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Assessment of selected cytokine levels and vitamin D concentration in children hospitalised due to simple febrile seizures

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ABSTRACT

Introduction: Febrile seizures remain a common issue with an unexplained pathomechanism. Increasing attention is being paid to the potential involvement of vitamin D and other immunological factors, including cytokines, as potential regulators of the seizure response during fever. Febrile seizures remain a common issue with an unexplained pathomechanism. Increasing attention is being paid to the potential involvement of vitamin D and other immunological factors, including cytokines, as potential regulators of the seizure response during fever. The aim of the study was to assess the serum levels of selected cytokines and vitamin D in children hospitalised due to febrile seizures.

Material and methods: The study group consisted of 31 children hospitalised due to simple febrile seizures. The control group included 30 children admitted due to febrile conditions without seizure incidents. Patients were treated in the General Paediatrics Department at the Independent Public Clinical Hospital No. 1, Zabrze. Blood samples were collected from selected patients upon admission to measure serum TNF- α , IL-1 β , IL-2, IL-4, IL-6, IL-10, IL-1RA, and vitamin D levels. Cytokine assays were performed at the Department of Medical and Molecular Biology, Faculty of Medical Sciences in Zabrze, Medical University of Silesia in Katowice, using the immunoenzymatic method. Vitamin D levels were measured in the hospital laboratory using the electrochemiluminescence method.

Results: Higher levels of all cytokines were found in children with febrile seizures compared to the children with fever. The vitamin D concentration was lower in the study group compared to the control group, but the difference was not statistically significant. Reduced levels of vitamin D were found in nearly one-third of the patients with febrile seizures and nearly one-fifth of the patients without febrile incidents. In both groups, the number of children with a deficiency was lower than the estimated population percentage.

Conclusions: The higher serum cytokine levels in children with febrile seizures, compared to febrile children without seizures, indicate the involvement of the immune system in their aetiopathogenesis. Confirmation of the relationship between vitamin D levels and febrile seizures requires further research.

KEY WORDS:

cytokines, vitamin D, febrile seizures.

INTRODUCTION

Febrile seizures (FS) are seizures that occur in association with febrile illnesses in the absence of central ner-

vous system (CNS) infections or other identifiable causes of seizures in children without previous afebrile seizures [1, 2]. They occur between 3 months and 5 years of age and are classified into simple and complex FS. Simple FS

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are generalised, last less than 15 minutes, and involve one seizure episode within 24 hours. Complex FS are focal, last more than 15 minutes, or involve more than one seizure episode within 24 hours [1, 3].

This condition is not uncommon, affecting about 2–5% of children aged 3 months to 5 years [1, 2]. FS are considered benign, but they are associated with a slightly increased risk of epilepsy, and very rarely a history of FS might be connected with sudden unexplained death [3]. Available data are insufficient to calculate their mortality risk. Gould *et al.* [3] estimated that 150 of 1,000,000 sudden deaths in children between one and 4 years of age may be associated with FS.

The pathomechanism of FS is still not fully understood. Increasing attention is being paid to the possible involvement of immunological factors. Pro-inflammatory cytokines (including tumour necrosis factor α [TNF- α], interleukin 1 β [IL-1 β], interleukin 2 [IL-2], and interleukin 6 [IL-6]) and anti-inflammatory cytokines (interleukin 4 [IL-4], interleukin 10 [IL-10], and interleukin 1 receptor antagonist [IL-1RA]) may play an important role in the mechanism regulating the seizure response during infections [1, 2, 4]. Besides affecting calcium-phosphate metabolism, vitamin D also participates in immunomodulation processes and exhibits anti-inflammatory effects [5, 6]. Many publications have addressed the potential role of vitamin D in the pathogenesis of various diseases, including neurological disorders such as epilepsy, autism, Alzheimer's disease, Parkinson's disease, and multiple sclerosis [7]. However, only a few studies have addressed its potential association with FS.

The aim of the study was to assess the levels of selected cytokines: TNF- α , IL-1 β , IL-2, IL-4, IL-6, IL-10, IL-1RA, and vitamin D concentration in children hospitalised due to FS.

MATERIAL AND METHODS

The study group consisted of 31 children hospitalised in the General Paediatrics Department at Independent Public Clinical Hospital No. 1 in Zabrze due to simple FS. The exclusion criteria included a positive personal or family history of other than simple FS episodes and lack of consent to participate in the study.

The control group included 30 children, comparable in age to those in the study group, hospitalised due to fever caused by infection, with no family or personal history of seizure episodes (including FS).

Upon admission to the department, venous blood samples were collected from patients in both groups for laboratory tests, including the measurement of serum vitamin D level. Serum samples for cytokine measurements were frozen and stored at -20°C until analysis at the Department of Medical and Molecular Biology, Faculty of Medical Sciences in Zabrze, Medical University of Silesia in Katowice.

In the study group, the time elapsed from the onset of the seizure to the collection of samples could not be precisely assessed; it included the child's transport to the hospital (estimated at 30–60 minutes from the onset of symptoms), registration, physical examination, and administrative procedures related to hospital admission. The time from registration in the emergency department to sample collection, except for one case, did not exceed 3 hours (from 22 to 652 minutes; mean 104.55 minutes, SD 110.62).

Vitamin D levels were determined using electrochemoluminescence method (ECLIA), and the concentration of respective cytokines was determined by immunoenzymatic methods (Appendix 1).

STATISTICAL ANALYSIS

Statistical analysis was performed using R language in the RStudio environment. The Shapiro-Wilk test was used to assess normality. Variables with normal distribution were compared between groups using Student's *t*-test, while variables with non-normal distribution were compared using the Mann-Whitney-Wilcoxon test. To evaluate the usefulness of the analysed parameters as predictors, ROC analysis was employed, presenting results as sensitivity, specificity, and AUC. Correlation analysis was conducted using Spearman's coefficient. Values of $p < 0.05$ were considered significant.

RESULTS

In 30.76% of patients in the study group, the vitamin D levels were below the normal range ($< 30 \text{ ng/ml}$). In 11.53% of the cases, it was classified as deficient ($< 20 \text{ ng/ml}$). In the control group, unsatisfactory vitamin D levels were found in 18.52% of patients, with deficiency observed in only 3.7% of children.

When evaluating the vitamin D levels in children diagnosed with FS, no statistically significant differences were found compared to the control group. However, significant differences were observed in the levels of the cytokines assessed. The concentrations of all cytokines were higher compared to those in the control group (Table 1).

In the ROC curve analysis, it was found that among the examined cytokines associated with FS, the highest sensitivity and specificity are characterised by the levels of IL-1 β and IL-10 (Table 2).

In the study group, no statistically significant correlation was found between the concentration of vitamin D and the concentration of the evaluated cytokines.

DISCUSSION

One of the considered factors influencing the occurrence of FS in infants and young children is the effect of fever on the still immature nervous system. Based on

TABLE 1. Vitamin D and cytokine serum levels in the study and control groups

Variable	Group	n	Mean	SD	Median	Q1	Q3	CI	p-value	Test
Age (months)	SG	31	26.80	11.10	26.00	18.00	33.00	4.080	0.593	Wilcoxon
	CG	30	25.70	12.40	23.00	17.00	31.00	4.620		
Vitamin D [ng/ml]	SG	26	35.60	12.00	35.30	28.60	42.30	4.850	0.157	Wilcoxon
	CG	27	42.20	16.00	40.20	33.20	48.20	6.310		
TNF- α [pg/ml]	SG	31	9.34	3.04	8.49	7.31	11.40	1.110	< 0.001	t-test
	CG	30	6.45	1.51	6.42	5.55	7.51	0.564		
IL-1 β [pg/ml]	SG	31	12.70	1.76	13.10	11.20	14.00	0.646	< 0.001	Wilcoxon
	CG	30	3.51	1.37	3.50	2.79	3.91	0.512		
IL-2 [pg/ml]	SG	31	8.12	2.12	8.22	7.46	9.13	0.777	< 0.001	Wilcoxon
	CG	30	4.50	1.02	4.20	3.94	5.10	0.383		
IL-4 [pg/ml]	SG	31	3.58	0.57	3.59	3.16	3.88	0.209	< 0.001	Wilcoxon
	CG	30	2.35	0.85	2.68	1.87	2.94	0.317		
IL-6 [pg/ml]	SG	31	12.80	1.81	13.30	11.40	14.10	0.664	< 0.001	Wilcoxon
	CG	30	9.43	1.04	9.39	8.58	9.99	0.389		
IL-10 [pg/ml]	SG	31	24.10	4.83	22.30	20.80	26.20	1.770	< 0.001	Wilcoxon
	CG	30	11.00	2.12	10.80	9.57	12.60	0.793		
IL1-RA [pg/ml]	SG	31	560.00	164.00	600.00	544.00	638.00	60.200	< 0.001	Wilcoxon
	CG	30	351.00	96.70	341.00	301.00	399.00	36.100		

SG – study group, CG – control group, n – number of patients, SD – standard deviation, Q1 – lower quartile, Q3 – upper quartile, CI – confidence interval

TABLE 2. Receiver operating characteristic (ROC) curve analysis of selected cytokines

Parameter [pg/ml]	Cut-off point	Specificity	Sensitivity	AUC
TNF- α	8.330	0.93	0.61	0.80
IL-1 β	9.055	1.00	1.00	1.00
IL-2	6.505	0.97	0.87	0.93
IL-4	3.125	0.90	0.77	0.90
IL-6	11.190	0.97	0.81	0.94
IL-10	16.300	1.00	1.00	1.00
IL-1RA	516.500	0.93	0.77	0.83

AUC – area under curve

previous research, it seems that not the fever itself, but the mechanisms associated with the systemic inflammatory response may play a significant role here. Higher concentrations of IL-2 and IL1-RA have been demonstrated only during infections leading to FS compared to the group of children with fever without accompanying seizures during the current infection, but with a positive history of FS [8]. Additionally, mild seizures in children with gastrointestinal disorders are characterised by afebrile seizures incidental to diarrhoea of viral aetiology, without accompanying significant disturbances in water-electrolyte balance. They occur most often between the sixth month and the third year of life in previously healthy children, and in this case, the association of afebrile seizures with an inflammatory reaction is considered [9, 10].

In recent years, there has been increasing interest in the role of the immune system in the aetiopathogenesis of FS. Although published data may be conflicting, it is possible to hypothesise, based on them, that the intensity of the inflammatory reaction is associated with the occurrence of FS in children during febrile infections. In the present study, significantly higher concentrations of all evaluated cytokines (TNF- α , IL-1 β , IL-2, IL-4, IL-6, IL-10, and IL-1RA) were demonstrated in the study group of children diagnosed with FS compared to the control group of feverish children.

IL-1 β exhibits strong pro-inflammatory properties, initiating the production of other cytokines, and its pro-seizure action is postulated [8]. Because it has a very short half-life, the timing of sample collection is significant for the obtained results and can significantly influ-

ence the occurrence of differences in studies. Similarly to the present study, higher IL-1 β concentrations in FS than in febrile-only patients were confirmed in studies by Choi *et al.* [11] and Elsaid *et al.* [12], but there are also publications presenting different results [8, 13]. In the presented analysis, it was also shown that IL-1 β and the anti-inflammatory IL-10 exhibited the highest sensitivity and specificity among the evaluated cytokines, but the small sample size does not allow for unequivocal conclusions.

IL-6 is one of the key pro-inflammatory cytokines exhibiting strong pyrogenic activity. In several reports, as in the above study, significantly higher IL-6 concentrations were demonstrated in the FS group compared to the group of feverish children [11, 14]. On the other hand, Hautala *et al.* [13] found no significant differences in IL-6 levels between patients with FS and children with fever. Conversely, Gupta *et al.* [15] and Vishal *et al.* [16] found significantly higher levels of this cytokine in the group of feverish children compared to children with FS. There are also publications assessing other pro-inflammatory cytokines, including TNF- α and IL-2, with conflicting results [8, 11, 13, 17].

As for IL-1 β , it stimulates, among other things, the production of IL-1RA, which is a protein with anti-inflammatory properties. It controls the action of IL-1 by blocking its activity in a negative feedback mechanism by binding to IL-1 receptors. Many studies confirm its possible association with FS. In some of them, significantly higher IL-1RA values in the studied groups were found compared to the control groups, with no such relationship in terms of IL-1 β concentration. Kim *et al.* [8] suggested that this may result from an excessive reactivity to IL-1 β in response to an epileptogenic environment, even though the IL-1 β concentration does not change significantly. Given the results of the present study, which showed higher concentrations of both IL-1 β and IL-1RA in the FS group compared to feverish children without seizures, combined with similar results from other authors, IL-1RA can be considered a surrogate marker for IL-1 β [12, 18]. It cannot be ruled out that conflicting results regarding the assessment of IL-1 β concentration may result from variable confounding factors such as sampling time, temperature intensity, fever duration, cytokine measurement difficulties, type of infection, and sample size [8, 13].

Interestingly, despite discrepancies regarding the assessment of other cytokines, researchers unanimously indicate the role of IL-1RA in FS, although some analyses differed in the timing of sample collection in the study group [8, 12, 13]. Hautala *et al.* [13] showed that serum levels of IL-1RA were significantly higher in patients with FS following their first episodes than during febrile episodes without seizures and healthy periods after FS in the same patients. Moreover, higher IL-1RA levels were found in patients with FS during first and recurrent episodes than in febrile control children. These observations

support the conclusion that patients with FS produced higher inflammatory responses than those with fever without seizures. Prolonged higher levels of this cytokine may be a protective factor, reducing the risk of subsequent seizures, and as known, simple FS do not recur within 24 hours of their occurrence. Support for this thesis may be provided by data presented by Shofijah *et al.* [18]. They found higher IL-1RA concentrations in patients with FS than in the group after a status epilepticus.

In previous studies, it was also pointed out that children with FS have higher levels of other anti-inflammatory cytokines, such as IL-4 and IL-10, than the control group of feverish children [8, 16, 17]. This is consistent with the results of the current study. These observations suggest that children with FS have a higher degree of inflammatory state intensity than in the group of feverish children without seizures, and higher levels of anti-inflammatory cytokines represent mechanisms of regulation of ongoing inflammation. There are also reports presenting conflicting results in the assessment of these cytokines [13, 14].

Currently, we have quite a lot of evidence that vitamin D interacts with the CNS. This occurs through VDR receptors, which are present in both neurons and glial cells. It has also been proven that tissues building the CNS contain 1 α -hydroxylase, responsible for the local formation of its active form [19]. Its rapid, non-genomic interaction with cells has also been demonstrated. All these mechanisms contribute, among other things, to neuroprotective and immunomodulatory effects, cytokine secretion, and neurotransmission [5, 7].

Despite the interest in the role of vitamin D in the pathogenesis of many CNS diseases, there are few studies evaluating its effect on FS. Additionally, available analyses do not provide unequivocal answers as to whether its deficiency or insufficient concentration is a possible risk factor for FS.

The results of our analysis showed a statistically insignificant difference in vitamin D concentrations in the control group compared to the study group, but with lower values in children with FS. These results were consistent with the results of 3 other previously published studies [20–22].

In 3 other works, the difference in serum vitamin D concentrations between the group of patients who experienced FS and the group of feverish children without observed seizure incidents was statistically significant [23–25]. Moreover, in the study conducted by Singh *et al.* [23], the risk of seizures in patients with a low value was 3 times higher than in patients with a satisfactory concentration. In this study, however, the norm for vitamin D concentration was defined as > 20 ng/ml, which differs from the cutoff point used in our study (> 30 ng/ml), which may affect the assessment of results.

It is worth noting that according to epidemiological analyses in Poland, vitamin D deficiency may affect up to

20% of infants and 85% of teenagers [26]. The difference in these values may be due to the mostly systematic supplementation in the first years of life. In our study, the percentage of children with vitamin D deficiency in both the control and study groups was lower than the estimated population percentage, at 3.7% and 11.53%, respectively.

CONCLUSIONS

The higher serum levels of cytokines in children with FS, compared to febrile children without seizures, indicate the involvement of the immune system in their aetio-pathogenesis.

Despite the observed lower serum vitamin D levels in a group of children presenting with FS compared to febrile children without seizures, these differences were not statistically significant. Therefore, it cannot be confirmed at present that its deficiency is a risk factor for such seizures.

DISCLOSURES

1. Institutional review board statement: The study project received approval from the Bioethics Committee of the Medical University of Silesia in Katowice (KNW/0022/KB1/10/19 dated 29 January 2019).
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4. Conflicts of interest: None.

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Appendix

DETAILED INFORMATION ON LABORATORY METHODS USED TO MEASURE THE SERUM LEVELS OF VITAMIN D AND SELECTED CYTOKINES

Vitamin D levels were determined using electrochemiluminescence (ECLIA), with the Elecsys Vitamin D total III test kit on a Cobas 6000 analyser (cobas e601 module). The analytical sensitivity of the test was 7.5 nmol/l (3.0 ng/ml).

The concentration of tumour necrosis factor α (TNF- α) in serum was determined by immunoenzymatic method using the R&D Systems (Minneapolis, USA) Quantikine Immunoassay DTA OOC test, following the manufacturer's instructions. A calibration curve was constructed using standards provided in the kit to determine the concentrations of the tested samples. Absorbance readings were conducted using the Universal Microplate Spectrophotometer- μ QUANT by Bio-Tek Inc. (Bio-Tek World Headquarters, California, USA) at a wavelength of 450 nm, and results were processed using KCJunior software (Bio-Tek, USA). The kit's sensitivity was 5.5 pg/ml, with intra-assay error at 5.2% and inter-assay error at 7.4%.

Interleukin 6 (IL-6) levels in serum were measured by immunoenzymatic method using the Thermo Fisher Scientific (USA) BMS213HS test, following the manufacturer's instructions. A calibration curve was constructed using standards provided in the kit to determine the concentrations of the tested samples. Absorbance readings were conducted using a Universal Microplate Spectrophotometer- μ QUANT by Bio-Tek Inc. (Bio-Tek World Headquarters, California, USA) at a wavelength of 450 nm, and results were processed using KCJunior software (Bio-Tek, USA). The kit's sensitivity was 0.03 pg/ml, with a method imprecision (reproducibility) of 3.9%.

Interleukin 1 β (IL-1 β) levels in serum were measured by immunoenzymatic method using the BioVendor LLC (Czech Republic) RD 194559200R test, following the manufacturer's instructions. A calibration curve was constructed using standards provided in the kit to determine the concentrations of the tested samples. Absorbance readings were conducted using the Universal Microplate Spectrophotometer- μ QUANT by Bio-Tek Inc. (Bio-Tek World Headquarters, California, USA) at a wavelength of 450 nm and a reference wavelength of 630 nm. Results were processed using KCJunior software (Bio-Tek, USA). The kit's sensitivity was 0.4 pg/ml, with intra-assay error at 4.2% and inter-assay error at 6.7%.

Interleukin 2 (IL-2) levels in serum were measured by immunoenzymatic method using the Abcam (Cambridge, UK) ab270883 test, following the manufacturer's instructions. A calibration curve was constructed using

standards provided in the kit to determine the concentrations of the tested samples. Absorbance readings were conducted using the Universal Microplate Spectrophotometer- μ QUANT by Bio-Tek Inc. (Bio-Tek World Headquarters, California, USA) at a wavelength of 450 nm and a reference wavelength of 630 nm. Results were processed using KCJunior software (Bio-Tek, USA). The kit's sensitivity was 0.31 pg/ml, with intra-assay error at 5.2% and inter-assay error at 6.1%.

Interleukin 4 (IL-4) levels in serum were measured by immunoenzymatic method using the MyBioSource (MyBioSource, Inc., CA, USA) MBS 590009 test, following the manufacturer's instructions. A calibration curve was constructed using standards provided in the kit to determine the concentrations of the tested samples. Absorbance readings were conducted using the Universal Microplate Spectrophotometer- μ QUANT by Bio-Tek Inc. (Bio-Tek World Headquarters, California, USA) at a wavelength of 450 nm. Results were processed using KCJunior software (Bio-Tek, USA). The kit's sensitivity was 1.61 pg/ml, with intra-assay error at 5.1% and inter-assay error at 6.3%.

Interleukin 10 (IL-10) levels in serum were measured by immunoenzymatic method using the Diaclone (France) MBS 850.950.096 test, following the manufacturer's instructions. A calibration curve was constructed using standards provided in the kit to determine the concentrations of the tested samples. Absorbance readings were conducted using the Universal Microplate Spectrophotometer- μ QUANT (Bio-Tek Inc., California, USA) at a wavelength of 450 nm. Results were processed using KCJunior software (Bio-Tek, USA). The kit's sensitivity was 5.7 pg/ml, with intra-assay error at 4.5% and inter-assay error at 6.1%.

Interleukin 1 receptor antagonist (IL-1RA) levels in serum were measured by immunoenzymatic method using the MyBioSource (MyBioSource, Inc., CA, USA) MBS 175836 test, following the manufacturer's instructions. A calibration curve was constructed using standards provided in the kit to determine the concentrations of the tested samples. Absorbance readings were conducted using the Universal Microplate Spectrophotometer- μ QUANT by Bio-Tek Inc. (Bio-Tek World Headquarters, California, USA) at a wavelength of 450 nm. Results were processed using KCJunior software (Bio-Tek, USA). The kit's sensitivity was < 2 pg/ml, with intra-assay error at 7.2% and inter-assay error at 8.1%.