RESEARCH ARTICLE

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The Systemic Cell Apoptotic-Based Neutrophil–Lymphocyte Ratio: Experience in Children Diagnosed with ADHD and Autism Spectrum Disorder

ABSTRACT

Objective: In recent years, the rate of Neutrophil/Lymphocyte ratio (NLR) has been shown to be a marker of systemic inflammation that associated with prognosis in many diseases like malignancies and chronic inflammatory diseases. Based on previous studies, there is not any finding about NLR and cellular morphological mechanism studied together in inflamation-related diseases; attention deficit hyperactivity disorder(ADHD) and autism spectrum disorders(ASD). We assessed the effect and association of these parameters on ethiopathogenesis of neurodevelopmental disorders.

Methods: 30 healthy and 30 each patients who were diagnosed with ADHD and ASD were evaluated at psychiatry department in tertiary hospital. The hemogram profile were analyzed and NLR parameter was statistically evaluated among groups. However, apoptotic stage of cells were staining with 2 different methods. Apoptotic mechanism of ADHD, ASD and control group were comparably displayed.

Results: NLR values in patients diagnosed with ADHD and ASD were significantly higher compare to control; lymphocyte count was found significantly lower level in patient groups. Apoptotic morphology becomes evident as degree of disease increment.

Conclusions: This parameter can be used as an easily applicable method is estimated to be risk for psychiatric diseases. The positive association of NLR with apoptotic imaging indicates a marker of cellular degradation with neurodegenerative disorders.

Keywords: Hemogram Profile, ASD, ADHD, Apoptotic Tunnel Variation, NLR

Otizm Spektrum Bozukluğu ile Dikkat Eksikliği Hiperaktivite Bozukluğu Olan Çocuklarda Apoptotik Hücre Tabanlı Sistemik Nötrofil-Lenfosit Oranı

ÖZET

Amaç: Son yıllarda, Nötrofil / Lenfosit oranının (NLR) sistemik inflamasyonun bir belirteci olduğu gösterilmiştir ve maligniteler ve kronik inflamatuar hastalıklar gibi birçok hastalıkta prognoz ile ilişkilidir. Önceki çalışmalara dayanarak, NLR ve hücresel morfolojik mekanizmanın inflamasyonla ilişkili hastalıklarda dikkat eksikliği hiperaktivite bozukluğu (DHB) ve otizm spektrum bozuklukları (ASD)'nın birlikte çalışıldığı bir bulgu yoktur. Araştırmamızda bu parametrelerin nörogelişimsel bozukluklar etiyopatogenezine etkisini ve ilişkisini değerlendirdik.

Gereç ve Yöntem: DHB ve ASD tanısı alan 30 sağlıklı ve 30 hasta, üçüncü basamak bir hastanede psikiyatri kliniğinde değerlendirildi. Tüm gruplarda hemogram profiline bakılarak NLR sayısı istatistiksel olarak karşılaştırıldı. Ayrıca hücrelerin apoptotik evresini belirlemek üzere 2 farklı yöntemle boyandı. DHB, ASD ve kontrol grubunun apoptotik mekanizması karşılaştırılabilir şekilde görüntülendi.

Bulgular: DHB ve ASD tanısı alan hastalarda NLR değerleri kontrole kıyasla anlamlı derecede yüksekti; lenfosit sayısı hasta gruplarında anlamlı olarak düşük bulundu. Apoptotik morfoloji, hastalık artışı derecesi ile korele olarak farklılık gösterdi.

Sonuç: Bu parametre, psikiyatrik hastalıklara yakalanma riski olan durumlarda, erken tanıda kolay uygulanabilir bir yöntem olarak kullanılabilir. Nötrofil/Lenfosit oranının apoptotik görüntüleme ile pozitif ilişkisi, nörodejeneratif patolojilerde hücresel bozulmanın bir belirtecini gösterir.

Anahtar Kelimeler: Hemogram Profili, ASD, ADHD, Apoptotik Tunel Değişimi, NLR

INTRODUCTION

Attention-deficit/hyperactivity disorder (ADHD) is a neurodevelopmental disorder which was diagnosed mostly on childhood and characterized by a persistent pattern of inattention, hyperactivity and impulsivity that leads to functional impairment in social and academic life (1). Recent systematic reviews report the prevalence of ADHD in worldwide as 5.29% (2). High heritability rates for ADHD were shown in genetic studies approximately 75% (3,4) but also gene-environment interaction plays an important role for emergence on clinical presentation of ADHD (5). Autism Spectrum Disorders (ASD) are also neurodevelopmental disorders characterized by impairments in social interaction and communication, and the presence of restrictive and repetitive behaviors that begin in early childhood (6). ASD as well as ADHD are both highly heritable neurodevelopmental disorders, commonly 70-80% the phenotypic variance of each disorder may be explained by genetic factors (3,7,8). However genetic factors seem to be very important for both ADHD and ASD, environmental factors contribute or exacerbate clinical manifestations so as to both disorders (9). Recent studies show high levels of comorbidity and symptom overlap between ASD and ADHD that might refer a common genetic and environmental pathway for these two neurodevelopmental disorders (10).

Both animal and clinical studies have reported a major role as an immune system alterations in the etiology of ASD. Studies show mostly complex interaction between genetic/environmental factors and the immune system such as; increased levels of pro-inflammatory cytokines and autoantibody production, high incidence of familial autoimmunity, maternal immune dysfunction, abnormal levels of immunological markers, cytokine/chemokine imbalance (11). Also there are some findings that immune dysregulation and free radical-mediated neuronal damage may play an important role in the immunopathogenesis of ADHD (12,13).

Neutrophil lymphocyte ratio (NLR) and platelet lymphocyte ratio (PLR) are cheap and easily calculated indexes correlating with the prognosis of systemic inflammatory diseases. Especially useful in inflammatory, cardiovascular and cancer diseases have been discovered (14). NLR analysis has been suggested as a clinical marker of systemic inflammation (15,16). This ratio has been investigated in various psychiatric disorders such as schizophrenia, bipolar disorder, and depression (17,18,19). So far, no study has been investigated NLR in patients with both ASD and ADHD.

The apoptosis mechanism deterioration may lead to pathological consequences such as neoplasia, viral infections, autoimmune diseases, AIDS. Apart from genetic control, extracellular factors (hormones, cytokines, chemical, physical and viral agents) are also involved in the initiation of apoptosis. Neutrophil apoptosis is essential for the recovery of acute inflammatory response in genetic diseases that constitute working position, to provide immunity and to prevent inappropriate tissue destruction (20). Histopathological studies have shown that the development of these cell-mediated diseases is characterized by the alteration of lymphocyte cell morphology, especially for inflammatory factors (21).

In this study, we aimed to investigate the relationship between NLR from whole blood count parameters and histopathologically changes of cellular apoptotic activity which lead the prognosis these disease analyzing with two different methods in ADHD and ASD diseases. The main goal is to demonstrate that full blood count parameters change markedly with number and quality during inflammation and this was stimulated by disruption of a single cell.

MATERIAL AND METHODS

Patient Material: This research evaluated the predictive value of Neutrophil/lymphocyte ratio on children diagnose with ADHD and ASD. 30 each children with ADHD and ASD children managed at Duzce University Hospital, between December 2016 and August 2017. Also 30 healthy children without any psychiatric history were included as control group. The ethics committee approved the study for ethics. Histological and biochemical analyzes were performed according to taking blood in different tubes considering the criteria of inclusion and exclusion at patients. Heparine and EDTA tubes were clasified and stocked at -20oC for later measurement.

Measurement of Blood Cell: The leukocyte count and neutrophil percentage were measured by an automated hematology analyzer (Coulter® LH 780 Hematology Analyzer, Beckman Coulter Inc., Brea, CA, USA). The upper limits of the reference interval for lympocyte counts were 10.0 – 48.0% of Leukocyte level 3.8 – 10.0 $x10^3/\mu L$. The calculation of the NLR (Neutrophil/Lympocyte ratio) may provide a sensitive parameter to differentiate complicated ADHD and Autism predisposing factors. Lymphocyte and Neutrophil counts were determined after hemogram measurement.

Tunnel Assay / Staining Method: The blood samples of Autism, ADHD and healthy experimental group were spread thinly over the lamellae. Prepared blood spreads were waited for drying at room temperature and then fixed with methanol for 3 minutes. After the fixation process, the lamellas left to dry in the room heat. Two different techniques with Tunnel painting and Giemsa painting procedures were applied to compare apoptotic changes.

Apoptosis staining was performed using Millipore ApopTag Plus Peroxidase. In Situ Apoptosis Detection Kit S7101 (Lot: 2325062). The tunnel slides were first incubated at 75°C for 40 minutes. Lames held in Xylene 3 times for 10 minutes. Laminates from the xylene were passed through 96%, 80% and 70% ethyl alcohols, respectively. Washed twice for 5 minutes in PBS. Slides were incubated for 15 minutes at 370C in Proteinase-K solution diluted to 20 µg / ml in PBS. Waited in the dark for 5 minutes in 0.3% H2O2 solution. The slides were equilibrated in buffer for 10 minutes as 130 microliters per cm2/ml. The sections at 370C were incubated with TdT enzyme for 1 hour in the presence of 110 microliters per cm2. The sections were agitated in the stop buffer for 15 seconds and left for 10 minutes. DAB chromogen was applied for 6-7 min. Antifouling was done for 7 to 8 minutes with 1% methylene green. The lamella was closed using Entellan.

Giemsa Assay / Staining Method: The lamellas in the study group were fixed with methanol for 3 minutes. Then the blood spots were dried in the room heat. Giemsa stock solution was diluted to 1/10 ratio and laid to cover the slides and applied to the slides for 30 minutes. The blood springs were thoroughly washed in distilled water and covered with lamellae using intellect.

Cell Morphology Detection: The staining methods were applied on an Olympus CX41 microscope where the Zeiss AxioCam ICc5 camera was attached. In order to detect changes in the morphology of cells belonging to control, ADHD and autism groups, cell images were obtained using the Zen 2 Lite program. Images were obtained by scanning the whole area with the Zen 2 Lite program.

Statistical Analysis: Statistical analysis was done with SigmaStat software version 2.03 (Jandel Scientific). Microscopic immunohistochemical analysis was done by Oxiron and photomicrographs were captured by Carl Zeiss AxioCam MRc5 camera. Quantitative data are presented as mean, SE, min and max. Statistical significance of difference between control, ADHD and autism group was determined by Kruskall–Wallis test Test and Mann–Whitney U tests was used for pairwise comparison of groups, respectively. The $P < 0.05$ was considered significant.

RESULTS

The average age of the pediatric patients included in the study was calculated and all criteria has designed for inclusion and exclusion requirements. Neutrophil/lymphocyte ratios for ADHD¸autism and control groups included in this research according to determined criteria were evaluated by Kruskal-Wallis test. There was a statistical difference (49,33 ± 2) between groups ($P < 0.001$) when they were evaluated in triplicate shown as Table 1.

Table 1. Statistical differences between groups duplicate comparations

NLR*	C /ADHD	C /ASD	ADHD/ASD
Z value	-5,994	-3,379	-5,491
P	,000	,001	,000

Paired comparisons were made according to the Mann-Whitney Test and it was found among the Control-ADHD groups (17,00 ± 5,99); between control and autism group (248.00 ± 3.37); between ADHD and autism (67.00 ± 5.49) values were determined. There was a statistically significant difference ($P < 0.001$) between the mean ratios obtained in comparative groups (Figure 1).

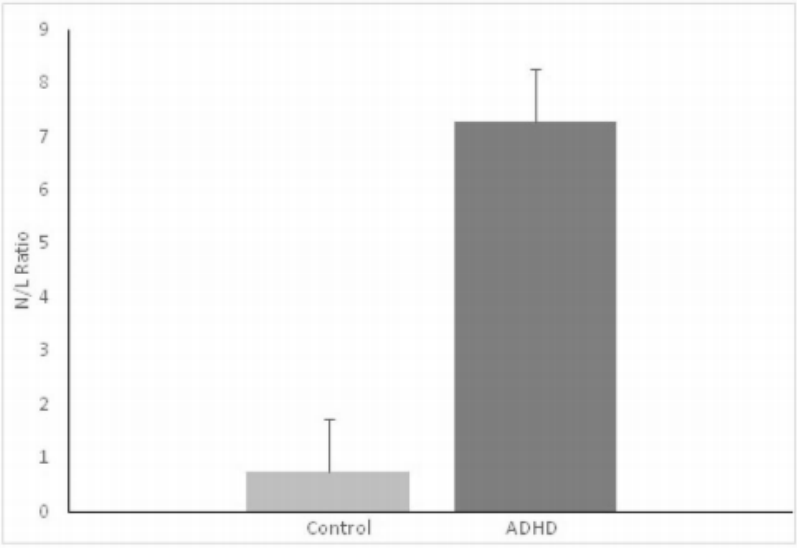

Figure 1. The variaton of Neutrophil/Lymphocyte ratio according to hemogram profile in ADHD patient group

When the hemogram analysis results of all groups were examined, it was aimed to determine the relationship between neutrophil/lymphocyte ratios and cellular apoptotic mechanism. Based of this hypothesis, datas of each group of datas were explained more detaily. The mean NLR in control group was 0.756 ± 0.50, while the min and max values ranged from 0.004 to 2.91, respectively. The mean NLR in ADHD group was 7,281 ± 4,98, the min and max value ranges are; 1,72 and 19,50; In the autism group, mean NLR was 1,662 ± 0,37, min and max values have been identified among 0,18 to 6,32, variably (Figure 2).

Significant difference was observed in the control, ADHD and ASD groups with reference to the obtained data. Moreover, this significant difference is related to the change of the cellular mechanism in the binary and triple comparisons of experimental groups (Figure 3).

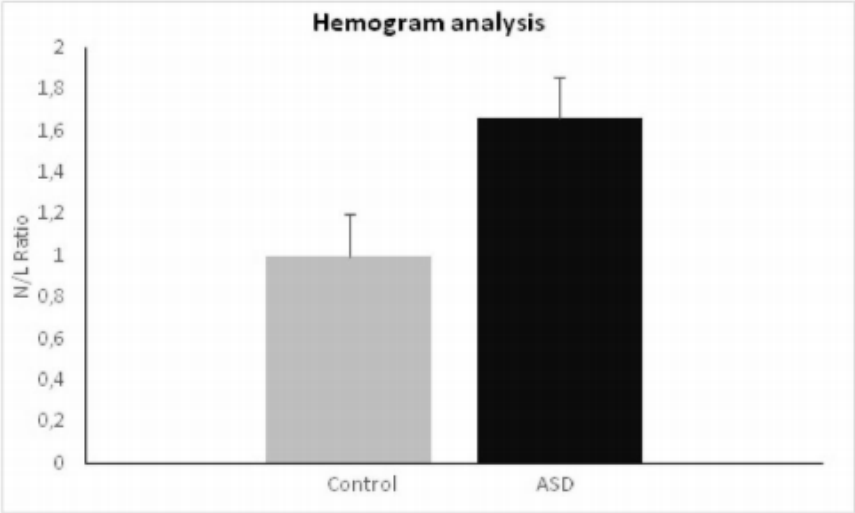

Figure 2. The variaton of Neutrophil/Lymphocyte ratio according to hemogram profile in Autism patient group

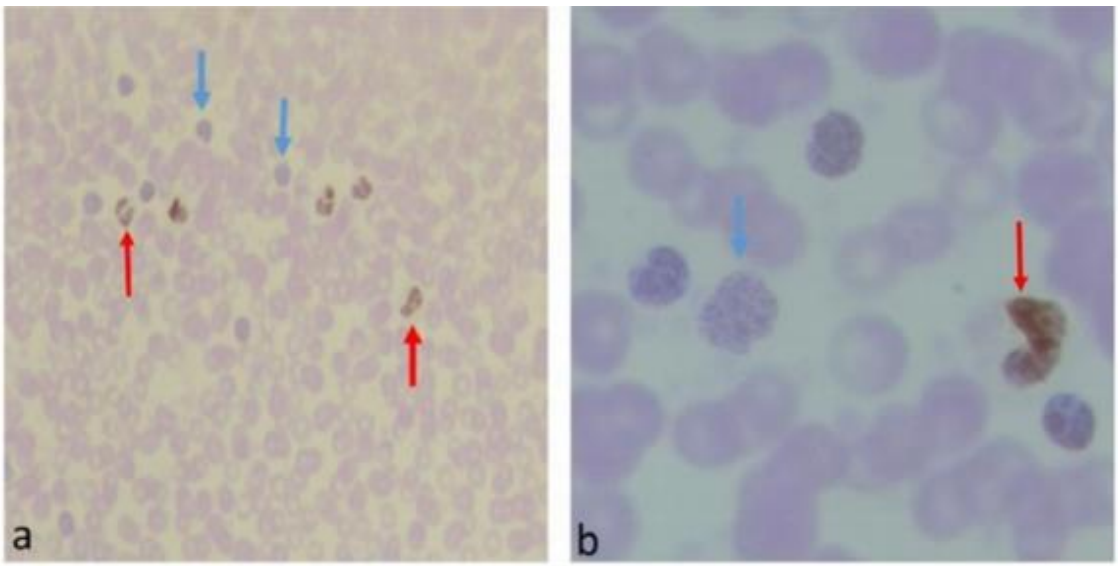

Figure 3. Cell morphological results by different (by Tunnel) staining [3a. Apoptotic leukocyte (red arrows) and normal leukocyte (blue arrows) (x40), 3b. Apoptotic leukocyte (red arrow) and normal leukocyte(blue arrow) (x100)]

The nuclei of apoptotic cells appear to be brown by Tunnel method. After the blood stains with Giemsa, cells which have chromatin condensation on the edge of the nucleus membrane and vacuolization in the nucleus were evaluated as apoptotic cells (20) (Figure 4, Figure 5).

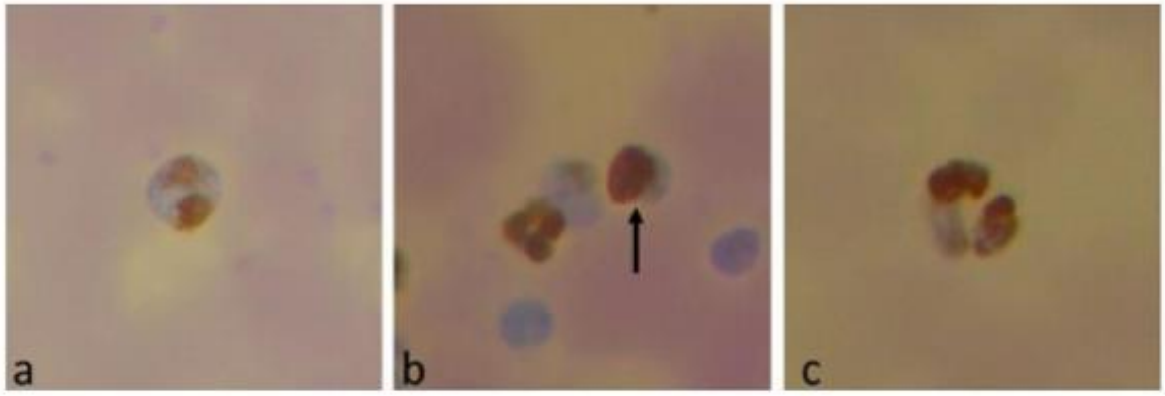

Figure 4. Apoptotic alteration of Leukocytes in Autism Group (Tunnel Method) [4a. Apoptotic eosinophil. 4b. Apoptotic lymphocyte (black arrow). 4c. Apoptotic neutrophil (x100)]

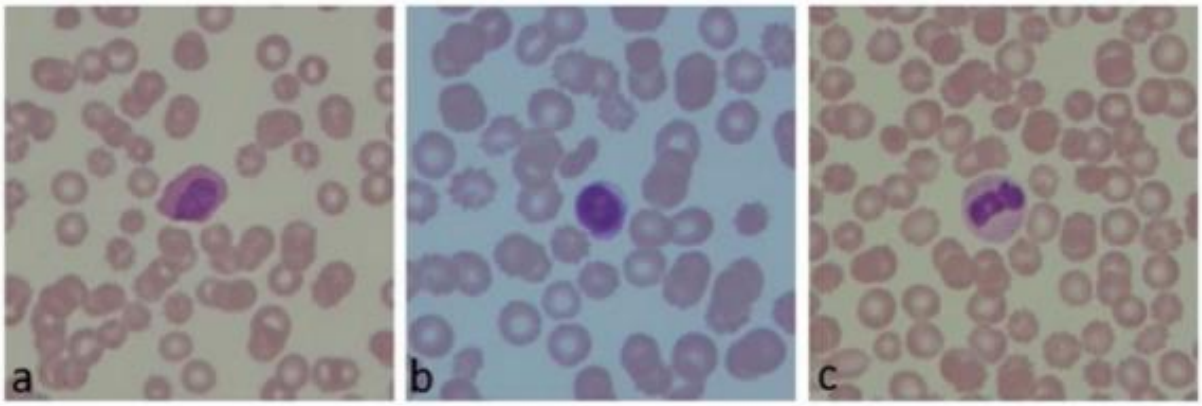

Figure 5. Apoptotic alteration of Leukocytes in Autism Group (Giemsa Method) [5a. Apoptotic eosinophil. 5b. Apoptotic lymphocyte 5c. Apoptotic neutrophil (x100)]

When the 3 major histologic experimental groups (healthy control, ADHD, autism) to be taken into consideration, we found a significant association between elevated N/L ratio corresponding increasement apoptotic sight in ADHD and autism.

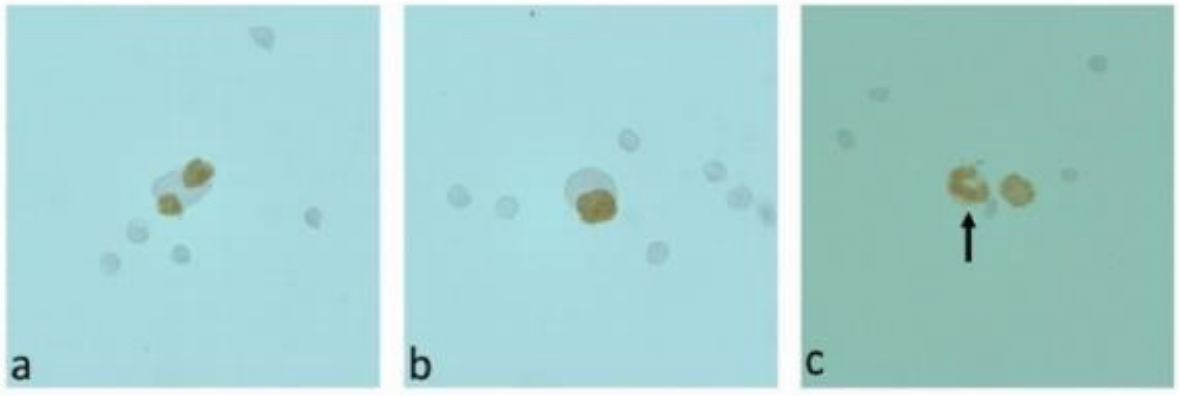

Figure 6. Apoptotic alteration of Leukocytes in ADHD Group (Tunnel Method) [6a. Apoptotic eosinophil. 6b. Apoptotic lymphocyte. 6c. Apoptotic neutrophil (black arrow) (x100)]

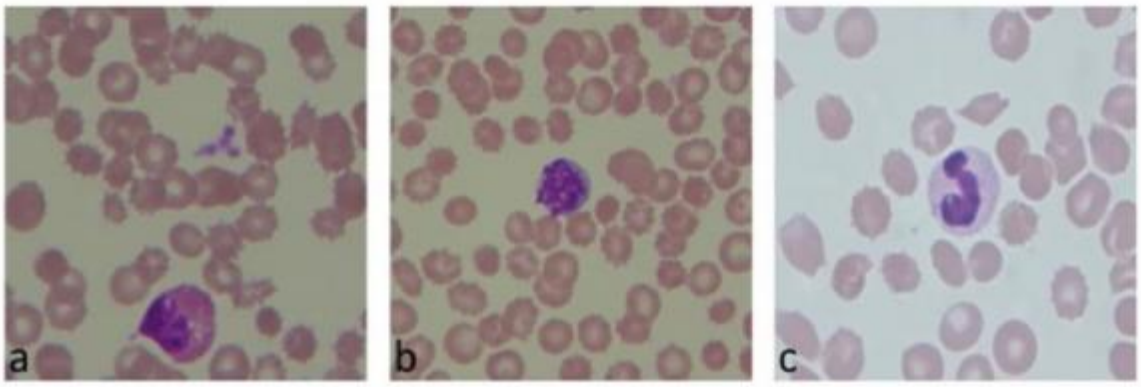

Figure 7. Apoptotic alteration of Leukocytes in ADHD Group (Giemsa Method) [7a.Apoptotic eosinophil.7b.Apoptotic lymphocyte. 7c.Apoptotic neutrophil (x100)]

Apoptotic appearance of blood-shaped components correlated with an increasement in NLR. These datas show that the degradation of cellular structure is effective in initiation and prognosis of the ADHD or ASD.

DISCUSSION

Apoptosis is perceived as a positive repair mechanism in some conditions; despite is used as a prognostic factor for diagnosis of disease. When apoptosis is inhibited in some situations, there is no explanation for the neutrophil status although it may cause an increase or prolongation of the inflammatory response. Another approach is that the neutrophils are followed by alternative cell death pathways leading to necrosis, in which an increase in the inflammatory response is encountered (22).

In the study conducted by Misso et al, apoptosis-induced neutrophils showed that intracellular glutathione concentration decreased, neutrophil apoptosis accelerated by increasing H2O2 production by exogenous glutathione (23) Turkmen et al. have found that NLR has a better association with inflammatory cytokines such as IL-6 and TNF-alpha in renal disease patients. Also NL Ratio has been shown to be associated with progression and metastasis of gastric cancer, non-small-cell lung cancer, ovarian cancer, intrahepatic cholangiocarcinoma, hepatocellular carcinoma, pancreatic cancer, CRC and nasopharyngeal cancer. They also found a significant association between an elevated N/L ratio and tumour size and the presence of histopathological tumour necrosis (24,25,26). Studies conducted by Rodriquez and colleagues showed a significant association between disease severity and acute phase markers and NLR levels in patients with romatoid artritis (27,28).

Another research on hepatocellular carcinoma, the mean survival time was 14 months with NLR values above 3, while the NLR value was reported as 26 months in below 3. In patients with non-small cell lung cancer, a strong association was found between the NLR values above 5 and the presence of advanced disease. Meta-analysis of 26 studies on primary liver tumors suggests that high NLR values are indicative of poor prognosis and vascular invasion of the tumor (29).

Lymphocytes levels are at the highest in the neonatal period, this proportion decreases deliberately up to 18 years. For Aydın et al, the lowest levels of NLR values were detected in the 0-1 age group. Datas observed that the NLR values continued to increase until the age of 20, then entered a plateau period, beside the NLR values tended to rise again after the age of 60 years. Also it was observed that there was a significant difference between male and female genders in all age groups after 30 years of age, when there was no significant difference between sexes in terms of NLR values between 10 and 30 years of age (30,31). The patients group includes 90 male whose age range was 8.2±2.8 in the current study. However, there is still no consensus on the range of the normal values of NLR in different age groups and different genders.

In the study, the tunnel assay in neutrophil and lymphocytes changes for patient group was statistically significant compare to control group cell morphology. The slowing down of apoptosis which the main key to cancer, the immune system and embryonic development, breaks the body's resistance to infection and inflammation. This formation indicates that neutrophile ratio variated with genetic diseases (32,33,34). At current study there are significant differences between the groups correlated with previous ones. Neutrophile/Lymphocyte ratio is consistent with cell apoptosis view between groups by Tunnel and Giemsa protocols in our study. The increasement in number of apoptotic cells indicates that the cell cycle genetic mechanism has changed significantly in ADHD and Autism patient group. On the other hand, we were compared 2 staining methods, Tunnel can be used more selectively during evaluate apoptosis while Giemsa was tested as more easier and quicker protocol than Tunnel (20, 35).

In autism literature, both prenatal and postnatal immune dysregulation which may lead to

neurological dysfunctions have been suggested (35,36). An altered immune cell ratio, decreased level of T lymphocytes, higher levels of monocytes as an indicator of chronic inflammation and decreased lymphocyte/monocyte ratio have been found in previous studies (37).

Latest researches indicate that the neutrophil lymphocyte ratio can also be used to calculate morbidity and mortality. This rate is regarded as a sign of lymphocyte proliferate function and activation. If NLR rates changes high level, that have been admissible to an independent risk and poor prognostic factor for many systemic disorders like acute myocardial infarction, renal artery stenosis, cancer, diabetes mellitus, hypertension and hyperlipidemia. In a study conducted by Tural et al, alterations in NLR from complete blood cell parameters in autism were investigated. They suggested increased levels of NLR and also a positive correlation of NLR with autistic symptom severity were found in ASD patients. NLR increase has been also reported in various psychiatric disorders associated with inflammation, such as schizophrenia,bipolar disorder and major depressive disorder (36,38).

CONCLUSION: Full blood count is simple, inexpensive but contains important follow-up parameters for many diseases. It is estimated that NLR values will be measured and NLR reference interval can be lightable for clinicians will form a source for other clinical work to be done.

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