

Determination of Dickkopf-1 in serum of physically disabled Saudi girls and its correlation with BMI and bone turnover markers

¹Zahra Ali AL-Balawi, ²Hala Salim. Sonbol, ³Mohammed Saleh Aradwi

¹ Department of Biochemistry, Faculty of Science, University of Tabuk, Tabuk, Saudi Arabia.

² Department of Biochemistry, Faculty of Science, King Abdul Aziz University, Jeddah, Saudi Arabia.

³ Centre of Excellence for Osteoporosis Research and Faculty of Medicine, King Abdul Aziz University, Jeddah, Saudi Arabia.

E-mail: hsunbol@kau.edu.sa

Abstract: Background: Physical disability is common in children. Dickkopf-1 (Dkk1) is a protein marker for the inhibition of bone formation. This study aimed to assess the level of Dkk1 in girls with physical disability and evaluate its correlation with bone turnover markers (BTMs) and body mass index (BMI). **Methods:** This study included 36 physically disabled Saudi girls recruited from the Disabled Children's Association in Jeddah, Saudi Arabia. Forty healthy Saudi girls were enrolled as a control group. The serum levels of Dkk1, the correlation between serum Dkk1 with N-terminal propeptide of type I collagen (P1NP), alkaline phosphatase (ALP), and C-terminal cross-linking telopeptide of type I collagen (β -CTX), as well as body mass index (BMI) were determined. **Results:** Dkk1, P1NP and ALP levels were significantly higher in the physically disabled girls than in the control girls. In the control girls, β -CTX were significantly higher than in the physically disabled girls. A moderately significant positive correlations between Dkk1, BMI and ALP in addition to a weak correlation with P1NP in the physically disabled girls were presented, however, the Dkk1 did not correlate with β -CTX. **Conclusions:** The results indicated that Dkk1 might have a role in bone changes in physically disabled girls. Dkk1 may assist in the assessment of patients with aberrant bone turnover. Further studies are needed to establish the exact role in bone metabolism.

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Keywords: Dickkopf-1; children disabilities; bone turnover markers; P1NP; β -CTX; BMI

1. Introduction

Physical disability is a condition caused by muscular and neurological impairments affecting the motor skill or mobility of an individual, such as muscular dystrophy and cerebral palsy [1, 2]. The most common causes of physical disability in children living in developing countries are cerebral palsy. It occurs in young children, in whom the damage to the brain causes impairment of the motor function [3]. Cerebral palsy is caused by birth injury such as hypoxia [4]. Children with cerebral palsy mainly develop a musculoskeletal disorder in terms of posture and muscle contraction [5]. Patients with physical disability lack mechanical loading due to the immobilization and paralysis of their limbs, which increases bone resorption and leads to a reduction in bone mass and size [6, 7], thereby increasing the risk of fracture, of developing osteopenia in the affected limb and of osteoporosis [8, 9]. It has been reported that patients with physical disability have low levels of vitamin D and high levels of calcium [10] as well as resorption markers such as CTX [11].

In Saudi Arabia, the Ministry of Health has reported that there are 1.5 million people with

disabilities [12]. Saudi Arabia confirmed its national commitment to the improvement of national systems that will protect the human rights of people with disabilities [13]. Accordingly, we decided to analyse and highlight physical disability in children by studying the potential protein biomarker Dkk1, which plays a key role in bone regulation. Dkk1 is a soluble inhibitor protein of wnt/ β -catenin signalling [14], that is essential pathway for bone development and remodelling [15]. Thus, increased serum Dkk1 is associated with a decrease in bone mass and density. Moreover, a high level of Dkk1 is also detected in metabolic bone diseases such as osteoporosis [16, 17].

Mechanical load is important inducer for bone formation through the wnt/ β -catenin pathway [18]. The expression levels of wnt/ β catenin inhibitors such as Dkk1 and sclerostin are high in cases of mechanical unloading, which usually result from a lack of physical activity [19, 20]. Mechanical unloading negatively affects the bone metabolism by increasing bone loss [21]. Studies conducted on patients with physical disabilities found that bone density was very low, with high levels of wnt/ β catenin suppressors such as Dkk1 and sclerostin [22]. However, wnt/ β

catenin inhibitor could also be an indicator for low bone mass, as it enhances bone resorption and suppresses bone formation [23, 24].

In this study, we aimed to assess the serum level of Dkk1 in disabled female girls and to correlate its level with BMI and bone turnover markers (PINP, ALP and β -CTX).

2. Material and Methods

Subjects

This study is a cross-sectional study, over a period of 18 months (March 2013 to September 2014). Thirty-six Saudi children girls (5-12 years old) with different physical disabilities, hemiplegia and quadriplegia, as a result of cerebral palsy and muscular dystrophy were recruited from the Disabled Children's Association in Jeddah, Saudi Arabia. In addition, 40 healthy volunteer Saudi children, sex and age matched, were enrolled as a control group from the Centre of Excellence for Osteoporosis Research (CEOR) in King Fahad Centre for Medical Research (KFCMR), King Abdulaziz University, Jeddah, Saudi Arabia.

We excluded patients with renal disease, liver disease, thyroid disorders, diabetes mellitus and any medications that affect bone metabolism. The laboratory work was conducted at CEOR and KFCMR. Written consent was obtained from the guardian of each child participating in the study. This research was approved by the Human Research and Ethics Committee at CEOR, King Abdulaziz University, Jeddah, Saudi Arabia.

Anthropometric Measurements

Weight was measured using a Seca Digital Chair (Seca GmbH & Co, Deutschland) a special medical scale designed to measure the weight of physically disabled patients. The subject's height was measured with a tape measure while they were lying on a bed. BMI was calculated as weight (kg)/height (m²).

Blood Sample Collection

Venous blood samples were collected at random times between 10 a.m. and 2 p.m. Serum was immediately separated and stored at -80 °C until analysis for biochemical and hormone determinations. All samples were stored in tubes labelled with the ID number.

Measurements of Serum Dkk1 Concentration

Serum Dkk1 levels were measured by enzyme immunoassay according to the manufacturer's instructions (Enzo Life Sciences AG, Switzerland). Using biotinylated polyclonal anti-Dkk1 antibody. The optical density was read at 450 nm using a microplate reader (ELx 808, Bio Tek. USA). The result was calculated using linear graph paper, and the average optical reading for each standard versus Dkk1 concentration was plotted. The unknown

concentrations of Dkk1 were determined by interpolation. The samples concentrations were multiplied by the dilution factor.

Measurement of Serum PINP Concentration

Bone formation was assessed by measurement of PINP in serum in accordance with the manufacturer's recommendations. The assay was based on the electrochemiluminescence immunoassay (ECLIA) using an Elecsys E411 autoanalyzer (Roche Diagnostics GmbH, D-68298 Mannheim, Germany). The results were determined via a calibration curve instrument-specifically generated by 2-point calibration and a master curve provided via the reagent barcode.

Measurement of Serum ALP Concentration

The serum ALP assessment method was used in accordance with the manufacturer's recommendations, with p-nitrophenyl phosphate as the substrate. The serum ALP concentration was measured using a Vitros 250 Chemistry System autoanalyzer (Ortho-Clinical Diagnostics-Johnson & Johnson Co, USA). The concentrations of alkaline phosphatase were automatically calculated on a Vitros 250 system at 400 nm.

Measurement of β -CTX Concentration

The serum concentration of bone resorption marker β -CTX was determined by the ECLIA technique using an Elecsys E411 autoanalyzer (Roche Diagnostics GmbH, D-68298 Mannheim, Germany). The β -CTX determination was undertaken by incubating the patient's serum (50 μ l) with biotinylated monoclonal anti- β -CTX antibody. The β -CTX concentrations were determined via a calibration curve, instrument-specifically generated by 2-point calibration, and a master curve provided via the reagent barcode.

Statistical Analysis

Data were analysed using the Statistical Package for Social Science software (Version 20, SPSS INC., Chicago, Illinois, USA). Descriptive data were given as the mean \pm SD (SD). The independent T-test was used to compare between two groups. P values < 0.05 were considered statistically significant. Graphs were made using Prism software version 5.

3. Results

Comparison of Demographic Characteristics and Biochemical Parameters Between the Controls and the Physically Disabled Girls

This study included 40 control girls and 36 physically disabled girls, with ages ranging from 5 to 12 years. The demographic characteristics and biochemical parameters of the study groups are presented in Table 1. There were no statistically significant differences in the age and weight of the two groups ($P = 0.076$, $P = 0.189$, respectively). The

mean \pm SD values for age and weight were 8.73 ± 1.43 vs. 9.39 ± 1.78 and 24.18 ± 2.75 vs. 26.21 ± 9.26 , respectively.

The mean \pm SD of BMI was higher in the physically disabled group than in the control group (18.12 ± 3.67 vs. 15.18 ± 0.66 kg/m²). The difference between the two groups was statistically significant ($P = < 0.001$).

Dkk1 protein was detected in all the serum samples. The mean Dkk1 serum level was 18067.31 ± 6116.66 pg/ml in the physically disabled girls but only 10190.35 ± 1950.24 pg/ml in the control girls. The serum Dkk1 levels of the physically disabled girls were significantly higher than for the control girls ($P < 0.001$).

With regard to the bone formation markers PINP and ALP, the serum levels were higher in the

physically disabled girls than in the control girls. The difference in PINP was statistically significant ($P < 0.001$). The mean for the physically disabled girls was 708.95 ± 270.89 ng/ml vs. 424.10 ± 228.6 ng/ml for the control group. On the other hand, there was no statistically significant differences in serum ALP level between the physically disabled girls and the control girls ($P = 0.065$). The mean ALP in the physically disabled girls was 237.81 ± 51.33 U/L vs. 218.15 ± 39.79 U/L for the control girls. The levels of the bone resorption marker β -CTX, in the control girls were significantly higher than in the physically disabled girls ($P < 0.001$). The mean of β -CTX in the physically disabled girls was 546.11 ± 383.08 vs. 879.62 ± 354.51 for the control group (Figure 1).

Table 1. Demographic Characteristics and biochemical parameters of the studied control and physically disabled girls.

Variables	Control girls (n=40), Mean \pm SD	Physically disabled girls (n=36), Mean \pm SD	P value
Demographic Characteristics			
Age (Years)	8.73 ± 1.43	9.39 ± 1.78	0.076
Weight (kg)	24.18 ± 2.75	26.21 ± 9.26	0.189
BMI (kg/m ²)	15.18 ± 0.66	18.12 ± 3.67	< 0.001*
Biochemical Parameters			
Dkk1 (pg/ml)	10190.35 ± 1950.24	18067.31 ± 6116.66	< 0.001*
PINP (ng/ml)	424.10 ± 228.6	708.95 ± 270.89	< 0.001*
ALP (U/L)	218.15 ± 39.79	237.81 ± 51.33	0.065
β -CTX (pg/ml)	879.62 ± 354.51	546.11 ± 383.08	< 0.001*

SD: Standard deviation, Dkk1: Dickkopf-1, BMI: Body mass index, PINP: Total procollagen type 1 N-terminal propeptide, ALP: alkaline phosphatase, β - CTX: C-terminal crosslinked telopeptide of type I collagen. Asterisk indicates statistical significance at $P < 0.05$.*

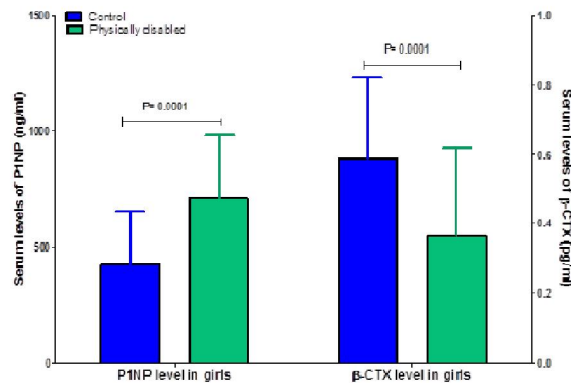


Figure 1. Levels of BTM in Physically Disabled and Control Girls. Data are expressed as the mean \pm SD. Significance of differences between patients and controls of the same gender was determined using the unpaired Student "t" test.

Correlation Between Dkk1 Level and Variables in Physically Disabled Girls

We found a significant moderate positive correlation between serum Dkk1 levels in physically disabled girls and BMI and ALP ($r = 0.388$, $P = 0.019$, $r = 0.396$, $P = 0.017$). On the other hand, there was a weak correlation between Dkk1 and PINP ($r = 0.197$, $P = 0.250$). No correlation was demonstrated between Dkk1 and β -CTX ($r = -0.270$, $P = 0.111$) (Table 2).

Table 2. Correlation Between Dkk1 Level and Variables in Physically Disabled Girls.

Variables	Dkk1	
	R	P
PINP (ng/ml)	0.197	0.250
ALP (U/L)	0.396	0.017*
β -CTX (pg/ml)	-0.270	0.111
BMI (kg/m ²)	0.388	0.019*

*Correlation is significant at the 0.05 level (2-tailed).r: Pearson's correlation (weak correlation if $r < 0.3$, moderate correlation if $0.3 < r < 0.7$, strong correlation if $r > 0.7$).

4. Discussions

According to the international classification of functioning, disability and health (ICF), the definition of childhood and youth disability is not merely biological or societal but the interaction among health surroundings, environmental and individual aspects [25]. WHO divides disability into three levels; damage to body function or structure, restriction of reading or movement and inability to perform daily activities. Some children can be disabled from birth, while others acquire disabilities later in life [26]. There have been several studies addressing the problems concerning children with physical disabilities in Saudi Arabia [27-29]. However, due to the lack of research conducted on protein biomarkers and their relation to physical disabilities specifically in children, we decided to analyse and highlight the role of Dkk1 as a potential protein biomarker in children (girls) with physical disabilities due to its key role in bone regulation. To our knowledge, this study is the first to analyse the Dkk1 level and address the relationship (correlation) between Dkk1 and mechanical loading in physically disabled girls in Saudi Arabia. Dkk1 has been considered as a bone turnover marker, among other major ones that were used for exploring the bone metabolism, such as P1NP, β -CTX and ALP [30, 31].

In this study, we assessed the serum level of Dkk1 in physically disabled girls. Our data provide clear evidence that Dkk1 is significantly elevated in physically disabled girls. It is known that the overexpression of Dkk1 is inversely proportional to bone mass and associated with metabolic bone disease [16]. Frings-Meuthen et al. conducted two studies on healthy young men; the first study conflicted with our findings, and the second agreed with them. The first study showed that Dkk1 decreased during 14 days of bed rest, increased during 21 days of bed rest, and returned to a normal level within 3 days after the bed rest ended. This result supports the suggestion that the expression of Dkk1 increases during mechanical unloading, i.e., immobilization leads to an increase in the Dkk1 level [32]. Furthermore, our results confirmed previous work that supported the increases in Dkk1 with mechanical unloading by showing that Dkk1 levels increased significantly in immobilized men with spinal cord injury [33]. Although the two studies were performed on different genders and ages, they found the same result. In Saudi Arabia, a similar work was conducted on a protein known as sclerostin that has a similar mechanism of action and function to Dkk1. However, the studies that have been performed focused on analysing the serum sclerostin levels in pre-and postmenopausal women and study its relationships to physical activity, osteoporosis related fracture, and bone turnover markers [34, 35].

Disease markers are required for many reasons: to achieve an initial diagnosis, identify the stage of disease and determine the response to medication. Markers of bone formation, serum P1NP and ALP levels show changes opposite from what was predicted for such disability. Both serum P1NP and ALP levels were higher in physically disabled girls than in the controls; however, only the elevation in serum P1NP levels was significant. β -CTX showed a significantly lower value in physically disabled girls than control girls. To explain these results, we have realized that the disabled girls were undergoing physical therapy during the blood collection period. The Disabled Children's Association conducts physical therapy for 3 weeks every several months, stops for 3-4 months, etc. The mechanical loading generated by the physical therapy could explain the high levels of P1NP and ALP and the low level of β -CTX in physically disabled girls. Accordingly, no matter how consistent the performance of physical activity, alteration in the levels of bone formation markers is very likely. According to Adami et al., small changes in physical activity can produce a clear effect on bone formation markers [36]. However, we could not confirm this opinion because we did not assess the P1NP, ALP and β -CTX levels before and after the physical therapy. A similar result has been reported in that P1NP was significantly reduced during mechanical unloading in young males [32]. Furthermore, a study conducted in postmenopausal women demonstrated that P1NP was significantly associated with the level of physical activity [36].

BMI is used to identify and describe obesity and assess physical growth in children, adolescents and adults [37-40]. The physically disabled girls in this study were underweight according to WHO BMI-for-age (5-19 years) (Overweight: equivalent to BMI 25 kg/m² at 19 years; Obese: equivalent to BMI 30 kg/m² at 19 years). However, by comparison to the controls, their BMI values were significantly higher. This result is in line with the report of Fong et al. [41]. The age (18.64 \pm 0.74 years) and gender (boys and girls) of the participants in their study, however, were different from ours. We demonstrated a significant moderate positive correlation between Dkk1 concentration and BMI in physically disabled girls. It has been mentioned in previous studies that Dkk1 promotes adipogenesis by enhancing the differentiation of adipose tissue in humans [42, 43]. This promotion might explain the association observed. Nevertheless, the correlation between Dkk1 and BMI in children is not yet clear, as two studies have shown contradictory results. The study conducted by Kanaka-Gantenbein and his group found that the Dkk1 level was negatively correlated with BMI in young girls [44],

whereas Brunetti and his colleagues revealed that Dkk1 levels in healthy obese children were high [45].

Our study revealed a weak positive and moderate correlation of the bone formation markers P1NP and ALP (respectively) with Dkk1 in physically disabled children and negative correlation with the bone resorption marker β -CTX. This result was unexpected since P1NP is an indicator for bone formation, while an increasing Dkk1 level indicates bone resorption. It is important to note that this study was conducted in children at an early age (5-12 years), and during this stage of human life, bone growth is highly active in favour of net bone formation over bone resorption until the optimum bone mass (peak bone mass) is achieved [46]. Although prolonged mechanical unloading (physical inactivity) before the physical therapy could be a reason for Dkk1 serum increase, we hypothesize that the increase in P1NP could be due to the increase in Dkk1 levels. Additionally, mechanical unloading increases the expression of Dkk1, which causes an increase in bone resorption. Therefore, P1NP expression may increase by a feedback mechanism to compensate for (neutralize) the increasing bone resorption.

The limitation of this study was the sample size, which was relatively small and might affect the statistical power. Additionally, we could not perform DEXA (dual energy X-ray scanning) on the children due to their health situation, which would have provided an overall picture of bone mass density status.

In summary, this cross-sectional study is the first study conducted in Saudi Arabia proposing the correlation of serum Dkk1 with BTM and BMI in physically disabled children. Our result showed an increase in the Dkk1 level in mechanical unloading cases that could indicate bone loss. Furthermore, we propose that physical therapy is an important factor in inducing bone formation and inhibiting bone resorption. The data could raise the issue of the compensation mechanism of bone formation markers along with the increase in Dkk1. Dkk1 may also promote adipogenesis by enhancing the differentiation of adipose tissue. We proposed that Dkk1 should be used as a diagnostic marker to find signatures that distinguish many types of physical disabilities, giving physicians and researchers another tool to improve detection, treatment, and patient outcomes.

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Conflict of Interest:

The authors have no conflicts of interest relevant to this article to disclose.

Disclosure Summary:

The authors have nothing to disclose.

Corresponding Author:

Dr. Hala Salim Sonbol
Department of Biochemistry
Faculty of Science
King AbdulAziz University
P.O. Box No. 122522,
Jeddah, 21332, Saudi Arabia.
E-mail: hsunbol@kau.edu.sa

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