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Pyridinium Crosslinks (*Pyd*) in the Urine is Associated with Stunting in Neonates

Aslis Wirda Hayati[1*], Alkausyari Aziz[1], Siti Rohaiza Ahmad[2] and Sri Widia Ningsih[3]

[1]Departmentof Nutrition, Poltekkes Kemenkes Riau, Indonesia.
[2]PAPRSB Institute of Health Sciences, Universiti Brunei Darussalam, Brunei.
[3]Department of Pharmacy, Poltekkes Kemenkes Medan, Indonesia.

Authors' contributions

This work was carried out in collaboration among all authors. Author AWH designed the study, performed the statistical analysis, wrote the protocol and wrote the first draft of the manuscript. Authors AA and SWN managed the analyses of the study. Author SRA managed the literature searches. All authors read and approved the final manuscript.

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ABSTRACT

Aims: The aim of the research is to evaluate the reliability of bone resorption biomarkers called Pyridinium Crosslinks (*Pyd*) in the urine of the neonates as an evaluation to bone growth of the neonate, as an indicator of stunting.
Study Design: A cross-sectional study.
Place and Duration of Study: Andini Mothers and Children Hospital (*Pekanbaru*, Indonesia). Duration of the study was between, August until September 2014.
Methodology: Subjects of study were 35 healthy neonates. Subjects were recruited at the first 3 days of life. Body length gauges, digital weighting scale, family socioeconomic questionnaires and *Pyd* kit were used to collect the data. Differences in the mean of the research variables were tested using an Independent t-test.

**Corresponding author: E-mail: asliswirda@yahoo.com;*

Results: Results showed that there were significant differences ($p<0.01$) in terms of height for age and *Pyd* in the urine of stunted (body length <48 cm) versus normal (body length ≥48 cm) neonates. The contents of *Pyd* in the urine of stunted neonates were 982.9±61.6 and normal neonates was 594.1±266.1 nmol/mmol.
Conclusion: Therefore, there is a possible association between height for age and *Pyd* in the urine as a potential early indicators to identify stunted and normal neonates.

Keywords: Body length; neonates; pyridinium crosslinks; stunting; height for age; normal neonates.

1. INTRODUCTION

Stunting is a condition by which an individual failed to reach the linear growth potential which could be resulted from the conditions of their health and nutrition [1]. Worldwide, childhood stunting declined from 39.7% in 1990 to 26.7% in 2010 [2]. This expected to incline towards 21.8%, or 142 million, in the year 2020 [2]. In Indonesia, stunting is one of the major nutrition problems in Indonesia, with the prevalence of 35.6% [3]. Stunting is resulted from poor standard of living, exposure to adverse conditions such as diseases and poor eating habits and practices [1]. The most common cause of stunting in the developing countries includes: Impaired absorption of nutrients due to intestinal infections or parasites; or combinations of these problems [4,5]. Stunting may also lead to several long-term effects when they reach adulthood period, which includes poor cognitive development, poor in academic, poor productivity towards the economy and negative impact on the reproductive health [4].

Improved early nutrition and care can compensate in part for stunted in utero. Stunting that began at a very early infancy age, leads to a more severe impairment to their cognitive development in later life [4]. Therefore, an early determination of stunting among neonates is very important in order to support for proper feeding support and the gain of better optimal health [6]. The use of classical anthropometric measurements for body length measurements is widely accepted since many decades ago [7]. However, the use of such measurements to determine stunting has drawbacks such as possible human error or issues with the devices. The use of biomarkers in radiology is being debated to measure the infant's bone density to monitor the growth of the baby; however this method is deemed unsuitable as it involves unnecessary invasive procedures for the babies. Therefore, the use of other readily obtained biological fluids or wastes such as urine can be used as a possible early indicator to stunting among neonates.

In this research, the quantification of a bone resorption marker called *Pyd* in the urine as a possible early indicator to stunting is proposed [8]. The condition of the urine is associated with health condition of the kidney. Stunted babies (that has been exposed to malnutrition) has shown to have disruption to their metabolic processes and increased risk of renal impairment [9]. In order to find out whether the babies are having these issues, the creatinine level can be measured from their urine. Creatinine is derived from the metabolism of proteins, either from food or from muscle. Human bone is formed through the process of pairing between bone resorption process (release of a cell or tissue with a gradual preparation of the compounds into smaller and dispersed in circulation) by osteoclasts and bone formation by osteoblast. This process happens normally in bone and skeletal growth. As many as 90% of the organic matrix of bone is made of Collagen Type I is a helical protein is stabilized by cross-linking between terminals N and C terminals on the basis of the formation of bone tissue. During maturation of collagen, *Pyd* formed by hydroxy Lysine or Lysine residues at the end of the C- and N- terminal telopeptide of collagen molecules and is released during the resorption of the matrix and is excreted through the urine. *Pyd* is expected to be specific and sensitive biomarker of bone resorption and are able to evaluate bone metabolism or disorder in neonates.

Based on the biological processes, *Pyd* seems to be of potential use to evaluate bone metabolism among neonates, which may indirectly indicate the possibility of stunting condition. Therefore, the main objective of the study is to test the possibility of *Pyd* level in urine as an early indicator to stunting.

2. MATERIALS AND METHODS

The study design was cross-sectional and was conducted between January to December 2014. Subjects of the study were 35 healthy neonates born at the Andini's Mothers and Children Hospital at Tuanku Tambusai street 55,

Pekanbaru (middle class hospital and population strategic location in Pekanbaru) between the 28th of August until 30th September 2014 (all babies born in a specific period of time that their mothers were willing to sign an informed consent). The minimum number of samples to compare two groups (stunted & normal) in this study is 30,29 neonates [10].

$$n = \frac{2\sigma^2(Z_{1-\alpha}+Z_{1-\beta})^2}{(\mu_1-\mu_2)^2}$$

n = Sample size
σ = Population standard deviation
μ_1 = Test value of the population mean
μ_2 = Anticipated population mean

The value of α = 5% (1.964) and β = 20% (0.842) [11]. In order to obtain a number of samples that reflect population characteristics, statistical parameters (eg mean and standard deviation) from previous studies were used in this study. The research was "Food Supplementation with Encouragement to Feed It to Infants from 4 to 12 Months of Age" carried out by [12]. The study showed that μ1 - μ2 = 0.4 cm (achievement of the subject body length increase), and standard deviation namely σ = 1.6 cm. In anticipation of the drop out subjects, a number of neonates were added so that n = 35 neonates. Subjects were recruited at around 1-3 days of neonatal life. Inclusion criteria were normal gestation (36 to 40 weeks), spontaneous and caesarean delivery. The study complies with the World Medical Association Declaration of Helsinki – Ethical Principles for Medical Research Involving Human Subjects and was approved by the Institutional Review Board of the Faculty of Medicine, University of Riau, Ministry of Education and Culture of Republic Indonesia. Parents of all subjects were given a written informed consent and signed them upon agreement to join the study.

Family socioeconomic questionnaires (e.g. name, gender, age, race, and height parent), body length gauges (BUTERFLY), digital weighting scale for baby (BABY SCALE TANITA), MicroVue™ PYD EIA kit, USA (Quidel Corporation, San Diego, CA 92121, USA, Cat: 8010, Lot: 015210, ED: 2015-07 and Spectrophotometer Microplate Reader 680 (Bio-Rad Laboratories, Inc., Hercules, CA 94547, USA), Creatinine measurements were performed with the use of Jaffe method and Spectrophotometer ADVIA 1800: ADVIA, Germany, baby urine bags (PEDIATRIC URINE COLLECTOR, Japan).

The 24-hours neonates' urine was collected using pediatrics urine bags by trained nurses, aliquoted to 6 mL. Mothers were briefed about the study, one day prior to neonates urine collection. Baby urine that has been collected was stored in the refrigerator at a temperature of -20°C at the Pekanbaru Prodia Clinical Laboratory and then was sent to Prodia Center in Jakarta for analysis.

Statistical analysis and results are reported based on the data. Statistical outliers, defined as outside the 95% confidence limits of the normal probability plots, two subjects were removed before the analysis. In all statistical tests performed, the null hypothesis (no effect) was rejected at the 0.01 level of probability. Differences in mean body weight, head circumference, concentrations of *Pyd* urine, age, weight-for-age-z-score (WAZ), length/height-for-age (HAZ), basal metabolic index (BMI) for age (BAZ), mother's height, mother's BMI, mother's weight before pregnancy, mother's prenatal weight, mother's pregnancy age, the number of children in family were evaluated by using a Independent t-test. Stunted neonates referred to babies with body length of <48 cm and normal babies referred to those with body length ≥48 [13]. All analyses were performed by using SPSS version 20 (IBM SPSS Statistics 20).

3. RESULTS AND DISCUSSION

The proportion of stunted neonates in the study was 22.9%. That is similar with reported by [13] that the proportion of stunting neonates in Indonesia is 20.2%.

All neonates were healthy and have received full enteral feeding (formula and/or breast feeding). 74.3% and 25.7% of the subjects involved in the study were male and female respectively (Table 1). It was found to be much easier to collect urine from male than female neonates, which reflected on the higher percentage of the subjects. Most of the mothers stayed at *Pekanbaru*, except for two of them. This is because their other family members also stayed at the same area and would like to be close to them when their baby is born. Apart from that, living at *Pekanbaru*, the mothers thought that they can get a proper medical attention when needed compared to outside the city. Most of the mothers were Malay and a small percentage was Chinese. 50% of the subject's mother was full housewife. Some of the mothers were also working as officers of the government and private

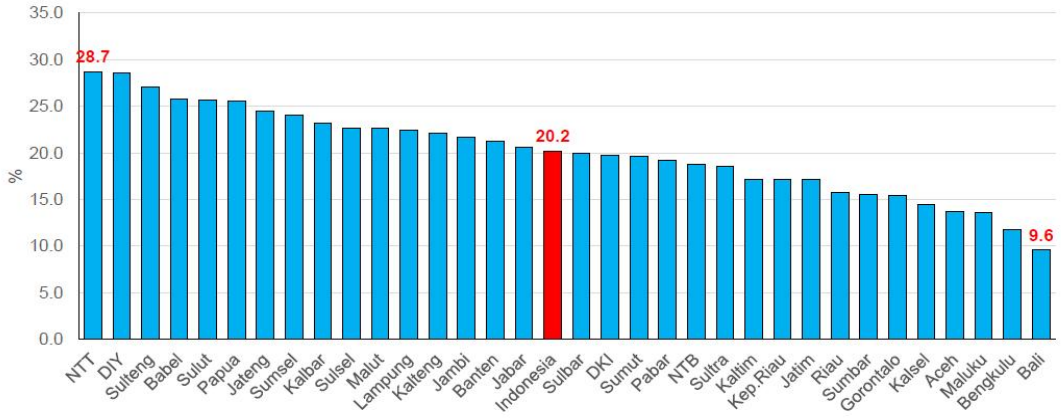

Fig. 1. The proportion of stunting neonates neonatal body length at birth < 48 cm) by province in Indonesia

Table 1. Growth problems

Z-score	Growth indicators			
	Length/height-for-age	Weight-for-age	Weight-for-length/height	BMI-for-age
Above 3	*See note 1*	*See note 2*	*Obese*	*Obese*
Above 2			*Overweight*	*Overweight*
Above 1			*Possible risk of overweight (See note 3)*	*Possible risk of overweight (See note 3)*
0 (median)				
Below −1				
Below −2	*Stunted (See note 4)*	*Underweight*	*Wasted*	*Wasted*
Below −3	*Severely stunted (See note 4)*	*Severely underweight (See note 5)*	*Severely wasted*	*Severely wasted*

Note 4: It is possible for a stunted or severely stunted child to become overweight [14]

sector. Overall, the education level among all the subjects mothers were from the Elementary to Scholar level.

Length for age males and female neonates in Indonesia is about same. [15] reported that length male neonatus is 49.9 cm and female neonatus is 49.1 cm. The growth of male and female neonates is almost the same in the early stages of life, differences will occur at the next stage of growth [16]. There were 42 neonates who were born during the urine collection period, as many as 5 neonates were not continued to be processed to the analysis stage of the urine Pyd content because the amount of urine collected was insufficient. The five neonates are female. Neonatal female urine collectors are relatively more difficult than men because female urine sometimes spills out of a pediatric urine collector. An addition, there were 2 female neonates whose Pyd content was in the form of outliers so they were not included in the data analysis.

The proportion of stunted neonates in the study was 22.9%. It was a similar findings as reported by Atmarita research which was at the rate of 20.2% [13]. The characteristics of the stunted and normal subject were recorded as Table 2. The length of stunted and normal neonates was 46.8±0.5 cm and 49.9±1.4 respectively. Their weight also differs, by which the normal neonates were much heavier compared to the stunted

neonates (a difference of about 300-400 g). The head circumference of stunted neonates and normal neonates were 33.3±1.0 and 33.6±1.2cm respectively. This study has shown that there was a major difference (statistically significance) in terms of the level of *Pyd* in the urine between the stunted neonates and normal neonates. Among stunted neonates, the amount of *Pyd* in the urine was 982.9±61.6 nmol/mmol Cr, compared to only 594.1±266.1 among normal neonates. The HAZ was also found to be statistically significant between the stunted neonates and normal neonates.

Mothers of subjects with small BMI's did not give birth small subjects (independent t test). The mean BMI of mothers of stunting and normal subjects were 22.2 and 20.0, respectively. The mother of the subject with a normal BMI was 48.3% (Table 4).

Classification of adult Asian body mass index (BMI) according to [14] is listed in Table 5.

The quantified amount of *Pyd* in the urine of the neonates had quadratic relationship with HAZ ('U-shaped' scatter plot) as shown in Fig. 1. The *Pyd* in the urine was negatively associated with body length in stunted neonates and can be used as biomarkers of linear growth. Neonates classified as stunted if their length <48 cm and the contents of Pyd>859.7 nmol/mmol Cr. Further study is recommended for infants aged 6 to 12 months to further confirm the hypothesis. In the previous studies, *Pyd* excretion were found to differ based on different age groups. *Pyd* excretion from elementary school children, for example, is about 50--500 nmol/mmol Cr [17]. *Pyd* excretion on children 3-5 year was 238.3±22.7 pmol/mumol Cr (male) and 261.8±14.2 pmol/mumol Cr (female) [18].

Table 2 .Familial socioeconomic status of the subjects

Variables	Criteria	Value*
Sex	Male	74.3 (26)
	Female	25.7 (9)
Residence	*Pekanbaru*	91.4 (32)
	Others	5.7 (2)
Ethnic group	Malay	97.1 (34)
	Chinese	2.9 (1)
Mother's job	Teacher	8.6 (3)
	Housewife	51.4 (18)
	Employed	31.4 (11)
	Entrepreneur	8.6 (3)
Mother's education	Elementary school	5.7 (2)
	Junior high school	2.9 (1)
	Senior high school	22.9 (8)
	Diploma 3	20.0 (7)
	Diploma 4	2.9 (1)
	Scholar	37.1 (13)

**% (n)*

Fig. 2. Pediatric urine collector

Table 3. Characteristics of the stunted and normal subject

Variables	Stunted	Normal
Length (cm)	46.8±0.5 (46:47)[a]	49.9±1.4 (48.0:53.0)[b]
Weight (g)	2846±360 (2400:3480)	3215±404 (2380:4080)
Head circumference (cm)	33.3±1.0 (31.0:34.5)	33.6±1.2 (31.0:35.0)
Pyd (nmol/mmol Cr)	982,9±61.6 (967,8:1049.6)[a]	594.1±266,1 (564,4:2550.8)[b]
Age (days)	1±1 (1:1)	1±1 (1:3)
WAZ	-1.03±0.82 (-2.15:0.33)	-0.21±0.87 (-2.15:1.74)
HAZ	-1.26±0.27 (-1.67:-1.00)[a]	0.23±0.66 (-0.56:1.97)[b]
BAZ	-0.69±1.29 (-2.27:1.66)	-0.60±1.11 (-3.21:1.34)
Mother's BMI (kg/m^2)	21.5±4.3 (18.0:30.5)	22.0±2.9 (16.9:26.7)
Mother's height (cm)	156±4 (150:165)	161±7 (150:185)
Mother's weight before pregnancy (kg)	53±13 (42:83)	57±8 (42:70)
Mother's prenatal weight (kg)	66±15 (53:101)	69±11 (50:86)
Pregnancy age (weeks)	38±1 (37:39)	38±3 (35:49)
Number of children (person)	2±1 (1:4)	2±1 (1:4)

Independent t test: [ab]$\rho<0.01$

Table 4. Nutritional status of subject mother

Category	Amount	
	n	%
Underweight	5	17.2
Normal	14	48.3
Overweight:	4	13.8
At Risk	5	17.2
Obese I	0	0.0
Obese II	1	3.4
Total	29	100.0

Table 5. Classification of adult Asian body mass index (BMI)

Category	BMI (kg/m2)	Risk of co-morbidities
Underweight	< 18.5 kg/m^2	Low (but the risk of other clinical problems increases)
Batas Normal	18.5 - 22.9 kg/m^2	Mean
Overweight:	$\geq$ 23	
At Risk	23.0 – 24.9 kg/m^2	Increase
Obese I	25.0 - 29.9 kg/m^2	Moderate
Obese II	$\geq$ 30.0 kg/m^2	Dangerous

Source: [14]

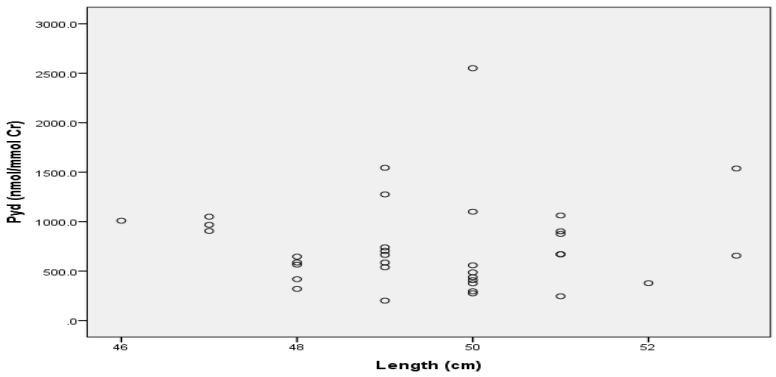

Fig. 3. Association of *Pyd* in the urine and body length in neonates

4. CONCLUSION

Pyd was significantly higher in the urine from stunted neonates than non-stunted neonates. Urine Pyd may become a candidate of a marker of stunted neonates. Further study on a large population is necessary.

CONSENT

All authors declare that written informed consent was obtained from the patient (or other approved parties) for publication of this case report and accompanying images. A copy of the written consent is available for review by the Editorial office/Chief Editor/Editorial Board members of this journal.

ETHICAL APPROVAL

All authors hereby declare that all experiments have been examined and approved by the appropriate ethics committee and have therefore been performed in accordance with the ethical standards laid down in the 1964 Declaration of Helsinki.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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Group Statistics

	Categorical of length	N	Mean	Std. Deviation	Std. Error Mean
Pyd (nmol/mmol Cr)	<48 cm	4	982.925	61.6458	30.8229
	>= 48 cm	28	594.118	266.1589	50.2993

Independent Samples Test

		Levene's Test for Equality of Variances		t-test for Equality of Means						
									95% Confidence Interval of the Difference	
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	Lower	Upper
Pyd (nmol/mmol Cr)	Equal variances assumed	3.316	.079	2.872	30	.007	388.8071	135.3688	112.3471	665.2672
	Equal variances not assumed			6.591	22.513	.000	388.8071	58.9921	266.6265	510.9878

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