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IS URINARY PYRIDINIUM CROSSLINKS ASSOCIATED WITH STUNTING IN STUNTING CHILDREN IN INDONESIA IN 2014-2020

ABSTRACT

Objective: The objective of this study was to analyze the correlation between pyridinium crosslinks (Pyd) urine and stunting among children. We also determined the effect of nutritional intervention on the Pyd content in urine among stunting children.

Methods: The study was a cross-sectional by involving 173 children in Pekanbaru and Payakumbuh, Indonesia in 2014 (children aged 0-3 days: n = 32), 2017 (children aged 4-6 years: n = 80), 2018 (children 4-6 years old: n = 25), and 2020 (children 12-15 years old: n = 36). Height gauges, family socio-economic questionnaires, pot urine and Pyd kit were used to collect the data. Nutritional interventions for children aged 4-6 years were given milk for 4 months (as additional energy, namely 25% of the recommended dietary allowance); nutritional intervention for children aged 12-15 years were provided brunch meals and milk for 34 days (as additional energy, namely 30% recommended dietary allowance). We used two indicators such as Pyd and height as a parameter in this study. Pearson correlation and t-test (significance $p < 0.05$ and $p < 0.01$) were applied for statistical analysis.

Results: The Pyd content of stunted children aged in 0-3 days, 3-5 years, 4-6 years, and 12-15 years were found to be 982, 16.4, 16.9 and 9.6 nmol / mmol creatinine, respectively. The Pyd content of stunted children aged 4-6 and 12-15 years before and after nutritional intervention were 16.9, 15.3, 9.81 and 5.33 nmol / mmol creatinine, respectively. Stunting neonatal urine Pyd content was found to be different from normal neonatal urine Pyd content ($p < 0.01$). There was a correlation found between urine Pyd content and height of children aged 4-6 years ($p < 0.05$) and $r = -0.242$. There was a difference observed in the urine Pyd content of children 4-6 years before nutrition intervention ($p < 0.01$). There was a difference between urine Pyd content of children aged 12-15 years before and after nutritional intervention ($p < 0.05$); as many as 19.4% of the subjects increased their nutritional status from stunting to normal.

Conclusion: Stunting children's urine Pyd content was found to be higher than normal. Pyd content in urine can be reduced by providing nutritional interventions to the stunting children. The Pyd urine was associated with stunting in children; therefore, can be used as a linear growth biomarker.

Keywords: stunting, children, pyridinium crosslinks urine, height

INTRODUCTION

Stunting is one of the major health problem in Indonesia and even in the world. The prevalence of stunting among children under five years of age in Indonesia is 30.87 % [1]. A review study in 36 countries found that the prevalence of stunting in children under one year was 40% and the prevalence of stunting

for children under two years reached 54% [2].

About 59.3% of children aged 3-5 years were included *stunting* [3]. The prevalence of global stunting of children aged 13-15 years is around 35.1% [4]. The results of Indonesia's basic health research in 2010 show that the prevalence of stunting in children aged 13-15 years is 35.2%., the

Prevalence is 36.6% in the Riau Province [5]. Public health problems are considered severe if the prevalence of stunting is 30-39% and serious if the prevalence of stunting is $\geq 40\%$ [4]. World Health Organization (WHO) established stunting standards based on anthropometrics measurement with Height for Age (HAZ)-score < -2 SD [6].

The biochemical markers of bone resorption can be analyzed clinically using conditions and treatments that affect bone metabolism. This bone formation marker is derived from type I collagen. About 90% of the bone organic matrix is made of collagen type I which is a helical protein, stabilized by cross-linking between the N terminal and C terminal in the formation of the base of bone tissue. The pyridinium crosslinks (Pyl) are formed by hydroxylysine or lysine residues at the C- and N-telopeptide terminals of the collagen molecule and released during matrix resorption, excreted in the urine. Pyl appears in urine that is characterized by peptide formation. There are several studies reported that the number of free crosslinks excreted in the urine is related to the rate of bone formation [7]. The absorption takes around 7-10 days, whereas the formation takes 2-3 months. Overall 10% of bone is replaced each year. The process of bone metabolism occurs in pairs (bone formation is related to bone resorption; occur in a balanced manner which indicates that the amount of bone removed will be completely replaced) [8]. There are two types of cells responsible for bone metabolism, namely osteoblasts and osteoclasts [9]. The function of osteoblast is influenced by calcium intake, which can cause low mineralization of the new bone deposit matrix; severe calcium deficiency in childhood can lead to stunting [10]. Calcium forms complex bonds with phosphate which can provide strength to bones [11].

Until now, there is no convincing stunting indicator reported in the literature. Anthropometric measurements of length or height to determine stunting have been inconclusive for many reasons. There are still many opportunities for errors in the

measuring instruments used and the ability of the enumerator to measure whose value can vary with other enumerators.

Radiological indicators are being debated to be used to measure children's bone density as biomarkers for their linear growth. Radiological results from the hospital can be used for medicinal purposes recommended by a doctor, but if only for research purposes it will not be permitted by the hospital. Biochemical indicators using blood are unethically carried out on children without any medical reason because they are invasive (painful).

Based on the above arguments, it is necessary to study a convincing and noninvasive biomarker to determine stunting in children using urine. The aim of this study was to assess the correlation between urine Pyl levels, height and the effect of nutritional interventions on the stunting status of children aged 0-3 days, 4-6 years, and 12-15 years, respectively.

MATERIALS AND METHODS

Study Design

It was a cross-sectional study conducted in 2014, 2017, 2018, 2020. The study was conducted in various Province including; Pekanbaru City, Riau Province and in 50 Kota district, West Sumatra Province, Indonesia.

Subject and Urine Collection

The study subjects consisted of neonates, children under the age of five and adolescents. The total number of study subjects was 173. In 2014, 32 neonates aged 0-3 days were selected. The subject was in Andini Mother and Child Hospital, Pekanbaru City, Riau Province. In 2017, 80 children aged 4-6 years were selected. Subjects were children who attend As-Shofa Kindergarten and Hidayatullah Kindergarten in Pekanbaru City, Riau Province and Al-Falah PAUD (Early childhood education programs) in 50 Kota District, West Sumatra Province. In the 2018 study, 25 children aged 4-6 years were

selected, who attended Al Falah PAUD, 50 Kota District, West Sumatra Province. In 2020, 36 teenagers aged 12-15 years were selected. These teenagers attended SMP (Junior high school) Negeri 3 Pekanbaru in Riau Province.

The study obeyed the Helsinki-Ethical Principles for Medical Research Involving Human Subjects and approved by the university review board (University of Riau), Ministry of Education and Culture of Republic Indonesia. (certificate number 067/UN.19.1.28/UEPKK /2014, 351/UN.19.5.1.1.8/UEPKK/2017; 073 /UN.19.5.1.1.8/UEPKK/2018, and 351/UN.19.5.1.1/UEPKK/2020).

Data Collection

All parent subjects gave written informed consent. At the time of informed parental consent, and race (Indonesian, expatriate). The urine were collected by using sterile pot, aliquot to 6 ml and stored in freezer at -20°C (GEA by Vestfrost-Denmark Type G.201 Serial No: 20021808005) until further analysis.

Equipment and Materials

In this study, body height gauges (microtoa) (STATURE METER 2M GEA, Indonesia), pot urine sterile (MERAH 60ml, Indonesia), sanitizing wipe, and household socio-economic questionnaires for the childrens (name, gender, age, race, height parents) were used.

Research Procedure

Childrens' urine was collected by a nurse who was trained by researchers at the kindergarten/nursery school. The mothers were briefly explained about the implementation of the study as well as pot urine collection. Urine was collected between 7:00 and 10:00 am. The minimum amount of urine taken from the subject was 10 ml. The urine samples were then stored in the freezer at a temperature of -20 °C in Prodia Clinical Laboratory PekanbaruBranch, and then sent to Prodia Center in Jakarta for

analysis. The analysis was carried out simultaneously [12].

Pyd Urine Measurement and Standardization

Pyd measurements were performed with the use of MicroVue™ PYD EIA kit, USA. Pyd analysis was performed according to Kiyati et al. [13] using a Spectrophotometer (Microplate Reader 680 series, Bio-Rad Laboratories, Inc, Hercules, CA 94547, USA).

Creatinine Urine Measurement and Standardization

Creatinine measurements were performed with the use of Jaffe reactions according to Jaffe method developed by Staden [14]. Creatinine is reacted with picric acid under alkaline conditions to form a red-orange compound. The absorbance of the compound formed was detected at a wavelength of 490- 520 nm using Spectrophotometer (ADVIA 1800: ADVIA, Germany).

Statistical Analysis

Statistical data analysis are reported based on the complete data. Pearson correlation and t-test with significance *p < 0.05 and **p < 0.01 was applied for statistical analysis. The analysis was performed using IBM SPSS Statistics version 20.

RESULTS

The socioeconomic characteristics of the child's family

All the respondents in this study were lived in cities. The average income of the respondent's parents was IDR 3,000,000 per month. The education of the respondents' parents was on average high school. Almost all respondent mothers were housewives (90%). The respondent father's job was usually entrepreneur, employee or laborer. The number of siblings of the respondent

was around 1-3 peoples. Almost all of the respondents parents' height was > 150 cm.

Urine Pyd content according to the age of the stunting child

The Pyd content of stunted children aged 0-3 days, 3-5 years, 4-6 years, and 12-15 years were found to be 982, 16.4, 16.9 and 9.81 nmol / mmol creatinine, respectively (Table 1).

Stunting children's urine Pyd content by sex

The Pyd content in urine of stunted girls was found to be higher than the Pyd content of stunted boys (Table 2). The Pyd content of stunted neonates urine for men and women were 988.45 and 641.40 nmol / mmol creatinine, respectively. Moreover, Pyd content of urine for stunted girls and boys aged 4-6 years were 18.70 and 16.27 and nmol / mmol creatinine, respectively.

Urine Pyd levels according to stunting status

The Pyd content of stunting neonates urine was 982.92 ± 61.64 , whereas normal neonates was 594.11 ± 266.16 nmol / mmol creatinine ($p < 0.01$). The Pyd content of urine in very stunting and normal children aged 4-6 years were found to be 18.4, 16.4 and 15.5 nmol / mmol creatinine. There was a negative correlation found between urine Pyd content and height of children aged ($p < 0.05$) ($r = -0.242$).

Urine Pyd content based on nutritional intervention

The Pyd content of stunted children aged 4-6 years before and after nutritional intervention were found to be 16.9 and 15.3 nmol / mmol creatinine, respectively. The same results were also observed among the older age group. Moreover, Pyd content in urine of stunting children aged 12-15 years before and after nutritional intervention were 9.81

and 5.33 nmol / mmol creatinine, respectively. A decreasing trends in the amount of urine Pyd indicated an increased in the linear growth of the child. The results also indicated that by providing nutritional interventions to stunting children reduced urine Pyd content (Table 2).

DISCUSSION

In this study, it was found that the urine Pyd content of stunting children decreased with increasing age. The same trend was also observed in the previous studies where Pyd urine excretion of children aged 0-3 days (neonates) was 10-100 times higher than that of children aged 3-16 years. The crosslink excretion in children was reported to be 20 times higher than in adults [8]. This was because of the day as we took neonates urine, the condition of neonates in dehydration. When the neonates just born, they were separated from their mother for hours without milk whether breastfeeding or formula. They were given formula milk (10-30 ml) then breastfeeding practice about 2-6 hours later [15]. Pyd excretion for neonates was reported to be 642.7 ± 281.3 nmol/mmol creatinine by Fujimoto et al. [7].

In another study, Pyd excretion among elementary school children was reported to be about 50-500 nmol/mmol creatinine [16]. The urine pyd of adults who have health problems is higher than normal adults. For example, Harvey et al. [17] used pyridinium cross-links as specific urinary markers for the measurement of bone collagen degradation in hyperthyroidism and during thyroxine replacement therapy. They reported that the urinary Pyd excretion was higher among postmenopausal female thyrotoxic patients compared to controls (median 131 vs 26 nmol/mmol creatinine ($p < 0.001$); in postmenopausal women urinary Pyd excretion was raised in those taking T4 yaitu 40.0 ± 2.7 nmol/mmol creatinine ($p < 0.05$).

Urine Pyc content has been used to determine the severity of osteoporosis in the elderly. The more urine Pyc content in the elderly group, the higher the level of osteoporosis. This means that more bone resorption occurs in this group of elderly people.

Reference interval Premenopausal adult female and male urine contained Pyc around 15.3-33.6 and 10.3-20.0 nmol / mmol creatinine. The target value for treated postmenopausal adult female was the same as the premenopausal reference interval [18]. The Pyc content of premenopausal women's urine ranged from 3.0 to 7.4, whereas their male peers ranged from 2.3 to 5.4 μ mol / mol of creatinine [19].

The growth spurt among boys occur more slowly than girls. Growth spurt in boys began to occur at the age of 10.5 years, whereas in girls it began to occur at the age of 9.5 years [17]. The increases in height occurred two years earlier in girls than boys. The peak height growth rate (percentage height velocity) in girls occurs around the age of 12 years, whereas in boys at the age of 14 years. In girls, growth will end at the age of 16 years while in boys in 18 years. After that age, in general, height gain is almost complete.

Sex steroid hormones also affect bone maturation in the epiphyseal plate. At the end of puberty the epiphyseal plate closes and height growth stops [21]. Relatively the same height at the age of 30-45 years. After 45 years there is a decrease in height [22].

In the age group of children and adolescents with normal nutritional status, there was more bone formation observed than bone resorption. Pyc in urine is a marker of bone resorption. This means that the Pyc content of urine in the age group of children and adolescents with normal nutritional status is less than the Pyc content of urine in the age group of children with stunting nutritional status.

Urine pyc is a specific constituent of skeletal collagen, released into the circulation and excreted in the urine. Their measurement in urine is a sensitive index of the ongoing rate of bone resorption. The clinical applications of urinary Pyc markers include many metabolic disorders of bone such as osteoporosis, primary hyperparathyroidism and metastatic bone diseases. Urine Pyc cross-link also shows great hope as a marker of therapeutic efficacy in bone disorders associated with accelerated bone resorption [23].

In this study, urine Pyc is expected to be a marker of the efficacy of nutritional care in bone growth disorders associated with bone resorption.

CONCLUSION

The Pyc content of stunting children's urine was found to be different from normal children. There was a negative correlation observed between urine Pyc content and children's height ($p < 0.05$). Pyc content showed a weak correlation with height $r = -0.242$. There was a difference found in the Pyc content of children's urine before the nutritional intervention. The data is in accordance with the toeri foundation.

It is necessary to do further research with more subjects in certain sex and age groups by providing nutritional interventions between the treatment and control groups at the same time and location.

ACKNOWLEDGEMENT

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Jakarta, and milk assistance from PT Indolakto Jakarta.

AUTHORS CONTRIBUTION

AWH, HA, YA contributed to design the study. AWH prepared the manuscript and research report. HA analyzed the data. YA management the data collection. All the authors reviewed the manuscript before submission. All authors read and approved the final manuscript.

CONFLICT OF INTEREST

The authors declare no conflict interest

AUTHOR DECLARATION

The manuscript has similarity index below 15%.

Table 1 . Pyd content in urine based on nutritional intervention (nmol /mmol creatinine)

| | Year | | | |
|--|---|---|---|---|
| | 2014 | 2017 | 2018 | 2020 |
| Location | Andini Hospital Pekanbaru in Riau Province | Al Falah PAUD, 50 Kota District (in West Sumatra), As-Shofa Kindergarten and Hidayatullah Kindergarten Pekanbaru (in Riau Province) | Al Falah PAUD, 50 Kota District (in West Sumatra), | SMP Negeri 3 Pekanbaru in Riau Province |
| Age | Neonatus 0 - 3 days | 4-6 years | 4-6 years | 12-15 years |
| Number of subjects | 32 | 80 | 25 | 36 |
| Sex | Boys (n=26) & girls (n=9) | Boy | Boys (n=16) & Girls (n=9) | Boys (n=18) & Girls (n=18) |
| Height (cm) | | | | |
| • Very Stunting | | 98±96(99:2) | | |
| • Stunting before nutritional intervention | 46.8±0.5 (46:47) ^a | 102±97(108:3) | 131.5 | 143,6±52(133,6:1549) |
| • Stunting after nutritional intervention | | | 133.2 | 144,9±51(134,7:155,2) |
| • Normal | 49.9±1.4(48.0:53.0) ^b | 109±97(121:5) | | |
| Nutritional status before intervention | Normal and stunting | Normal and stunting | Stunting | Stunting |
| Intervention | - | - | Milk every day and four eggs per week for 4 months. Additional energy, namely 25% nutritional adequacy rate / RDA | Brunch meals and milk daily for 34 days. Additional energy, namely 30% nutritional adequacy rate / RDA. |
| Pyd urine (nmol/mmol creatinine) | | | | |
| • Very Stunting | - | 18.1 | - | - |
| • Stunting | 982 | 16.4 | - | - |
| • Normal | 594 | 15.5 | - | - |
| • Before nutritional Intervention | - | - | 16.9 | 9.81 |
| • After nutritional Intervention | - | - | 15.3 | 5.33 |
| Conclusion | The Pyd content in the urine of stunted neonates was found to be different from the Pyd content in normal neonatal urine (p <0.01). The pattern of Pyd content in urine according to height was like the letter "U" | There was a negative correlation observed between urine Pyd content and the subject's height (p <0.05). Urine Pyd content showed a weak correlation with height at r = -0.242 | There was a difference in Pyd content in the urine of subjects before and after nutritional intervention (p <0.01). However, all subjects were still in the stunting category | There was a difference observed in Pyd content of subjects before and after the nutritional intervention (p <0.05). Approximately, 19.4% of subjects increased their nutritional status from stunting to normal |

Table 2. Pyd content in urine of stunted children by sex

| Year | Status nutritional intervention | Pyd (nmol/mmol creatinine) | | | |
|------|---------------------------------|----------------------------|-----------------------------------|----|---------------------------------|
| | | n | Girl | n | Boy |
| 2014 | No intervention | 2 | 988.45±29.20 (967.80: 1009.10) | 7 | 641.40±257.73 (319.80: 1049.60) |
| 2018 | Before nutritional intervention | 7 | 18.70±7.73(9.73: 29.79) | 11 | 16.27±5.98(6.23:27.71) |
| 2018 | After nutritional intervention | 7 | 18.55±10.91 (9.84: 43.56) | 11 | 14.64±3.63(9.07:20.80) |

^saverage ± standard deviation (minimal: maximal)

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