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DICKKOPF-1 SERUM LEVELS IN SAUDI BOYS WITH CEREBRAL PALSY AND CORRELATIONS WITH BODY MASS INDEX AND BONE TURNOVER MARKERS

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ABSTRACT

Cerebral palsy (CP) is a common disorder in children. Dickkopf-1 (Dkk1) inhibits the canonical Wnt signaling pathway. The study aimed to assess the serum levels of Dkk1 in boys with CP and to evaluate its correlations with body mass index (BMI) and bone turnover markers (BTMs).This study included 32 Saudi boys with different physical disabilities as a result of CP and muscular dystrophy and 39 healthy Saudi boys were enrolled as a control group. The serum levels of Dkk1, N-terminal propeptide of type I collagen (P1NP), bone alkaline phosphatase (B-ALP), and C-terminal cross-linking telopeptide of type I collagen (β-CTX) were measured using enzyme link immunosorbent assay (ELISA) kits. Serum Dkk1 levels were higher in boys with CP than in controls. Further, level of PINP, TSH, FT4, calcium, and phosphorus were also reported significantly higher in CP boys than in controls. While, level of BMI, β-CTX, 25-hydroxyvitamin D and PTH were significantly lower in patients than in controls. Serum Dkk1 levels were moderately correlated with calcium (r=0.361, P=0.0240) in the controls. Dkk1 may play a role in bone loss in boys with CP experiencing weight loss. Dkk1 could aid in the assessment of boys with CP and abnormal bone turnover.

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1 Introduction

The Dickkopf (Dkk) family is a Wnt signaling pathway antagonist that has many roles in human physiological and pathological processes through both Wnt pathway-dependent and Wnt-independent mechanisms (Shao et al., 2017). This gene family encodes secreted glycoproteins that are composed of five members: Dkk1–4 and Dkkll (soggy) (Choi et al., 2012). The Dkk family has a master role in bone regulation (Ray et al., 2017). Dkk1decreases bone formation and increases bone resorption by inhibiting Wnt/β catenin signaling through the formation of a tertiary complex with a low density lipoprotein receptor-related protein, LRP 5 and 6, and kermen 1 and 2 receptors (Niida et al., 2004). Therefore, high serum Dkk1 is associated with a decrease in bone mass and density (Butler et al., 2010; Tella & Gallagher, 2014). The Dkk1 protein is encoded by the Dkk1 gene. In humans, the Dkk1 gene is located on chromosome 10 (10q11.2) (Roessler et al., 2000). Dkk1 is believed to be expressed in a wide variety of human tissues including bone tissue; Dkk1 is primarily expressed by osteocytes (Bonewald, 2011). The chromosomal region containing the Dkk1 gene has been associated with the regulation of bone mass in men (Ralston et al., 2005). Morvan and colleagues proved that Dkk1 expression *in vitro* negatively affects bone formation by inhibiting osteoblast differentiation and inducing adipocyte differentiation (Morvan et al., 2006).

In bone tissue, osteocytes are the most sensitive bone cells to mechanical load. Osteocytes respond to mechanical load by producing a signaling molecule called Wnt that activates the canonical Wnt signaling pathway, inducing bone formation (Bonewald & Johnson, 2008; Santos et al., 2009; Klein-Nulend et al., 2013). The canonical Wnt/β-catenin signaling increases bone mass by inducing the differentiation of osteoblasts (Galli et al., 2012). However, the absence of mechanical load in cases of bed rest, disused limbs, and microgravity increases bone loss due to the apoptosis of osteocytes and osteoblasts, while osteoclast activity increases as a result of increased Wnt signaling antagonist expression (Aguirre et al., 2006; Robling &Turner, 2009). Additionally, mechanical load causes a significant reduction in Wnt signaling antagonists, such as sclerostin and Dkk1 (Holguin et al., 2016).

Childhood and adolescence are important periods for bone mineralization. Although peak bone mass and bone mineral density (BMD) are primarily determined genetically, they are also related to age, weight, height, nutrition, physical activity, hormones, drugs, and environmental factors related to lifestyle (Baroncelli et al., 2005; Goksen et al., 2006; Verrotti et al., 2010). The Ministry of Health in Saudi Arabia has reported 1.5 million people with disabilities (Ministry of Health Saudi Arabia, 2016).

Cerebral palsy (CP) is a clinical syndrome caused by nonprogressive disorders in the development of the brain. CP is the most common form of chronic motor disability in children (Aronson &Stevenson, 2012). Children with severe CP are at risk for developing low BMD and low-impact fractures due to the restriction of mobilization and paralysis of their limbs, which increases bone resorption and reduces bone mass and size (Lin & Henderson, 1996; Unay et al., 2003; Aronson &Stevenson, 2012).

Bone turnover markers (BTMs) are used to assess bone metabolism. BTMs are molecules released from bone cells during bone metabolism and are used with limitations as a diagnostic tool in osteoporosis (Civitelli et al., 2009). They can also predict rapid bone loss in clinical studies and monitor drug efficacy during healing of fractures (Henriksen et al., 2015). BTMs are classified into bone formation markers and bone resorption markers (Szulc & Delmas, 2008). Many hormones and minerals, including parathyroid stimulating hormone (PTH), thyroid stimulating hormone (TSH), glucocorticoids, calcium, phosphorus and vitamin D, affect bone mass. In addition, external factors influence bone such as physical activity, sun exposure, and nutrition (Hunter et al., 2001; Quarles, 2008).

Currently, there is no information on protein biomarkers and their relation to physical disabilities specifically in boys with CP. To our knowledge, this study is the first to analyze the serum levels of Dkk1 and address the relationship between Dkk1 and different measured parameters and mechanical loading in physically disabled boys in Saudi Arabia. We decided to address the role of Dkk1 as a potential protein biomarker in boys with CP due to its key role in bone regulation. Our hypothesis was that Saudi boys with diverse physical disabilities with increased serum Dkk1levels have a greater risk for osteoporosis and osteoporosis-related fractures. The aim of this study was to determine the serum levels of Dkk1 in Saudi boys with CP and to study the correlation between serum Dkk1 levels and BMI as well as BTMs (P1NP and CTX).

2 Materials and methods

2.1 Study subjects

Thirty-two (32) Saudi boys (5–12 years old) with diverse physical disabilities, such as hemiplegia and quadriplegia, as a consequence of CP and muscular dystrophy, were involved in this cross-sectional study over a period of 18 months (March 2013 to September 2014). They were recruited from the Disabled Children's Association in Jeddah, Kingdom of Saudi Arabia. Thirty-nine (39) healthy Saudi volunteers, age and sex matched with the patients, were employed as a control group from the

Center of Excellence for Osteoporosis Research (CEOR) at King Fahad Center for Medical Research (KFCMR), King Abdulaziz University (KAU), Jeddah, Kingdom of Saudi Arabia. Written consent to participate in the study was obtained from each child's guardian. This research was approved by the Human Research and Ethics Committee at CEOR, KAU, Jeddah, Kingdom of Saudi Arabia. Children with chronic illnesses, such as renal diseases, liver diseases, thyroid disorders and diabetes mellitus, or those on medications that affect bone metabolism, such as steroids and antiepileptic medications, were excluded from the study.

2.2 Anthropometric measurements

Height and body weight measurements were recorded. The weight was measured by a Seca Digital Chair (Seca GmbH &Co, Germany), a special medical scale designed to measure the weight of physically disabled patients. Height was measured by using a tape measure while the children were lying on a bed. BMI was calculated as weight (kg)/height (m²).

2.2.1 Blood sample collection

Venous blood samples were randomly collected. Immediately, serum was separated and stored at -80°C until further use. All samples were stored in tubes labeled with the ID number of the participants.

2.2.2 Biochemical analysis

Serum Dkk1 levels were measured as described by AL-Balawi et al. (2017) by enzyme immunoassays (Betts et al, 2010) according to the manufacturer's instructions (Enzo Life Sciences AG, Switzerland). 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 serum concentrations of PINP and β-CTX were measured (Ardawi et al., 2010) by electrochemiluminescence immunoassays (ECLIAs) using an Elecsys E411 autoanalyzer (Roche Diagnostics GmbH, D-68298, Mannheim, Germany). TSH and thyroxin hormone were determined by Vitros ECI (Immunodiagnostic Systems, UK). PTH was measured using an ECLIA Elecsys autoanalyzer (Roche Diagnostics GmbH, Sand hofer Strasse 116, D-68305, Mannheim, Germany). The assessment of 25-hydroxyvitamin D [25(OH)D] (Ardawi et al., 2010) was conducted using chemiluminescent immunoassay (CLIA) technology for the quantitative determination of 25(OH)D and other hydroxylated vitamin D metabolites in human serum, and the assay was performed using the LIAISON fully automatic immunoassay system analyzer. Serum calcium phosphate and bone alkaline phosphatase (B-ALP) were assessed using the Vitros slide method with a Vitros 250 Chemistry System autoanalyzer (Ortho-Clinical Diagnostics-Johnson & Johnson Co., USA).

2.3 Statistical analysis

Statistical analyses were performed by using the Statistical Package for Social Science package (Version 20, SPSS INC., Chicago, Illinois, USA). Descriptive data are given as the median (interquartile range). Mann-Whitney tests were used to compare patients and controls. Correlations between different variables and Dkk1 were assessed using Pearson's correlation as appropriate. A *P* value <0.05 was considered significant.

3 Result

3.1 Comparison of the physical and biochemical characteristics of the control and CP boys

The comparison between the control and CP boys is presented in Table 1. There were no significant differences in age (*P*=0.986). The BMI of CP boys was significantly lower than that of the control group (*P*=0.016). Further, CP boys had a higher median Dkk1 level (177719pg/mL) than control boys (15292pg/mL). However, this increase was not significant (*P*=0.234). With regard to the bone formation markers, P1NP was significantly increased (*P*=0.0001), while β-CTX was significantly decreased in CP boys compared with control boys (*P*=0.0001). The bone formation marker B-ALP showed no differences between patients and controls. According to the data, both PTH and 25(OH)D levels were obviously significantly higher in the control boys than in the CP boys (*P*=0.0001 for both). TSH, FT4, calcium and phosphate serum levels were significantly higher in CP boys than in control boys (*P*=0.0001, *P*=0.0001, *P*=0.0001 and *P*=0.002, respectively).

3.2 Correlations between Dkk1 and measured parameters

Serum levels of Dkk1 had a positive moderate correlation with calcium in controls group (r=0.361, P=0.024). Moreover, there were weak negative correlations identified between Dkk1, β-CTX, (r=-0.133, P=0.420), PTH (r=-0.199,P=0.225) and phosphate (r=-0.137-0.199P=0.405). However, did not reach significance value.

Weak correlation was also demonstrated between Dkk1 and and PINP (r= 0.332, P= 0.063), β-CTX (r= 0.155, P=0.397), B-ALP (r=0.270, P=0.135), TSH(r=0.123, P= 0.502) and phosphate (r=0.259, P= 0.152) in patients group. This correlation did not reach significance values.BMI (r=-0.219, P= 0.229) was negatively weakly correlated to Dkk1 in patients group.

4 Discussion

A comparison between boys with CP and control boys showed that the BMI in CP boys was significantly lower than that in the control boys (*P*=0.016). Low BMI and underweight in boys with CP in our study could be a consequence of feeding problems resulting either from the children's pathological symptoms

Table 1Comparison between the physical and biochemical characteristics of patients and control boys

Variables	Controls (n=39)	Patients (n=32)	P value using Mann-Whitney Utests
Age (years)	8.00 (7.00 – 9.50)	8.00 (7.00 – 9.00)	0.986
BMI (kg/m²)	16.84 (16.15 – 18.05)	15.63 (13.98 – 17.19)	0.016
Dkk1 (pg/mL)	15291.92 (13263.40– 17518.15)	177719.00 (11772.50 – 26396.25)	0.234
PINP (ng/mL)	328.00 (294.00 – 401.00)	548.90 (460.90 – 712.45)	0.0001
β-CTX (pg/mL)	888.00 (811.00 – 949.00)	438.30 (248.55 – 604.30)	0.0001
B-ALP (U/L)	213.00 (199.00 – 231.00)	231.50 (196.75 – 264.75)	0.252
25(OH)D (nmol/L)	49.10 (44.80 – 60.13)	29.00 (19.13 – 37.13)	0.0001
PTH (pmol/L)	4.05 (3.66 – 4.58)	1.36 (0.70 – 2.16)	0.0001
TSH (uIU/mL)	1.98 (1.79 – 2.16)	3.04 (2.01 – 4.58)	0.0001
FT4 (pmol/L)	10.23 (9.02 – 11.11)	18.48 (16.50 – 20.20)	0.0001
Calcium (mmol/L)	2.40 (2.36 – 2.41)	2.62 (2.39 – 2.70)	0.0001
Phosphate (mmol/L)	1.39 (1.30 – 1.45)	1.65 (1.39 – 1.92)	0.002

Data are represented as the median (interquartile range). P values were calculated using Mann-Whitney Utests. BMI: body mass index; PINP: total procollagen type 1 amino-terminal propeptide; β-CTX: C-terminal cross-linking telopeptide of type I collagen. PTH: Parathyroid hormone, 25(OH)D: 25-hydroxyvitamin D, TSH: thyroid stimulating hormone, FT₄: thyroxine,B-ALP:alkaline phosphatase

Table 2Pearson's correlation between Dkk1 (pg/mL) level and measured variables in controls and CP patients

Variables	Controls Dkk1		Patients Dkk1	
	R	P	R	P
Age (years)	-0.076	0.647	-0.039	0.832
BMI (kg/m²)	-0.080	0.629	-0.219	0.229
PINP (ng/mL)	0.003	0.984	0.332	0.063
β-CTX (pg/mL)	-0.133	0.420	0.155	0.397
B-ALP (U/L)	0.044	0.793	0.270	0.135
25(OH)D (nmol/L)	0.208	0.204	-0.160	0.381
PTH (pmol/L)	-0.199	0.225	-0.152	0.407
TSH (uIU/mL)	0.055	0.738	0.123	0.502
FT4 (pmol/L)	-0.010	0.951	0.026	0.886
Calcium (mmol/L)	0.361	0.024	0.101	0.584
Phosphate (mmol/L)	-0.137	0.405	0.259	0.152

R: Pearson's correlation (weak correlation if R<0.3, moderate correlation if 0.3<R<0.7, strong correlation if R>0.7). PINP: total procollagen type 1 amino-terminal propeptide. β-CTX: C-terminal cross-linking telopeptide of type I collagen. BMI: Body mass index.

associated with CP or the subjects being in a household with multiple children, which could lead to a decreased ability to manage the disease. In this respect, children with CP are often viewed as undernourished and believed to show growth impairment (Melunovic et al., 2017). Diwan et al. (2014) analyzed the BMI of 50 children (1–18 years old) with different clinical types of CP with respect to their gross motor functions using a gross motor function classification system. The researchers concluded that underweight is prevalent in children with spastic quadriplegic CP. They stated that poor nutrition in CP children is the result of various factors that directly or indirectly affect food intake, such as oro-motor dysfunction and/or poor appetite (Diwan et al., 2014). Feeding problems are common in children with CP (Dahl et al., 1996). Disabled children or children who required an aid to walk also had a greater chance of having eating and swallowing problems, thus needing food mashing or liquidization than those who were not disabled (Sullivan et al., 2000). Moreover, children suffering from CP also had medical and social issues, restricting their participation in physical activities (Azcue et al., 1996).

However, conflicting studies have shown that children with CP have a high rate of overweight, mostly among ambulatory children (Hurvitz et al., 2008). Duran et al. (2018) reported that the prevalence of excess body fat in CP children was 17.0%. Other researchers studied 348 boys and 240 girls with a mean age of 11.2 (standard deviation 3.2) years (Pascoe et al., 2016) and stated that 19.4% of ambulant CP children were overweight or obese due to reduced physical activity (Pascoe et al., 2016). The BMI of CP children and adolescents (n=73) of different genders, with a median age of 9.6 years (range 2.6 to 19.4), was measured in a study conducted by Delalić et al. (2014). Twenty-nine percent of the participants were under weight and were found to have quadriplegia. The rest of the participants who were overweight (11%) and obese (9.6%) had diplegia. Approximately 40% of children and adolescents with CP, were shown to have nutritional problems. Delalić et al. (2014) stated that immobile children and those with quadriplegia have an increased risk for malnutrition, whereas mobile children and those with diplegia CP have an increased risk for over nutrition (Delalić et al., 2014).

This study showed that serum levels of Dkk1 in boys with CP were nonsignificantly higher than those in the controls. Similar results were achieved in our previously published research, indicated that the serum Dkk1 levels in the physically disabled girls were significantly higher than control girls (AL-Balawiet al., 2017). In this respect, Ke et al. (2012) reported that Dkk1 serum levels were increased in patients with bone diseases. A study conducted by Dovjak et al. (2014) demonstrated that Dkk1 levels increased with age and in patients with hip fractures. Significant increases in Dkk1 in cases of mechanical unloading were observed in immobilized men with spinal cord injury (Gifre et al., 2015). Moreover, a study on rodents showed that mRNA expression of Dkk1 was upregulated in fracture healing, and the levels peaked around the 14th post-traumatic day (Li et al., 2011). Butler and colleagues also stated that the over expression of Dkk1 was inversely proportional to bone mass and associated with metabolic bone disease (Butler et al., 2010). Dkk1 contributes to bone lesions by impairing osteoblast activity (Rachner et al., 2014).

In the present study, serum levels of the bone formation markers P1NP and B-ALP were significantly higher, while β-CTX levels were significantly lower in boys with CP than in controls. The mechanical loading created by physical therapy could explain the high levels of P1NP and B-ALP and the low level of β-CTX in boys with CP. This finding indicates that bone collagen synthesis in mature osteoblasts is diminished by rapid unloading in young people. The BTMs could be affected by prolonged wheelchair use in boys with CP but not in their counterparts. Therefore, no matter how consistent the performance of physical activity is, alterations in the levels of bone formation markers are very likely (AL-Balawi et al., 2017). In children, bone turnover can be 10 times higher than that in adults due to three physiologic processes related to the skeleton: bone modeling, remodeling, and growth. The levels of bone formation and resorption markers are consequently much higher in children than in adults (Rauchenzauner et al., 2007). A study was performed by Buehlmeier et al. (2017) on old (60±2 years) and young men (23±3 years) who experienced bed rest for 14 days. There were higher P1NP levels in young men than in old men ($P<0.001$), but these levels decreased during bed rest in the young group ($P<0.001$) (Buehlmeier et al., 2017). The increase in bone resorption markers during bed rest was comparable among the age groups. Greater urinary β-CTX levels at baseline were observed in young than in old subjects, while the increases during unloading or bed rest were similar. The authors concluded that during bed rest, bone formation markers were reduced only in young men, whereas resorption markers were elevated to a comparable extent. The researchers suggested that older men did not have a higher risk of losing bone when immobilized (Buehlmeier et al., 2017). Furthermore, a study conducted in postmenopausal women demonstrated that PINP but not β-CTX was significantly associated with the level of physical activity (Adami et al., 2008). According to Adami, PINP could be more sensitive to the mechanical load than Dkk1 and β-CTX.

In the current study, the serum levels of vitamin D in boys with CP were significantly lower than those in the controls, and calcium levels were significantly higher than those in the controls. The same results were reported by others in patients with physical disabilities (Sato et al., 1998). Calcium is an essential mineral for babies and young children to ensure the

growth of strong bones. In addition, vitamin D plays a vital role in calcium metabolism and bone mass during growth and development (Henderson et al., 2002). However, children with physical disability lack calcium and vitamin D due to feeding problems and may have less exposure to sunlight than healthy children due to their prolonged wheelchair use, which limits their accessibility and ability to go outdoors (Henderson et al., 2002; Hillesund et al., 2007; Dahlseng et al., 2012). Accordingly, this status could contribute to poor bone mineralization. Studies conducted in Kingdom of Saudi Arabia showed that the Saudi population has a low level of vitamin D due to lifestyle and inadequate exposure to sunlight during the day (Mansour & Alhadidi, 2012). In this study, the increase in serum calcium level could be due to a low degree of calcium incorporation in the skeleton of physically disabled patients as result of decreased bone mineralization as well as increased bone resorption markers that release calcium from bone storage.

Serum PTH levels in the present study were significantly lower in CP patients than controls. In this regard, a study was performed with 16 boys and 8 girls (aged 10 months to 12 years) and showed that PTH levels were similar in CP patients and controls (Tasdemir et al., 2001). Moreover, others have indicated that PTH levels in CP patients depend on ambulatory status; thus, patients with nonambulatory CP showed lower PTH levels than patients with ambulatory CP (Kim et al., 2015; Shin et al., 2017). The increase in the calcium level may be responsible for the significant low levels of PTH observed in our patients by a negative feedback mechanism.

According to our results, the level of T4 hormones was significantly higher in patients than in controls. Similar results were achieved by Vasilieva et al (2005). The researchers studied the plasma levels of T3 and T4 in 142 children with CP and other neurological pathologies. They showed that there was a significant increase in T3 and T4 in many cases compared to the healthy controls. The changes in thyroid hormone content depended on the patient's age and severity of the pathological condition (Vasilieva et al., 2005). Mild thyroid dysfunction, with elevated TSH that was below the clinical threshold (5–15 mIU/L), was found in boys with CP. However, it may not be associated with their impaired psychomotor development.

We found no significant correlation between Dkk1 and BMI or osteoblast or osteoclast activation markers in the CP group. However, there was a moderate positive relationship between Dkk1 and calcium in the control group. Notably, 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 favor of net bone formation rather than bone resorption until the optimum bone mass (peak bone mass) is achieved (Davies et al., 2005). The

prolonged absence of mechanical unloading prior to physical therapy could be a reason for the Dkk1 serum increase.

In summary, our study showed a nonsignificant increase in the Dkk1 levelin boys with CP, parallel to an increase in bone formation markers, P1NP and B-ALP. Measurement of serum Dkk1 levels may be useful predictive tool in the study of CP children who complain of bone fragility. Moreover, Dkk1 may in part mediate bone loss among patients experiencing weight loss.

The strength of this project is that Dkk1 could be used as a potential diagnostic marker in CP patients with rickets and osteoporosis. Further research is needed to determine the specific role Dkk1 plays in the bones of children with CP. The mechanisms for weight loss-induced bone loss could lead to more effective management to avoid bone loss, especially in physically disabled children who suffer from rickets and osteoporosis.

Declarations of interest

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

Disclosure summary

The authors have nothing to disclose.

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