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Effects of circulating endothelial progenitor cells, serum vascular endothelial growth factor and hypogammaglobulinemia in Perthes disease

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Objective: The aim of this study was to investigate Legg-Calvé-Perthes disease (PD) pathogenesis by comparing absolute circulating endothelial progenitor cell (EPC) counts, serum levels of vascular endothelial growth factor-A (VEGF-A) and immunoglobulins between PD patients and controls.

Methods: The study included 28 PD cases (mean age: 8±3.8) and 25 healthy age-matched control subjects. EPC, serum VEGF-A and immunoglobulin levels were measured in peripheral blood samples. Comparisons and correlation analysis were performed.

Results: In the PD group, 17 subjects were in the fragmentation stage and 11 in the healing stage. Four patients had bilateral disease and 14 had hypogammaglobulinemia. Median EPC count of the PD group was 80 and was significantly higher than those of the control group (p=0.011). No significant difference was determined in serum VEGF-A levels (p=0.354). EPC count were inversely correlated with serum IgG levels of the PD group (r=0.403, p=0.03). Absolute EPC count was also significantly higher in the fragmentation stage than in the healing stage and were also greater in bilaterally affected than in unilaterally affected patients. Circulating EPC count was correlated to the serum VEGF-A levels in patients with fragmentation stage of PD (r=0.605, p=0.01) and in those with hypogammaglobulinemia (r=0.599, p=0.001).

Conclusion: High EPC count at the fragmentation stage of PD and relatively higher counts in bilateral disease suggest that EPC may be a valuable marker in the diagnosis and follow-up of PD. Additional studies are needed to explain the strong correlation between EPC and serum VEGF-A level in the fragmentation stage and in the presence of hypogammaglobulinemia.

Key words: Circulating endothelial progenitor cells; hypogammaglobulinemia; Perthes disease; serum vascular endothelial growth factor.

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Osteonecrosis of the femoral head is one of the most serious conditions affecting the hip joint in the pediatric population. Legg-Calvé-Perthes disease (PD) is a common pediatric form of osteonecrosis that affects children between 2 and 12 years of age and has a cumulative incidence of 1 in 740 boys and 1 in 3700 girls.[1]

Histological studies show ischemic damage to the deep layer of the epiphyseal cartilage where endochondral ossification normally takes place.[2] The ability to recover from such damage has an important implication for maintaining the normal growth and development of the immature femoral head. The biological factors responsible for the restoration of endochondral ossification and resumption of femoral head growth after ischemic necrosis have not been investigated.[3] Although research has been performed on many factors such as environmental factors, clotting impairment (lack of protein C, thrombophilia), trauma, vascular anomalies and hyperactivity that may be related to the disease etiology, no evident relationship has been found.[4-9] However, there are studies in the literature showing a relationship between endothelial progenitor cells (EPC) and idiopathic avascular necrosis in the adult femoral head.[10]

When taken from primitive hematopoietic stem cells, endothelial progenitor cells have the ability to transform into endothelial cells. The number of endothelial progenitor cells in circulation increase during many pathological and physiological events. Studies have shown regional ischemia to be a powerful endogenous stimulator in the mobilization of EPC. Mediators such as vascular endothelial growth factor (VEGF) and granulocyte macrophage-colony stimulating factor (GM-CSF) expressed from damaged tissue accelerate the change to EPC by stimulating the leading endothelial progenitor cells in the silent area of bone marrow.[11]

Hypogammaglobulinemia is defined as reduced levels of one or more immunoglobulin isotypes below 2 standard deviations of the mean values for age. When physiological hypogammaglobulinemia persists beyond 6 months of age, the condition is referred to as transient hypogammaglobulinemia of infancy. Despite a defined upper limit for normalization of immunoglobulins of 3 years of age, several studies have reported that normalization may be delayed. Such cases have been classified as undefined/unclassified hypogammaglobulinemia.[12] The results of a recent study indicate that the functions of circulating EPCs improved after intravenous immunoglobulin therapy related to tumor necrosis factor alpha (TNF-alpha) and C-reactive protein (CRP).[13]

The aim of this study therefore was to investigate the etiopathogenesis of PD in terms of the number of circulating EPCs, the level of serum VEGF-A and the major inflammatory pathways that could be clinically related such as hypogammaglobulinemia, coagulation abnormalities and complement abnormalities.

Patients and methods

The Local Ethics Committee approved the study and informed consent was obtained from all subjects. The study included 28 patients (mean age: 8±3 years, median age: 8, range: 2 to 12 years) with PD and 25 healthy age-matched subjects (HS) (mean age: 7.6±3.2 years, median age: 8 years, range: 2 to 12 years) randomly chosen from local residents who underwent health examinations at the hospital between June 2011 and December 2012. Diagnosis of PD was performed by radiography. Patients with known or symptomatic cardiovascular disease, active or chronic infection or malignancy, history of drug consumption, demonstrable history of direct injury of the hip or possible combined causes were excluded. A pediatric immunologist evaluated all patients including physical examination, laboratory tests, clinical diagnosis and the questionnaire on illness and medical treatment history. Patients were classified using radiographs obtained at presentation according to the Waldenström staging in fragmentation and healing stages. The appearance of the femoral head to fragment or dissolve was defined as the fragmentation stage and remodeling of the femoral head as the healing stage. Demographic data are defined in the Table 1.

A total of 8 cc peripheral venous blood sample was taken from the PD group subjects: 0.5 cc for blood count, 2 cc for EPC count of flow cytometric evaluation, 4 cc for immunoglobulin, CRP, CH50 and VEGF-A levels and 1.5 cc for prothrombin time (PT) and activated partial thromboplastin time (aPTT) levels. The serum was separated from the blood sample in the biochemistry tube and placed in three Eppendorf Tubes® (Eppendorf AG, Hamburg, Germany). One tube was analyzed at the university's Central Laboratory to determine serum immunoglobulin and CRP levels. PT and aPTT samples were

Table 1. Demographical data of the children in the study.

	PD (n=28)	HS (n=25)
	Mean±SD	Mean±SD
Age (years)	8±3	7.6±3.2
Gender (M/F)	23/5	19/6
Weight (kg)	30±15	32.2±13.2
Height (cm)	123±23	128±22

HS: Healthy age-matched subjects; PD: Legg-Calvé-Perthes disease; SD: Standard deviation.

also assayed on the same day at the central laboratory of university. The remaining 2 tubes were stored at -80°C until the study day for serum VEGF-A and CH50 levels at the Pediatric Immunology-Allergy Department.

While the evaluation of CH50 levels of the PD group was planned, we were unable to run the commercial CH50 kit and discarded the complementary system branch of the study.

In the HS group, a total of 4.5 cc blood was taken: 0.5 cc for blood count, 2 cc for EPC count of flow cytometric evaluation and 2 cc for serum VEGF-A level. As the children in the control group were healthy, the reference values for their age were used for immunoglobulin, CRP, PT and aPTT levels for comparisons with those of the PD group.

Hypogammaglobulinemia was defined as reduced levels of one or more immunoglobulin isotypes (IgG, IgA and IgM) below 2 standard deviations of the mean values for age.[12]

A total of 5 cc peripheral venous blood was collected from all subjects into the EDTA tubes. Three cc was used for enumeration of circulating EPCs. Another 2 cc was used to identify the absolute count of lymphocytes tubes simultaneously. Mononuclear cells (MNC) were separated using a Ficoll-Paque density gradient centrifugation according to standard protocols. MNCs were washed twice and re-suspended in 0.2 ml of phosphate buffer solution (PBS) and incubated with a 10 µl panel of the conjugated antibodies CD45-peridinin-chlorophyll-protein complex (PerCP) (BD Pharmingen™; BD

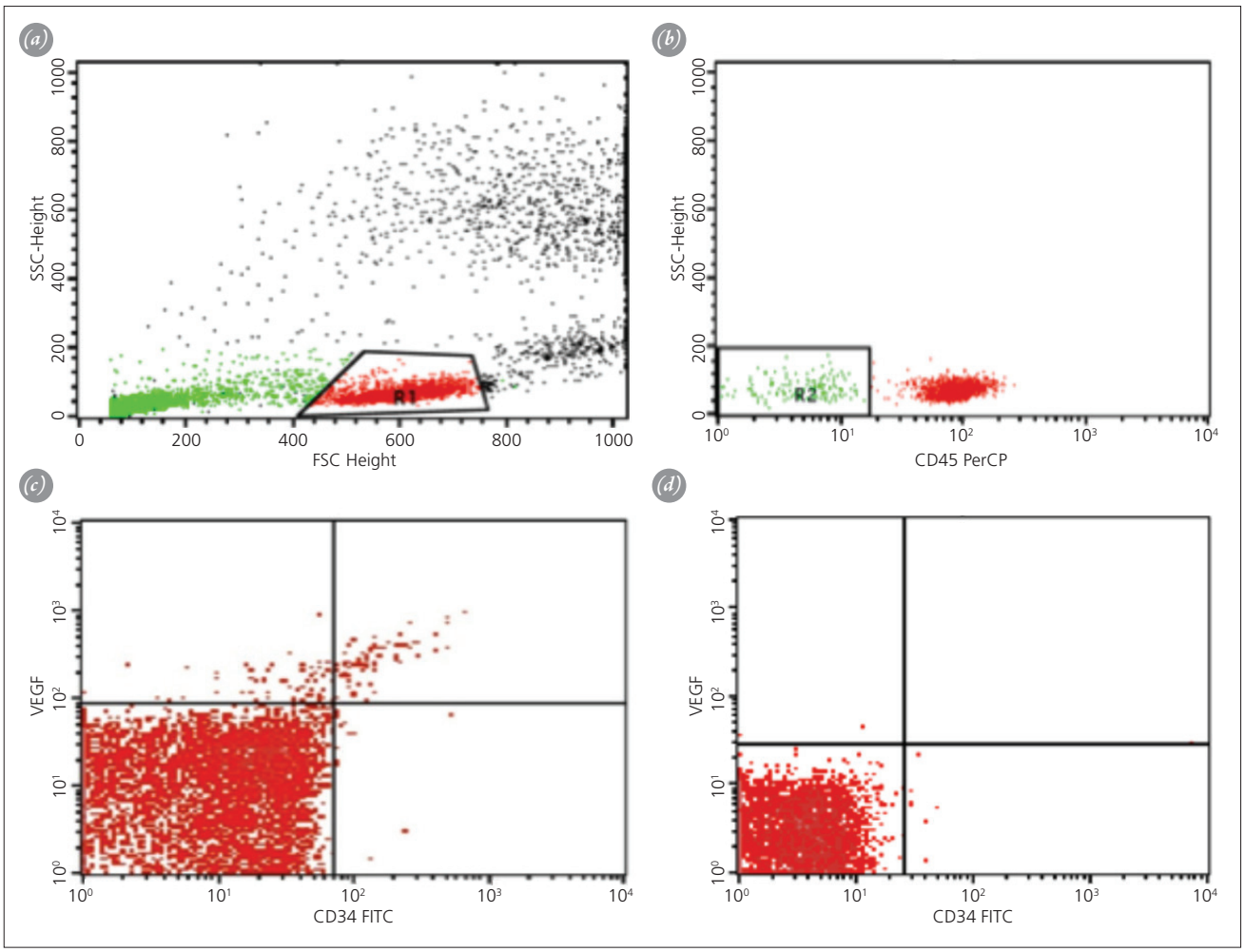

Fig. 1. MNCs were separated from peripheral blood by centrifugation on density gradient media. The cytometer was set to acquire 100,000 events, and analyses were performed within the lymphocyte gate. Within the $CD45^{low}$ gates, dual expression of CD34+ and VEGFR2+ cell was EPCs. On the basis of the lymphocyte counts, the absolute numbers of circulating EPCs per ml were calculated. **(a)** R1 was the lymphocyte gate. **(b)** Within the lymphocyte gate, R2 was $CD45^{low}$ gates. **(c)** The absolute number of CD34+/VEGFR2+ (EPC) cells was proportionated with the absolute number of lymphocytes. **(d)** The number of CD34+/VEGFR2+ cells from HS. EPCs: Endothelial progenitor cells; HS: Healthy age-matched subjects; MNCs: Mononuclear cells; VEGF: Vascular endothelial growth factor. [Color figure can be viewed in the online issue, which is available at www.aott.org.tr]

Biosciences, San Jose, CA, USA), CD34-fluorescein isothiocyanate (FITC) (BD Biosciences, San Jose, CA, USA) and VEGFR2-phycoerythrin (PE) (R&D Systems Inc., Minneapolis, MN, USA) for 20 min at room temperature away from light. Samples were then lysed by incubating in a FACS lysing solution for 15 minutes. Cells stained with isotypic controls for IgG1-FITC or PE was performed as negative controls. After appropriate gating with lymphocytes, $CD45^{low}$/CD34+/VEGFR2+ cells (EPC) were identified by the dual expression of CD34+ and VEGFR2+ in the $CD45^{low}$ gates (Fig. 1).[10] The cytometer was set to acquire 100,000 events. Data were processed using CellQuest™ (BD Biosciences, San Jose, CA, USA) software. These analyses were executed on all subjects and were performed by the same well-trained operator, who was unaware of the patients' clinical status throughout the study. On the basis of the absolute lymphocytes count, the absolute number of circulating EPCs was proportioned as the number of cells per 1 ml of whole blood.

Serum VEGF-A was quantified by commercially available enzyme-linked immunosorbent assay kits (R&D Systems Inc., Minneapolis, MN, USA) according to the manufacturer's instructions. Each measurement was made 5 times. The VEGF-A level was determined from a standard curve generated for each set of samples assayed.

Results were expressed as mean±standard deviations or as median values. As the data of neither group showed normal distribution, the Mann-Whitney U test was used to evaluate all data. Correlation analysis was used to determine the presence of a relationship between parameters. P values of less than 0.05 were accepted as significant.

Results

In the PD group, 17 patients were determined to be in the fragmentation stage and 11 in the recovery stage.

Although total lymphocyte count and serum VEGF-A levels of both groups were similar, absolute EPC count was significantly higher in the PD group (Fig. 2A, Table 2). In the PD group, hemoglobin levels were also high (p=0.028) and related with serum VEGF-A levels (p=0.013, r=0.463).Serum IgG levels were related with INR levels (p=0.012, r=0.466) but inversely related with absolute EPC count (p=0.033, r=-0.403) in the PD group.

When the PD patients with bilateral involvement (n=4) were compared with those with unilateral involvement (n=24), absolute EPC count and serum VEGF-A levels were significantly higher in the bilateral group (Table 3). Serum IgA levels were also significantly higher in bilaterally involved PD patients. Similarly, in the fragmentation stage of PD, absolute EPC count was higher than those of healing stage of disease while serum VEGF-A levels were not (Table 4).

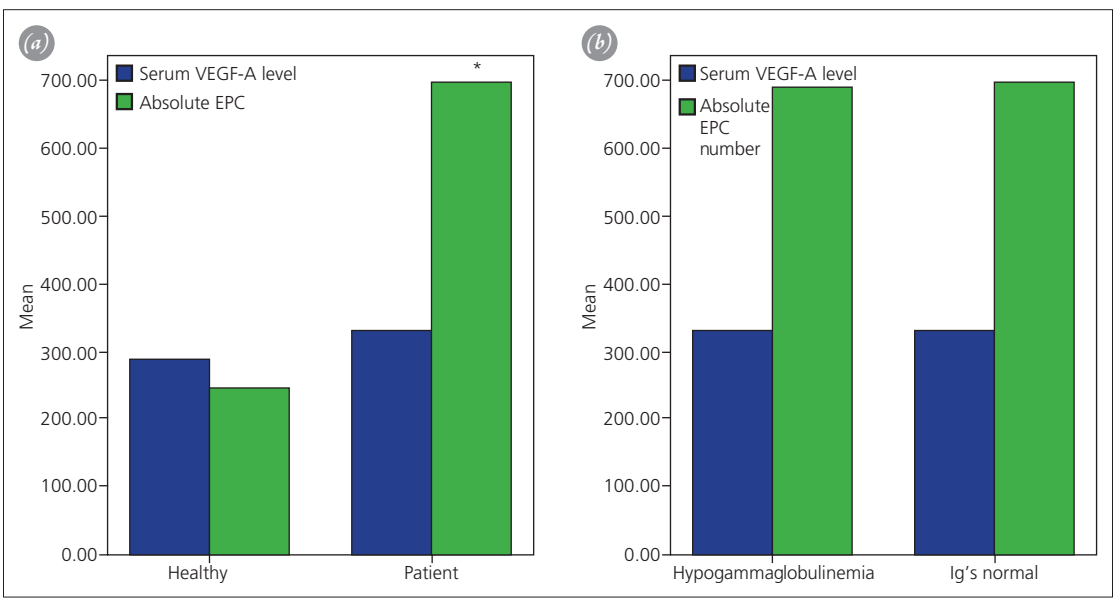

Fig. 2. Number of circulating EPCs. **(a)** CD45low/CD34+/VEGFR+ cells were detected in peripheral blood from 28 PD patients and 25 age-matched healthy subjects. EPC count was higher in the PD group. **(b)** Number of circulating EPCs in presence of hypogammaglobulinemia. Data are presented as median values, compared with each other. EPCs: Endothelial progenitor cells; PD: Legg-Calvé-Perthes disease; VEGF: Vascular endothelial growth factor. *p<0.05. [Color figure can be viewed in the online issue, which is available at www.aott.org.tr]

Table 2. Total lymphocyte count, absolute endothelial progenitor cells count and serum vascular endothelial growth factor levels of both group were as follows.

	Legg-Calvé-Perthes disease (n=28)	Healthy age-matched subjects (n=25)	p
Total lymphocyte count (/mm^3)			
Mean±SD	2989±1305	2901±664	0.232
Median	2625	2870	
Min-Max	1700-6830	1390-5000	
Absolute endothelial progenitor cells (/mm^3)			
Median	80	0.011	35
Min-Max	1-1289	0-120	
Vascular endothelial growth factor (pg/ml)			
Mean±SD	323±170	278±125	0.354
Median	304	272	
Min-Max	83-854	67-553	

Table 3. Comparison of total lymphocyte count, absolute endothelial progenitor cells count and serum vascular endothelial growth factor levels of bilateral vs unilateral Legg-Calvé-Perthes disease.

	Bilateral Legg-Calvé-Perthes disease (n=4)	Unilateral Legg-Calvé-Perthes disease (n=24)	p
Total lymphocyte count (/mm^3)			
Mean±SD	2710±808	3035±1378	0.924
Median	2416	2660	
Min-Max	2110±3900	1700±6830	
Absolute endothelial progenitor cells (/mm^3)			
Median	944	49	0.005
Min-Max	295-1242	0-1289	
Vascular endothelial growth factor (pg/ml)			
Mean±SD	447±122	302±170	0.07
Median	450	258	
Min-Max	325-564	83-854	

Table 4. Comparison of total lymphocyte count, absolute endothelial progenitor cells count and serum vascular endothelial growth factor levels of fragmentation vs healing stages of PD.

	Fragmentation stage (n=17)	Healing stage (n=11)	p
Total lymphocyte count (/mm^3)			
Mean±SD	3327±1545	2465±533	0.134
Median	2910	2500	
Min-Max	2000±6810	1700±3680	
Absolute endothelial progenitor cells (/mm^3)			
Median	141	36	0.033
Min-Max	10-1289	1-815	
Vascular endothelial growth factor (pg/ml)			
Mean±SD	357±196	271±109	0.264
Median	375	225	
Min-Max	83-854	96-454	

Hypogammaglobulinemia was determined in 14 of the PD patients. EPC count and serum VEGF-A levels in PD patients with hypogammaglobulinemia were not different from those of the PD patients with no hypogammaglobulinemia (Fig. 2B, Table 5). CRP levels were higher in hypogammaglobulinemic PD patients

Table 5. Comparison of total lymphocyte count, absolute endothelial progenitor cells count and serum vascular endothelial growth factor levels of hypogammaglobulinemic PD versus normal immunoglobulin PD patients.

	Hypo Ig Legg-Calvé-Perthes disease (n=14)	Normal Ig Legg-Calvé-Perthes disease (n=14)	p
Total lymphocyte count (/mm^3)			
Mean±SD	3266±1616	2711±871	0.329
Median	2730	2545	
Min-Max	1900-6830	1700±4740	
Absolute endothelial progenitor cells (/mm^3)			
Median	80	89	0.839
Min-Max	1-1242	10-1289	
vascular endothelial growth factor (pg/ml)			
Mean±SD	326±188	320±157	0.701
Median	275	364	
Min-Max	119-854	83-541	

(p=0.024). In hypogammaglobulinemic PD patients, serum IgG levels were related with INR levels (p=0.015, r= 0.455) and inversely related with absolute EPC count (p=0.042, r=-0.387).

Circulating EPC count was correlated to the serum VEGF-A levels in patients at the fragmentation stage of PD (r=0.605, p=0.01) and in those with hypogammaglobulinemia (r=0.599, p=0.001).

Discussion

Endothelial progenitor cells may contribute to vascular homeostasis such as vascular repair and new blood vessel growth.[10] This study aimed to clarify the etiopathogenesis of PD with the number of circulating EPCs, the level of serum VEGF and the major inflammatory pathways that could be clinically related such as hypogammaglobulinemia, coagulation abnormalities and complement abnormalities. Unfortunately, we were unable to evaluate the complement system. Our findings suggest markedly higher levels of absolute EPCs in the PD group although serum VEGF level was not informative with these data. In hypogammaglobulinemic PD patients, serum IgG levels were inversely related with absolute EPCs. Additionally, EPC count was correlated to the serum VEGF-A levels in patients at the fragmentation stage of PD and in those with hypogammaglobulinemia. This study was the first to evaluate EPC and serum VEGF-A immunoglobulins in children with PD.

Recently, Feng et al.[10] compared the EPC numbers and functions of 54 patients (mean age: 45 years) with steroid-induced, alcohol-induced or idiopathic non-traumatic avascular necrosis of the femoral head with those of healthy controls. The authors found that EPC numbers and functions (namely migratory capacity) were reduced in the non-traumatic avascular necrosis patients, suggesting that risk factors of this disease may alter EPCs biology in angiogenesis and vascular repair. These findings and EPC numbers differ greatly from our study's findings. This discrepancy may be explained by the different etiology, especially in steroid-induced patients and an older study population.

Physiological hypogammaglobulinemia persisting beyond 6 months of age is referred to as transient hypogammaglobulinemia of infancy. Although the upper limit for normalization of immunoglobulins has been set at 3 years, cases in which this time is delayed are classified as undefined/unclassified hypogammaglobulinemia. In addition to infections, these primary immunodeficiencies have a wide spectrum of clinical manifestations including autoimmune diseases, unregulated inflammation and predisposition to malignancies.[12,14] We attempted to evaluate the relationship between PD and childhood hypogammaglobulinemia due to their overlapping periods. Serum IgG levels were found to be inversely related with absolute EPC count. In addition, EPC count was correlated to the serum VEGF-A levels in patients at the fragmentation stage of PD and in those with hypogammaglobulinemia.

Xu et al.[13] sought to determine the effects of treatment with intravenous immunoglobulin (IVIG) and aspirin on the functions of EPC in patients with Kawasaki disease. The authors found that the functions of circulating EPCs improved after treatment with IVIG and aspirin, which may be related to decreased concentrations of TNF-alpha and CRP. These findings support our findings in hypogammaglobulinemic patients.

In conclusion, while radiologic examinations are the main diagnostic tool in PD, the high level of EPC in

the fragmentation stage of PD and the relatively higher counts in bilateral disease than those of unilateral disease suggest that EPC may be a valuable marker for the follow-up of PD. Additional studies are needed to explain the strong correlation between EPC and serum VEGF-A level in the fragmentation stage and in the presence of hypogammaglobulinemia.

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Conflicts of Interest: No conflicts declared.

References

1. Molloy MK, MacMahon B. Incidence of Legg-Perthes disease (osteochondritis deformans). N Engl J Med 1966;275:988-90. CrossRef
2. Kim HK, Su PH, Qiu YS. Histopathologic changes in growth-plate cartilage following ischemic necrosis of the capital femoral epiphysis. An experimental investigation in immature pigs. J Bone Joint Surg Am 2001;83-A:688-97.
3. Kim HK, Bian H, Randall T, Garces A, Gerstenfeld LC, Einhorn TA. Increased VEGF expression in the epiphyseal cartilage after ischemic necrosis of the capital femoral epiphysis. J Bone Miner Res 2004;19:2041-8. CrossRef
4. Almeida Matos M. The role of protein C deficiency in the etiology of Perthes disease. Ortop Traumatol Rehabil 2008;10:274-8.
5. Daniel AB, Shah H, Kamath A, Guddettu V, Joseph B. Environmental tobacco and wood smoke increase the risk of Legg-Calvé-Perthes disease. Clin Orthop Relat Res 2012;470:2369-75. CrossRef
6. Hailer YD, Montgomery S, Ekbom A, Nilsson O, Bahmanyar S. Legg-Calvé-Perthes disease and the risk of injuries requiring hospitalization: a register study involving 2579 patients. Acta Orthop 2012;83:572-6. CrossRef
7. Loder RT, Schwartz EM, Hensinger RN. Behavioral characteristics of children with Legg-Calvé-Perthes disease. J Pediatr Orthop 1993;13:598-601. CrossRef
8. Perry DC, Green DJ, Bruce CE, Pope D, Dangerfield P, Platt MJ, et al. Abnormalities of vascular structure and function in children with Perthes disease. Pediatrics 2012;130:126-31. CrossRef
9. Yilmaz D, Karapinar L, Karapinar B, Ozturk H, Kavakli K. Evaluation of anticoagulant system in Turkish children with Perthes disease. Pediatr Int 2005;47:43-8. CrossRef
10. Feng Y, Yang SH, Xiao BJ, Xu WH, Ye SN, Xia T, et al. Decreased in the number and function of circulation endothelial progenitor cells in patients with avascular necrosis of the femoral head. Bone 2010;46:32-40. CrossRef
11. Tepper OM, Sealove BA, Murayama T, Asahara T. Newly emerging concepts in blood vessel growth: recent discovery of endothelial progenitor cells and their function in tissue regeneration. J Investig Med 2003;51:353-9. CrossRef
12. Keles S, Artac H, Kara R, Gokturk B, Ozen A, Reisli I. Transient hypogammaglobulinemia and unclassified hypogammaglobulinemia: 'similarities and differences'. Pediatr Allergy Immunol 2010;21:843-51. CrossRef
13. Xu MG, Men LN, Zu Y, Zhao CY, Meng XC, Wang T. The functions of endothelial progenitor cells were significantly improved after treatment with intravenous immunoglobulin and aspirin in children with Kawasaki disease. Pediatr Cardiol 2011;32:455-60. CrossRef
14. Al-Muhsen SZ. Gastrointestinal and hepatic manifestations of primary immune deficiency diseases. Saudi J Gastroenterol 2010;16:66-74. CrossRef