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Ca^{2+}-Mg^{2+}-dependent ATP-ase activity in hemodialyzed children. Effect of a hemodialysis session

Dorota Polak-Jonkisz · Leszek Purzyc · Danuta Zwolińska

Abstract In the course of chronic kidney disease (CKD) the intracellular erythrocyte calcium (Ca$_i^{2+}$) level increases along with the progression of the disease. The decreased activity of Ca^{2+}-Mg^{2+}-dependent ATP-ase (PMCA) and its endogenous modulators calmodulin (CALM), calpain (CANP), and calpastatin (CAST) are all responsible for disturbed calcium metabolism. The aim of the study was to analyze the activity of PMCA, CALM, and the CANP-CAST system in the red blood cells (RBCs) of hemodialyzed (HD) children and to estimate the impact of a single HD session on the aforementioned disturbances. Eighteen patients on maintenance HD and 30 healthy subjects were included in the study. CALM, Ca$_i^{2+}$ levels and basal PMCA (bPMCA), PMCA, CANP, and CAST activities were determined in RBCs before HD, after HD, and before the next HD session. Prior to the HD session, the level of Ca$_i^{2+}$ and the CAST activity were significantly higher, whereas bPMCA, PMCA, and CANP activities and the CALM level were significantly lower than in controls. After the HD session, the Ca$_i^{2+}$ concentration and the CAST activity significantly decreased compared with the basal values, whereas the other parameters significantly increased, although they did not reach the levels of healthy children. The values observed prior to both HD sessions were similar. Ca$_i^{2+}$ homeostasis is severely disturbed in HD

children, which may be caused by the reduction in the PMCA activity, CALM deficiency, and CANP-CAST system disturbances. A single HD session improved these disturbances but the effect is transient.

Keywords Children · Hemodialysis · Cytosolic calcium · Ca^{2+}-Mg^{2+}-dependent ATP-ase

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Introduction

Chronic kidney disease (CKD) involves intracellular calcium (Ca$_i^{2+}$) accumulation in numerous tissues, which leads to intensification of various clinical symptoms. With respect to red blood cells (RBCs), these features are related to increased deformation and morphological changes in erythrocytes, which in turn shortens their life span, and hence increases anemia.

Normal calcium level in erythrocytes is assured by the influx of those ions through the cell membrane and active efflux outside the cell by an energy-dependent calcium pump: Ca^{2+}-Mg^{2+}-dependent–ATP-ase (PMCA), plasma membrane Ca^{2+}-Mg^{2+}-transporting adenosine triphospha-tase: ATP-ase 3.6.1.38. Human PMCA is encoded by at least four genes (phenotypes denoted as PMCA 1–4) [1, 2]. PMCA 4b is the main type in mature RBC. The activity of the pump is modified by a network of protein factors—calmodulin (CALM) and the calpain-calpastatin system (CANP-CAST) in particular [3, 4].

Our earlier studies have revealed that children and adolescents with CKD stages 2–4 are characterized by a reduced PMCA activity in erythrocytes accompanied by a CALM deficit and disturbances in the CANP-CAST system [5, 6]. The changes result in a high intracellular calcium level. There are no studies concerning children on renal

replacement therapy. In the population of adults with end-stage kidney disease (ESKD), reduced PMCA activity and calcium accumulation in erythrocytes were observed, both in patients on hemodialysis (HD) and peritoneal dialysis as well [7–10]. However, the data concerning the impact of the HD session on these disturbances are rare and the results of the few studies are not uniform and do not involve endogenous modulators of PMCA activity [7–9].

The aim of our study was to analyze the activity of PMCA, CALM, and the CANP-CAST system in erythrocytes of hemodialyzed children and to estimate the impact of a single HD session on the above-mentioned disturbances.

Subjects and methods

Patients

Eighteen hemodialyzed children from the Dialysis Unit of the Paediatric Nephrology Department, Medical University in Wrocław, were enrolled in the study. The control group consisted of 30 healthy, age-matched subjects, hospitalized because of nocturnal enuresis, with normal kidney function. Table 1 shows the biochemical characteristics of the groups examined. The reasons for ESRD were as follows: chronic glomerulonephritis (10 patients), chronic pyelonephritis (6 patients), and abnormalities of the urinary tract (2 patients).

The duration of HD therapy ranged from 5 months to 48 months (mean: 15 ± 4 months). Dialysis was carried out for 4 h three times weekly, using polysulphone membranes and dialysate with bicarbonate, calcium 1.5 mmol/L, potassium 2 or 3 mmol/L, blood flow 150–300 ml/min.

Eleven patients were treated effectively with antihypertensive drugs (calcium channel blockers—nifedipine [Polfa-Grodzisk, Grodzisk, Poland], angiotensin-converting enzyme inhibitors, beta-blockers). Other drugs used were: erythropoietin, vitamin D_3 analogs, vitamins C, B complex, folic acid, iron, and ranitidine hydrochloride.

All CKD patients were kept on a phosphate-poor diet (phosphate intake did not exceed 500–800 mg/day) and treated with compounds binding phosphate in the intestine (calcium carbonate at a dosage of 35–200 mg/kg/day). The doses of these therapeutic agents were individualized, so as to maintain the serum concentrations of phosphate at 4.5–5.52 mg/dl and calcium at 8.8–10.8 mg/dl. Dietary calcium intake was defined as 500–600 mg of elementary calcium per day.

Laboratory investigations

The following parameters were determined in the serum: inorganic phosphate (P_i), total calcium (t-Ca), creatinine (cr), urea, intact parathormone (iPTH); whereas Ca_i^{2+}, CALM concentrations, and activities of PMCA, basal PMCA (bPMCA), CANP, and CAST were assessed in red blood cells.

Serum P_i, and t-Ca were tested by the direct potentiometry method using ion-selective ISE electrodes in the automatic analyzer Konelab (bioMérieux, Craponne, France) with the reference ranges: 8.80–10.80 mg/dl and 4.5–5.52 mg/dl respectively. Serum creatinine was determined by the Jaffe method (reference range: 0.3–1.0 mg/dl) and urea was measured using the enzymatic-colorimetric method (reference range: 10.00–45.00 mg/dl).

Intact parathormone (iPTH) was estimated by the immunoenzymatic ELISA microassay of the solid phase from Roche Diagnostic (Warsaw, Poland) on Immulite 2000 apparatus (reference range: 11–67 pg/ml).

Alcohol dehydrogenase (yeast), casein, CANP I from human erythrocytes, DTT (dithiothreitol), PBS and Ultra Gel AcA34 were purchased from Calbiochem (Warsaw, Poland) and the remaining reagents and materials from Sigma-Aldrich (Poznan, Poland).

In the control group, morning blood samples were obtained from fasted children from the antecubital vein in the sitting position, whereas in hemodialyzed patients blood samples were collected from the arterial line immediately prior to HD and immediately after the HD session. The third blood sample was obtained before the following HD session (pre-hemodialysis), which was performed 48 h after the first session. Serum was separated within 1–3 h of blood collection and the samples were stored at −70°C.

Table 1 Selected clinical and biochemical parameters in investigated patients

	Hemodialyzed	Control	p
Gender (male and female)	10/8	17/13	NS
Age (years)	16.1 ± 2.0	14.7 ± 2.7	<0.001
iPTH (pg/ml)	233 ± 59	46.69 ± 9.22	<0.001
P_i (mEq/l)	5.97 ± 0.65	4.95 ± 0.33	<0.001
t-Ca (mg/dl)	9.05 ± 0.78	9.70 ± 0.63	<0.01
cr (mg/dl)	7.29 ± 0.92	0.81 ± 0.15	<0.001
GFR (ml/min/1.73 m)	<10 ml	129 ± 8.7	
Kt/V	1.25 ± 0.04		

NS non-significant

All parents of younger children and adolescents enrolled in the study gave their informed consent for the procedures performed. The investigation protocol was approved by the local ethics committee.

Preparation of red blood cells (RBCs)

The preparation of RBCs was performed according to Beutler et al. [11]. The methodology was described in detail in our previous study [5].

Measurement of PMCA and bPMCA activity in the suspension of erythrocyte membranes

Hemoglobin was removed from erythrocyte hemolysates according to the Lehotsky et al. method [12]. The methodology concerning the PMCA and bPMCA activity assessment was described in detail in our previous study [5]. bPMCA activity measurement is based on its ability to hydrolyze ATP to ADP and P_i. P_i was determined using the colorimetric method of Fiske and Subbarow [13].

The intra-assay (within 1 day) and inter-assay (between different days) coefficients of variation (%CV) for PMCA at the quantification limit were <8.5% and <6.7% respectively.

Determination of CALM

Calmodulin concentration in erythrocyte hemolysate (in core extract) was assessed with the modified spectrophotometric method established by Garg et al. [14]. CALM concentration was measured according to the methodology described in detail in our previous study [5].

Determination of Ca_i^{2+} in human erythrocytes

The measurement of Ca_i^{2+} was conducted in intact erythrocytes according to Soldati et al. [15] with minor modifications (after incubation with Fura-2, erythrocytes were diluted with HBS solution without Ca^{2+} content). Fluorescence measurements were conducted on Perkin Elmer LS50 spectrometer, and calculations were performed according to Grynkiewicz et al. [16] and Poenie et al. [17]. The determination of free cytoplasmic calcium in human erythrocytes was described in detail in our study [5].

The intra-assay and inter-assay %CV for Ca_i^{2+} at the quantification limit are 4.5 and 2.0% respectively.

Determination of CANP and CAST activities

Hemoglobin was removed from erythrocyte hemolysates according to the method of Lehotsky et al. [12]. The activities of CANP and CAST were measured by the method described by Kosower et al. [18].

Determination of protein concentration

Protein concentration was measured using the method of Bradford et al. [19] with bovine albumin as a standard.

Statistical analysis

Results are expressed as mean values and standard deviation: x±SD. A repeated variance analysis (repeated ANOVA) and a post-hoc test (LSD test) were applied in order to compare means between intervals. Comparisons between the study group and controls were performed using Student's t test. The associations between variables were analyzed by Pearson correlation coefficients. A p value of less than 0.05 was considered statistically significant.

Results

Ca_i^{2+} (nmol/l)

In HD patients the intracellular Ca_i^{2+} concentrations were significantly higher than in controls, regardless of the timing of blood sampling. A significant decrease in Ca_i^{2+} concentration was observed after the HD session compared with the basal values (152.33±11.80 nmol/l vs 58.94± 4.45 nmol/l; $p<0.001$), whereas the values observed prior to both HD sessions were similar.

Serum t-Ca levels before and after HD were significantly different ($p<0.05$). There was no significant difference between the levels of t-Ca in patients after HD and children from the control group.

No correlation was found among Ca_i^{2+}, t-Ca , P_i, and iPTH.

The mean Ca_i^{2+} concentrations in RBCs of the investigated groups are shown in Fig. 1.
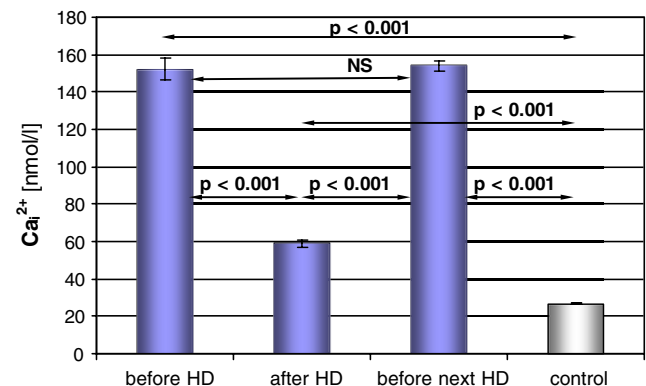
Fig. 1 The mean values of intracellular calcium (Ca_i^{2+}) concentrations in the red blood cells of the groups investigated

PMCA (μmol/P$_i$/mg/min)

Despite a significant increase in the PMCA activity after the first HD session (1.22±0.28 μmol/P$_i$/mg/min vs 2.55± 0.31 umol/P$_i$/mg/min; $p<0.001$), its concentration before the second one decreased to the values similar to those observed before the first HD session. However, the PMCA activity remained at all times significantly lower in the study population than in the controls.

The mean values of PMCA activity in RBCs of the groups investigated are shown in Fig. 2.

bPMCA (umol/P$_I$/mg/min)

The HD session had no effect on bPMCA, as opposed to PMCA. The activity of bPMCA measured at different time points in hemodialyzed children showed no statistical differences and remained significantly lower than in healthy children ($p<0.001$).

The mean values of bPMCA activities in RBCs of the groups investigated are shown in Fig. 3.
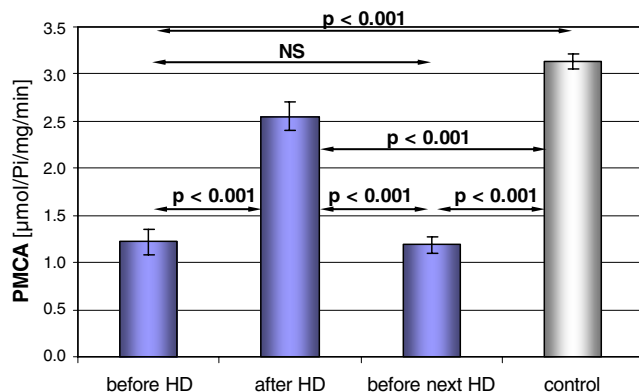
CAST (U/mg)

Calpastatin (CAST) activity was significantly higher in HD subjects compared with controls, regardless of the timing of sampling. After the HD session, CAST activity decreased significantly in comparison to the basal values (28.58± 2.11 U/mg vs 64.90±3.76 U/mg; $p<0.001$). However, the activity increased again before the following session and was significantly higher than prior to the previous one (69.93±3.04 U/mg vs 64.90±3.76 U/mg; $p<0.001$).
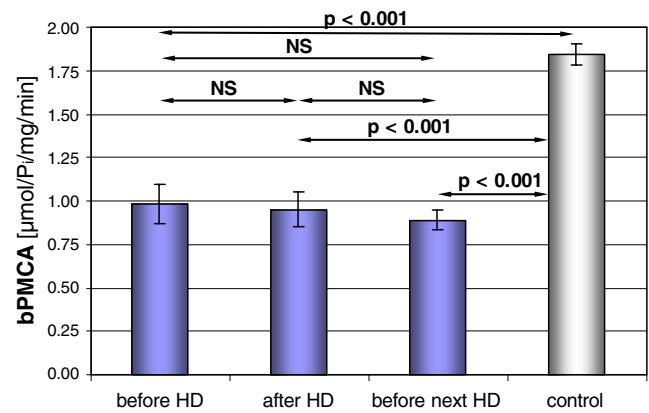
The mean CAST activities in the RBCs of the groups investigated are shown in Fig. 4.

CANP (U/mg)

Prior to the HD session, CANP activity was significantly lower than in the control group (28.37±3.91 U/mg vs

Fig. 3 The mean values of basal PMCA (*bPMCA*) activity in the red blood cells of the groups investigated

44.13±4.23 U/mg; $p<0.001$). However, the activity increased after the HD session and was not only significantly higher than initially, but also in comparison to the controls. Prior to the next HD session CANP activity was comparable to the value observed before the previous one.

The mean CANP activities in the RBCs of the groups investigated are shown in Fig. 5.

CALM (mg/l)

Calmodulin concentration was significantly lower in the study group prior to HD and after the HD session compared with the controls. However, a transient significant increase in the concentration of CALM was observed after the HD session, compared with the values from before HD. There was no significant difference in CALM levels before each HD session.

The mean CALM activities in the RBCs of the groups investigated are shown in Fig. 6.

Fig. 2 The mean values of Ca^{2+}-Mg^{2+}-dependent ATP-ase (*PMCA*) activity in the red blood cells of the groups investigated

Fig. 4 The mean values of calpastatin (*CAST*) activity in the red blood cells of the groups investigated

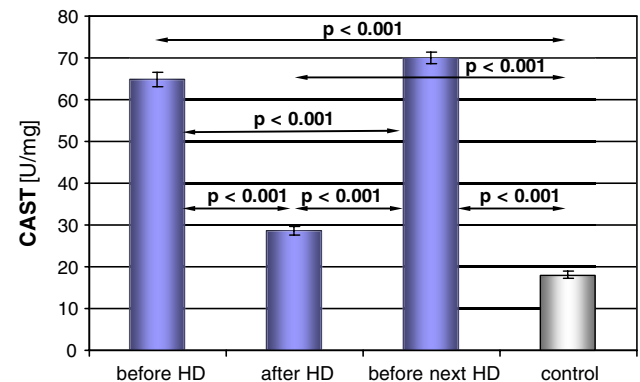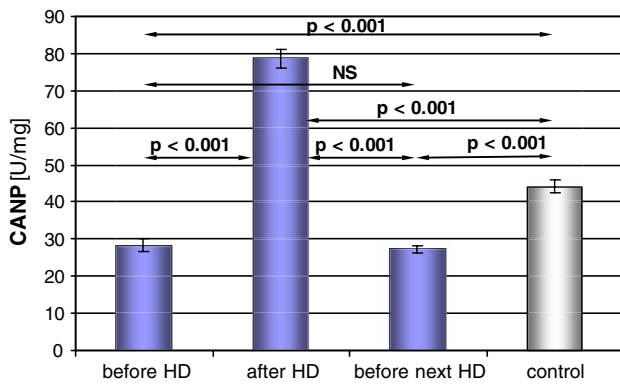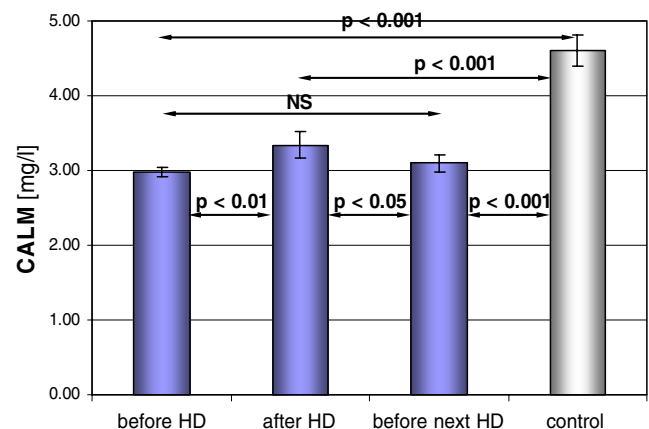**Fig. 5** The mean values of calpain (CANP) activity in the red blood cells of the groups investigated

Discussion

Not surprisingly, the concentration of intracellular calcium in the red blood cells of children and adolescents on long-term hemodialysis was significantly higher in comparison to healthy children. Similar disorders were observed in adults with ESRD on maintenance HD and on continuous ambulatory peritoneal dialysis [7, 8, 20]. Calcium accumulation in erythrocytes has negative consequences, as it leads to the membrane deformation of RBCs and increased osmotic fragility. These disorders, in turn, result in morphological changes of erythrocytes, mainly echinocytes, and reduction of their lifetime, which intensifies anemia, which accompanies uraemia [10, 20, 21]. Increased calcium level in erythrocytes is undoubtedly caused by disturbed balance between Ca^{2+} influx into the cell and its efflux from the cell. Increased erythrocyte membrane permeability for calcium in patients with ESRD was proven many years ago [10, 20, 21]. It was found that the Ca_i^{2+} level was twice as high as in healthy subjects, which led to the conclusion that pore-forming toxins could be responsible for this phenomenon. Parathormone could be one of the toxins that intensify calcium influx into the cell [7, 22]. However, no direct correlation between these parameters was found in hemodialyzed adults [7, 10]. On the other hand, such a correlation was observed by Karapati et al. with regard to polymorphonuclear leukocytes (PMNs) [22]. The authors have shown that after iPTH decrease following parathyroidectomy, the calcium level in PMNs returns to normal values [22]. All children in our study had features of hyperparathyroidism, which might have been one of the reasons for increased calcium influx into erythrocytes. Increased Ca_i^{2+} level in erythrocytes may also result from reduced PMCA activity, which was documented for both children and adults with CKD treated conservatively, as well as in adults on renal replacement therapy [5, 6, 10]. Results of the present study are compatible with the above observations. Activity of basal and CALM-stimulated

PMCA was significantly lower than in the control group. The pump activity may be indirectly influenced by external factors, but its endogenous protein modulators play the main role. In our previous study, we observed that CALM deficit appears as early as CKD stages 2–4 and it increases along with CKD progression [5]. The aim of the present study was to analyze the impact of a single HD session on CALM and the CANP-CAST system and, if there is one, how long the effect lasts.

We have shown that the intracellular calcium concentration after dialysis is lower than before dialysis, although it remains significantly higher than in the control group. These results are comparable with those obtained by other authors [7, 8, 23, 24]. The above-mentioned phenomenon may be caused by increased PMCA activity, which in turn is stimulated by the CANP-CAST system. Contrary to our findings and those of Yang, Gafter et al. observed that the calcium level in erythrocytes was similar before and after HD sessions [8, 24]. These differences may result from the different dialysis techniques used. Subjects studied by these authors were dialyzed on less biocompatible dialysate (octane) and it may be presumed, given the year of the study (1989), that the membranes were less biocompatible too (no data in the publication) [8].

In the present study, we have shown that the effect of a reduced Ca_i^{2+} level after single HD session is transient, as both the intracellular calcium level and PMCA activities before the second HD session were similar to the values seen before the previous one. Aside from biological modulators, PMCA activity may also be influenced by external factors, such as pore-forming toxins. So far, the following plasma toxins in erythrocyte hemolysates have been shown to act as PMCA inhibitors: dimethylguanosine, phenylethylamine, phenyl acid, and para-hydroxy-hippuric acid [25–28]. The low lipophilicity of these factors limits their activity within the cell. Therefore, their influence on the activity of the intracellular system that we studied is questionable.

Fig. 6 The mean values of calmodulin (CALM) levels in the red blood cells of the groups investigated

It is known that a physiologically high intracellular CALM level results in permanent PMCA stimulation and keeps the enzyme in the "open state" conformation [29, 30]. This allows maintaining PMCA activity, regardless of the pulsatile changes in the intracellular calcium concentration. The present study has revealed a significantly reduced CALM concentration before the HD session. CALM level as well as PMCA activity both significantly increased after the HD session, but did not reach normal values. This effect, similar to calcium pump activity, was transient, which indicates that CALM deficit plays an important role in PMCA deactivation. However, we have not observed any correlation between these parameters, contrary to the results obtained from CKD children treated conservatively [5].

Modulation of PMCA activity in erythrocytes, with CALM deficit and excessive calcium accumulation, is supported by other endogenous modifiers, such as Ca-dependent kinases, caspases, and the CANP-CAST system. According to Pontremoli et al., increased CANP activity is the last line of defense against excessive cytotoxic concentration of Ca_i^{2+} accompanied by CALM deficit [31]. Activity of the CANP-CAST system is sensitive to changes in intracellular calcium level and dependent on reversible phosphorylation by specific kinases and phosphatases. Multi-stage sequential CANP activation begins with the appearance of a high local concentration of Ca_i^{2+}. Then, CAST dissociation takes place. CANP undergoes autolysis and its shortened molecules with protease activity begin to attack proteins both in the cytoplasm and the cellular membrane, including PMCA. In consequence, the molecules of native and shortened CANP affect PMCA and increase the number of shortened and still active calcium pump molecules.

In our studies, CANP activity was significantly lower before hemodialysis and was almost 2.5 times higher after the HD session. However, it did not reach normal values. This effect was also transient. CANP activity is related to its endogenous inhibitor—CAST. In comparison to controls, CAST activity in dialyzed patients was significantly higher before HD and it was normalized partially after the session, indicating a reduced number of active molecules, which may have resulted from digestion by active caspases in erythrocytes.

Typical features of chronic kidney disease, such as oxidative and carbonyl stress, accompanied by the appearance of uremic toxins in plasma, leads to structural changes in the membrane of erythrocytes and other cells. Persisting high calcium concentration in erythrocytes, both before and after the HD session, leads to scramblase stimulation and in consequence to the appearance of phosphatidylserine outside the erythrocyte membrane.

We are aware of the study limitations that arise from the potential impact of the therapy. According to some authors, antihypertensive drugs (calcium channel blockers, angiotensin-converting enzyme inhibitors) have a tendency to reduce calcium levels with a concomitant drop in blood pressure [32, 33].

We have used nifedipine, a blocker of the membrane voltage-dependent cation channel present mainly in excitable cells. They are also encountered in erythrocytes, among other channels that transport $CAST^{2+}$ into the cells, such as $CAST^{2+}$ channels, unselective cation channels TRPC6 or vanade induced $CAST^{2+}$ entry pathway. In 11 of our patients the anti-hypertensives were used with good therapeutic effect, but the concentration of intracellular calcium was still increased. Vitamin D_3 analogs had virtually the same effect. There are no studies investigating their impact on the erythrocyte Ca_i^{2+} level, but it has been documented that $25(OH)D_3$ supplementation reduced the Ca_i^{2+} level in leukocytes [34], and most probably the same effect may be expected with respect to erythrocytes.

Irrespective of the drugs used, the intracellular calcium was increased in all the patients examined. Therefore, even though the drugs might have influenced the erythrocyte Ca_i^{2+} homeostasis, the mechanisms leading to its normalization were still disturbed.

Conclusion

Children and adolescents subjected to maintenance hemodialysis demonstrate a greatly disturbed intra-erythrocyte calcium homeostasis. Significant Ca_i^{2+} accumulation is caused by, among others, decreased PMCA activity, probably due to a CALM deficit, CANP-CAST system disorders, as well as a permanent activation of calcium-dependent erythrocyte proteolytic enzymes. A single HD session improves these disturbances, but the effect is transient. The results of our study suggest that daily dialysis might be the optimal solution for maintaining the proper calcium homeostasis. Nevertheless, such a hypothesis remains to be verified and requires further investigations.

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