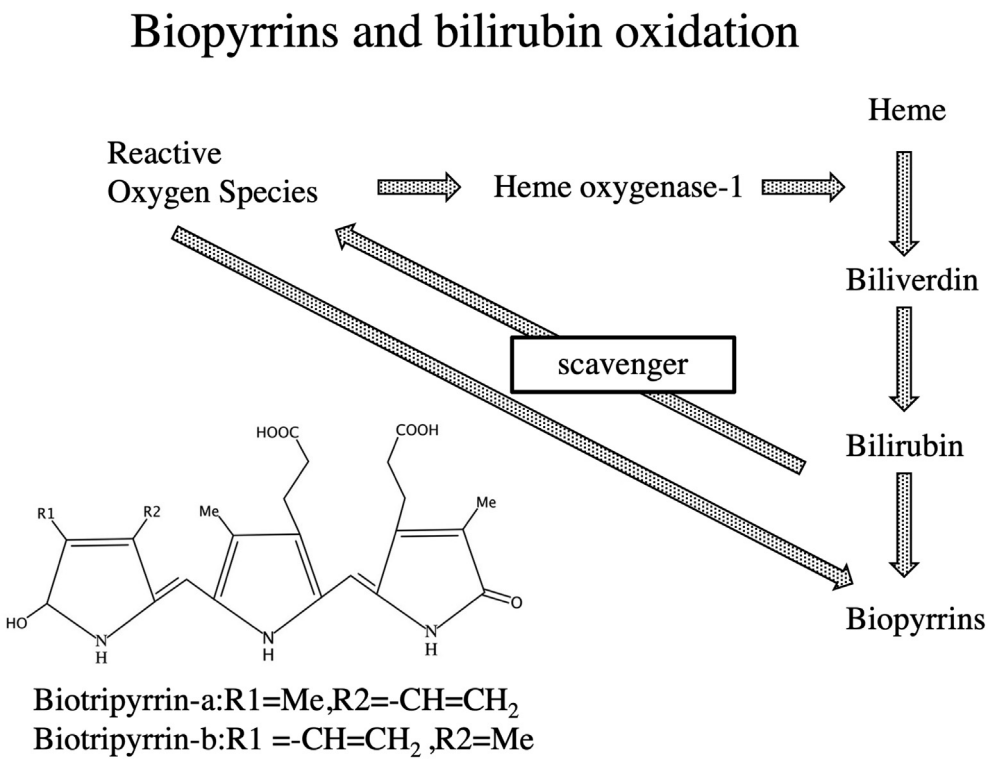

Figure 1. The reactive oxygen species (ROS) induce hemoxigenase-1, which generates bilirubin from heme and biliverdin. Bilirubin reacts as a suicide radical scavenger with ROS to form several different biopyrrins.

characteristics, UBP could be expected to serve as a real-time oxidative stress marker indicating the redox state of the body [13]. The UBP levels have been reported to be responsive to various types of stress, such as postoperative stress [14], atopic dermatitis [15], psoriasis [16], psychosomatic disease and psychological/social stress [17], and septic patients [18]. However, reference UBP levels in healthy adult populations have not been extensively studied, and no reports are available on intra-month variation, particularly on the relationship between menstrual stress and the UBP levels in healthy adults. In the present study, we examined the reference UBP level in healthy young adults and evaluated the differences in UBP levels between grades in Kagawa Prefectural University of Health Sciences to investigate the feasibility of using UBP levels to detect subtle stress in the daily life of healthy individuals. The authors have also measured 8OHdG as a stress marker [19, 20, 21, 22]. The results obtained were compared with data on 8OHdG levels. We also examined the effect of menstrual pain stress on the UBP level.

2. Materials and methods

2.1. Subjects

A total of 40 healthy young adult volunteers who consented to participate in the study (35 female and 5 male aged 20–23 years; body mass index <25.0 kg/m^2) were recruited from among third- and fourth-year non-smoking students at our university and were included in the present study. For fourth-year students, the UBP and 8OHdG levels in the urine samples collected when they were in third-year grade (2017 urine samples) and collected when they were in fourth-year grade (2018 urine samples) were measured.

2.2. Measurements of UBP and 8OHdG

UBP and 8-hydroxy-2-deoxyguanosine (8OHdG) levels could be considered as oxidative stress markers. The UBP levels were measured with a competitive ELISA kit using biopyrrin antibody 24G7 (Metallogenics, Chiba, Japan), according to the manufacturer's protocol [23, 24]. 8OHdG levels were measured with a Highly Sensitive 8-OHdG Check kit (JaICA, Fukuroi, Japan). This kit can measure extremely low levels of 8OHdG, and the specificity of the monoclonal antibody has been established [19, 20, 21, 22]. UBP and 8OHdG measurements were performed in triplicate and means values calculated. For both parameters, creatinine (Cr) correction was performed using urinary creatinine levels measured by an enzymatic method. All data in this study are presented as mean ± SD.

Midstream urine samples collected in 2018 were used to investigate the reference level, whereas morning urine samples were used to investigate the intra-month variation and effect from menstrual stress. All urine samples were stored at −80 °C while protected from light; they were used for measurement immediately after being thawed with light protection.

2.3. Statistical analyses

Correlations between variables were calculated using Spearman's correlation coefficients. Mann–Whitney U test or Wilcoxon signed-rank test was used for comparison between two groups not normally distributed having quantitative variables. StatFlex ver. 6 software (ArTec, Osaka, Japan) was used for all statistical analyses and P value of <0.05 was considered statistically significant.

Ethical approval

The present study was conducted with approval from the ethical committee of Kagawa Prefectural University of Health Sciences (No.214). The objectives of the study were explained to all of the subjects, and written consent was obtained in accord with the Declaration of Helsinki.

3. Results

3.1. UBP and 8OHdG levels in healthy volunteers

A significant correlation was found between UBP and 8OHdG levels with r = 0.378 (P < 0.02) (Figure 2). The UBP and 8OHdG levels (mean ±

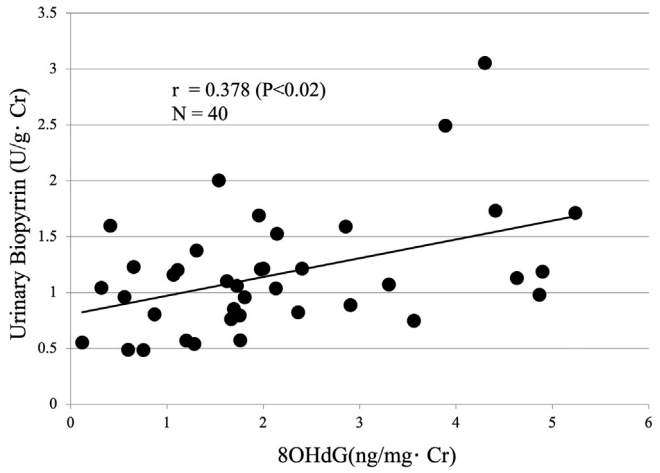

Figure 2. Correlation between the UBP and 8OHdG levels in healthy volunteers (n = 40). The UBP and 8OHdG levels in the healthy volunteer group were low, and a significant correlation was found between the two (Spearman's correlation coefficients).

SD) were 1.21 ± 0.61 U/g·Cr and 2.14 ± 1.40 ng/mg·Cr (Figure 3), respectively. The UBP level in the 40 healthy volunteers was lower than the value reported (3.16 ± 1.05 U/g·Cr) [16], and the 8OHdG level was also lower than the value reported for healthy individuals (15.4 ± 5.60 ng/mg·Cr) [25]. These results could be used as a normal stress level healthy volunteer group in the present study. UBP is not affected by gender differences [16].

3.2. The UBP and 8OHdG levels in different-year students

The UBP and 8OHdG levels measured with spot urine samples collected during the 2018 spring health checkup were compared between third-year and fourth-year students from our university (Figure 4). The 8OHdG levels in third-year and fourth-year students were 2.03 ± 1.39 ng/mg·Cr and 2.25 ± 1.45 ng/mg·Cr, respectively, showing no significant difference (P = 0.64: Mann–Whitney U test). Meanwhile, the UBP level in third-year students (1.31 ± 0.61 U/g·Cr) tended to be higher than that in fourth-year students (1.11 ± 0.60 U/g·Cr); however, no significant difference was observed (P = 0.09: Mann–Whitney U test). We therefore tested urine samples collected when the current fourth-year students were third-year students (2017 urine samples) and those collected in the current year (2018 urine samples) to assess the annual variation (Figure 5). The UBP level in the 2017 urine samples (1.79 ± 1.54 U/g·Cr) was significantly higher than that in the 2018 urine samples (1.11 ± 0.60 U/g·Cr) (P < 0.05: Wilcoxon signed-rank test).

3.3. Effect of menstrual stress on the UBP levels

The UBP levels of each healthy volunteer varied from 0.1 to 1.8 U/g·Cr. The intra-month variation in the whole group of four healthy volunteers was 0.68 ± 0.40 U/g·Cr. Next, we compared the UBP levels during a menstrual period versus those during a non-menstrual period (Table 1). No volunteers showed significant differences between the UBP levels during menstrual and non-menstrual period. Therefore, as no effects of menstruation on UBP measurements were observed, the menstruation cycle should not be considered in urine collection for UBP assays.

4. Discussion

Bilirubin, one of bile pigments representing heme metabolites, is a physiologically important, potent low-molecular-weight antioxidant, and has been reported to have an antioxidant capacity exceeding that of α-tocopherol [11].

Biopyrrins are end products of the oxidation reaction between the suicide antioxidant bilirubin and reactive oxygen. Unlike other antioxidative nutrients, such as vitamins C and E, biopyrrins are not reduced to bilirubin in the redox environment in the body and rapidly excreted into urine. Increased bilirubin consumption due to oxidative stress is indicated by an increased UBP level. From these characteristics, the UBP level is expected to serve as a real-time oxidative stress marker indicating the redox state of the body [13, 14]. However, biopyrrin levels in healthy adults have not been reported, and we investigated the UBP levels in healthy adult volunteers recruited from among students of our university. Results showed that the UBP level in students at our university was 1.21 ± 0.61 U/g·Cr, which was lower than the UBP level previously reported (3.16 ± 1.05 U/g·Cr) [16] [Figure 3]. Similarly, 8OHdG, which is another oxidative stress marker, had a measured level of 2.14 ± 1.40 ng/mg·Cr, which is lower than the previously reported reference value (15.4 ± 5.60 ng/mg·Cr) [25], demonstrating that the stress level in the

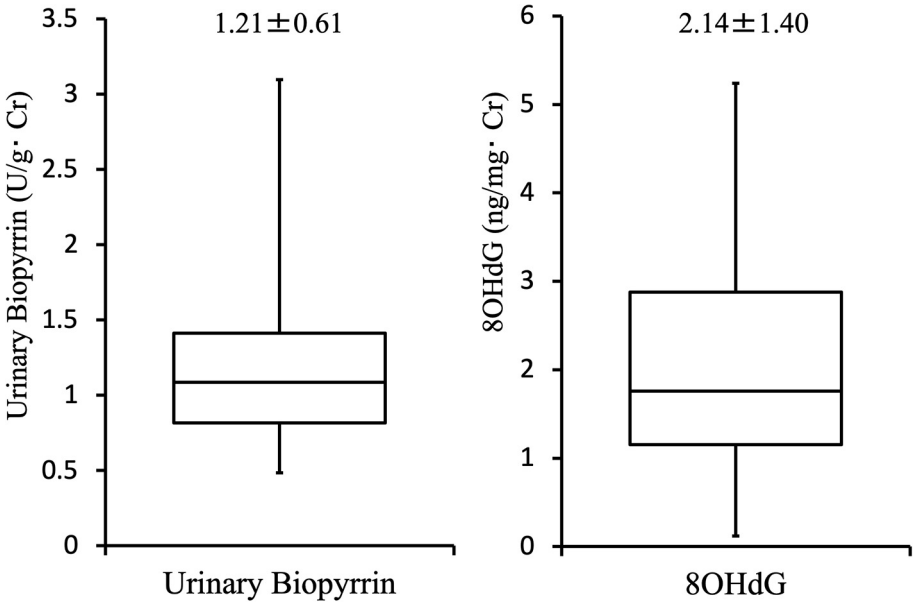

Figure 3. UBP and 8OHdG levels in healthy volunteers (n = 40).

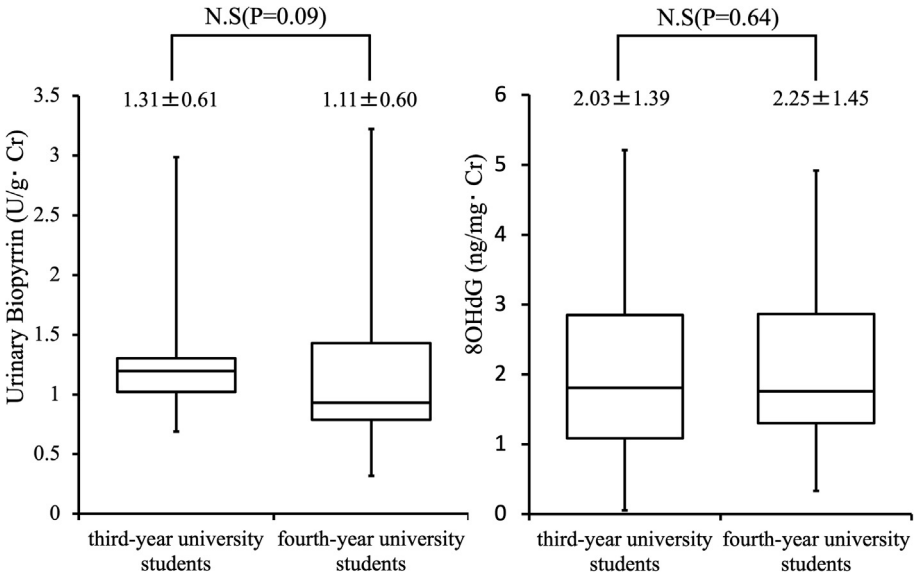

Figure 4. Comparison of UBP and 8OHdG levels between third-year (N = 20) and fourth-year students (n = 20). No significant differences in the UBP and 8OHdG levels were observed between third-year and fourth-year students (Mann–Whitney U test).

healthy volunteers of the present study was normal. In addition, comparison of the UBP and 8OHdG levels between third-year and fourth-year students revealed that third-year students tended to show a higher level (1.31 ± 0.61 U/g·Cr) than fourth-year students (1.11 ± 0.60 U/g·Cr) [Figure 4]. The UBP and 8OHdG levels were compared between third-year and fourth-year students. Although the comparison showed no significant difference with respect to 8OHdG levels (P = 0.64), The UBP level in third-year students (1.31 ± 0.61 U/g·Cr) tended to be higher than that in fourth-year students (1.11 ± 0.60 U/g·Cr) (P = 0.09).

We then examined the annual variation from the third-year (2017 urine samples) to the fourth-year (2018 urine samples) in fourth-year students (Figure 5). The UBP level of 2017 urine samples (1.79 ± 1.54 U/g·Cr) was significantly higher than that in urine samples from fourth-year students (1.11 ± 0.60 U/g·Cr) (P < 0.05). Third-year students have practicums and other classes that require them to acquire more specialized skills, and they were suggested to experience a higher level of school stress than fourth-year students. In particular, it has been reported that students in grades with clinical training should be stressful [26, 27, 28, 29]. In the present study, there is clinical training in the third grade. In fact, it has been confirmed that a student whose 2017 urine sample showed an extremely high UBP level (7.75 U/g·Cr) actually had various levels of stress. Therefore, the UBP level may be useful as an index for objective evaluation of school stress, and this is considered to be a future study subject. No significant difference was observed in 8OHdG levels (P

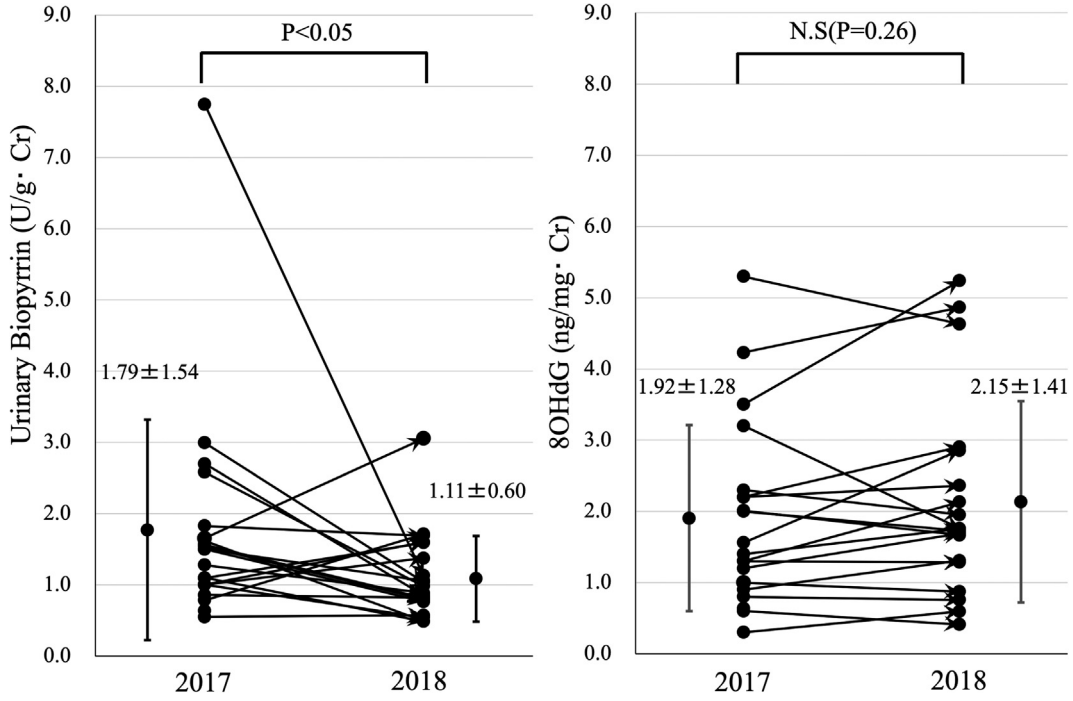

Figure 5. Annual changes in the UBP and 8OHdG level in healthy fourth-year students (n = 20). The UBP levels in 2017 were significantly higher than those in 2018 (Wilcoxon signed-rank test).

Table 1. Monthly variation in the UBP levels.

	During non-menstrual period	During menstrual period	P value*
Volunteer 1	0.51 ± 0.38	0.75 ± 0.30	0.202
Volunteer 2	0.50 ± 0.35	0.42 ± 0.32	0.660
Volunteer 3	0.77 ± 0.32	0.96 ± 0.27	0.190
Volunteer 4	0.88 ± 0.52	0.70 ± 0.20	0.416

Each value represents the mean ± S.D. (U/g·Cr).
* during non-menstrual period versus during menstrual period (Student's t-test).

= 0.26). In patients with chronic schizophrenia, a recent report has shown an increase in the UBP level, but no increase in the urinary 8OHdG level [30]. This finding suggests that patients with chronic schizophrenia are under a certain kind of oxidative stress, and our results also suggest that the UBP level is more associated with school stress than the 8OHdG level. 8OHdG is produced through reactions between nuclear DNA and ROS [31], whereas UBP is produced through reactions between bilirubin and ROS [12]. This difference in their production processes might underlie the greater promise of UBP as a mental stress marker [32]. However, the sample size in our study was small, and further studies are also necessary to evaluate sex difference.

It is interesting to know the changes in the 8OHdG levels in the four cases that showed significant changes in the UBP levels. In addition, the results showed that the UBP levels of third-year students were higher than those of fourth-year students, indicating that the stress levels of third-year students were higher than those of fourth-year students. It is necessary to discuss what type of characteristic learning stress third-year students had. However, this could not be identified because the survey was conducted using a blind test. In the further, it is important to investigate the relationship between the test results and stress levels using approaches such as encryption processing.

We examined the effect of menstrual stress in women to establish the reference UBP level. The UBP levels measured with morning urine samples collected daily for one month from four healthy volunteers varied from 0.1 to 1.8 U/g·Cr, and the intra-month variability in all four healthy volunteers was of 0.68 ± 0.40 U/g·Cr. We compared menstrual and non-menstrual UBP levels, but found no significant differences in any of the volunteers (Table 1). Therefore, the menstruation cycle appeared to be unnecessary to be considered in UBP measurement. These findings suggest that the UBP level may serve as an index of school stress, warranting studies on its clinical significance as an objective indicator of stress in various social environments.

5. Conclusion

The present study was conducted in healthy adult volunteers recruited from among students of our university, and showed that the UBP level in these students was 1.21 ± 0.61 U/g·Cr. In addition, the comparison of the UBP level between third- and fourth-year students revealed that the third-year students tended to have higher UBP levels than the fourth-year students, suggesting that the UBP level may reflect school stress. No effects of menstrual stress were observed on UBP measurements.

Declarations

Author contribution statement

Satoshi Tada, Takehiro Nakamura: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Atsuko Shiota, Hidehiro Hayashi: Analyzed and interpreted the data.

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Competing interest statement

The authors declare no conflict of interest.

Additional information

No additional information is available for this paper.

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