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Original Article

Elevated visfatin levels illuminate the inflammatory path in bronchopulmonary dysplasia

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ABSTRACT

Background. Bronchopulmonary dysplasia (BPD) is a chronic lung disease in premature infants caused by an imbalance between lung injury and lung repair in the developing immature lungs of the newborn. Pulmonary inflammation is an important feature in the pathogenesis of BPD. The aim of this study was to evaluate the relationship between the inflammatory microenvironment and the levels of visfatin and nesfatin-1, which are among the new adipocytokines, in BPD patients.

Methods. The groups consisted of 30 patients with BPD and 30 healthy children. Plasma levels of visfatin and nesfatin-1 and inflammation-related markers including interleukin-4 (IL-4), interleukin-10 (IL-10), nuclear factor kappa B (Nf-κB) and matrix metalloproteinase-9 (MMP-9) were determined by enzyme-linked immunosorbent assay (ELISA). RT-PCR was performed to evaluate the change in mRNA expression of visfatin and nesfatin-1 in the groups.

Results. Visfatin levels were significantly higher in the BPD group compared to the healthy control (7.05±4.07 ng/ml vs. 2.13±1.66 ng/ml, $p<0.0001$). There was a 1.36±0.12 fold increase in visfatin mRNA expression ($p<0.05$) in the BPD group. There was no significant difference in plasma levels of nesfatin-1, IL-4, and IL-10 between the groups. Although MMP-9 and Nf-κB levels were significantly higher in the BPD group ($p<0.0001$), there was no correlation between visfatin levels and MMP-9 and Nf-κB levels in BPD patients.

Conclusions. This study showed that significant changes in visfatin levels in BPD patients might be associated with the risk of developing inflammation in BPD.

Key words: adipokine, bronchopulmonary dysplasia, inflammation, nesfatin-1, visfatin.

Bronchopulmonary dysplasia (BPD) is a chronic lung disease in premature infants, resulting from an imbalance between lung injury and repair during development.[1] First described by Northway et al. in 1967, BPD was associated with infants treated for respiratory distress syndrome (RDS) using high levels of oxygen and positive pressure ventilation.[2] The disease is multifactorial, influenced by both prenatal and postnatal factors, though its molecular pathogenesis remains unclear and effective prevention methods are lacking. Despite advances in treatment, BPD continues to be a common late morbidity in preterm infants.[3]

Pulmonary inflammation plays a central role in the pathogenesis of BPD, driven by risk factors like mechanical ventilation, infections, and hyperoxia.[4] This inflammation is characterized by the presence of inflammatory cells, cytokines,

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and mediators in the lungs, which damage lung structure and induce cell death.[5]

Adipokines, proteins secreted by adipose tissue, are involved not only in energy metabolism but also in inflammatory responses in chronic diseases.[6,7] While much is known about adipokines like adiponectin and leptin in chronic obstructive pulmonary diseases, the roles of newer adipokines such as nesfatin-1 and visfatin in inflammatory lung diseases are less studied.

Nesfatin-1 is a polypeptide expressed from nucleobindin-2 (NUCB2) in the hypothalamus.[8,9] NUCB2 has also been shown to be secreted outside the central nervous system, mainly in the gastric mucosa and white adipose tissue, as well as in small amounts in the periphery, especially in adipose tissue, pancreatic endocrine beta cells and testicular tissues.[9,10] Nesfatin-1 has been linked to higher plasma levels in lung cancer patients with fat mass changes and in cystic fibrosis patients with low fat mass.[11,12] Visfatin, also known as nicotinamide phosphoribosyltransferase (NAMPT), inhibits neutrophil apoptosis[13] and plays a role in nicotinamide adenine dinucleotide (NAD) biosynthesis.[14] It is synthesized not only in visceral fat but also in various tissues, including lymphocytes, hepatocytes, and pneumocytes.[13]

In the light of the existing body of knowledge, we aimed to investigate the relationship between novel adipocytokines such as nesfatin-1 and visfatin and the inflammatory microenvironment, in patients diagnosed with BPD.

Materials and Methods

Subjects

The study included thirty patients diagnosed with BPD who were being followed up in the Pediatric Pulmonary Diseases Department of our hospital. The inclusion criteria were being followed with a diagnosis of BPD and being between 0-3 years of age. Patients with infections and/or known chronic diseases were excluded from the study. We included BPD patients in the study when they visited our pediatric pulmonary diseases outpatient clinic for follow-up. The control group consisted of 30 age- and sex-matched children without infection and acute/chronic disease, who applied to our hospital's Social Pediatrics child health follow-up outpatient clinic for routine follow-up and vaccinations. The study was conducted with the approval of the Clinical Research Ethics Committee of Ankara University, and informed consent forms were obtained from each participant prior to enrollment.

Pulse oximetry saturations were measured in room air. We recorded whether they received oxygen support at home and whether they used inhaled steroids. Following the examination of the BPD patients, blood samples for a complete blood count (CBC) and C-reactive protein (CRP), and a chest X-ray were obtained. Pathological findings such as peribronchial thickness or infiltration and hyperinflation were recorded. Venous blood samples were taken from healthy controls and BPD patients for measurement of visfatin, nesfatin-1, nuclear factor kappa B (Nf-κB), matrix metalloproteinase-9 (MMP-9), interleukin-4 (IL-4), interleukin-10 (IL-10) and CRP levels, CBC and gene expression analysis.

ELISA Measurements

The plasma levels of visfatin and nesfatin-1 were determined by commercially available enzyme-linked immunosorbent assay (ELISA) kits (FineTest). The analytical sensitivities of the kits were 0.188 ng/mL, and the detection range were between 0.313-20 ng/mL. The coefficients of variation (CV) within and between tests were less than 8% and 10%, respectively. The analytical sensitivity of the ELISA kit (FineTest) used for the determination of plasma levels of IL-4 was 18.75 pg/mL and the detection range was between 31.25-2000 pg/mL. The plasma levels of IL-10 were determined by the ELISA kit (FineTest), while the analytical sensitivity was 4.68 pg/mL, the detection range

was between 7.81-500 pg/mL. The intra-assay and inter-assay coefficients of variation for interleukin kits were less than 8% and 10%, respectively. The analytical sensitivity of the kit (FineTest) which was used for the detection of plasma Nuclear Factor kappa B levels was less than 0.188 ng/mL, while the detection range was between 0.313-20 ng/mL. The intra-assay and inter-assay coefficients of variation were less than 8% and 10%, respectively. The plasma levels of Matrix metalloproteinase-9 protein were also determined by a commercially available ELISA Kit (eBioscience) and the limit of detection of human MMP-9 defined as the analyte concentration resulting in an absorbance significantly higher than that of the dilution medium (mean plus 2 standard deviations) was determined to be 0.05 ng/mL (mean of 6 independent assays). The calculated overall intra-assay and inter-assay coefficients of variation were 7.3% and 10.2%, respectively.

Gene expression analyses

Total RNA was isolated from whole blood samples collected into EDTA-containing tubes using the RNA isolation kit (Qiagen). cDNA samples were generated with the PCR system (Qiagen RotorGene) using cDNA synthesis kit (Qiagen). Quantitative real-time PCR (RT-PCR) was performed using the Qiagen RotorGene system. ß-actin was used as the housekeeping gene for normalization. The PCR reaction mix were prepared according to the manufacturer's recommendations. Samples were analyzed in duplicate. The forward and reverse primer sequences were as follows: 5′-GGGAGGCTAAGCAAAGAACTG-3′ forward,5′TCCATGCCTATATCTTGAAGGGA-3′ reverse for nesfatin-1; 5′-AATGTTCTCTTCACGGTGGAAA-3′ forward, 5′-ACTGTGATTGGATACCAGGACT-3′ reverse for visfatin; 5′-TGTACCGCTATGGTTACACTCG-3′ forward, 5′-GGCAGGGACAGTTGCTTCT-3′ reverse for MMP-9. The results were calculated with the $2^{-\Delta\Delta Ct}$ method and expressed as fold change in the BPD group normalized with the controls.

Statistical analysis

Statistical analyses were performed using the GraphPad Prism (GraphPad Inc., Version 6) statistical program. Demographic data, plasma cytokine levels, oxygen saturation levels, plasma CRP levels, blood white blood cells (WBC), neutrophil and lymphocyte counts were expressed as mean±standard deviation and compared with Mann-Whitney U test. Plasma cytokine levels of BPD patients grouped according to receiving inhaled steroid treatment at home, oxygen supplementation at home and having pathological findings on chest radiography were compared with the student-t test. Real-time PCR results were analyzed using a one-way ANOVA test. Correlation analyses between plasma cytokine levels and oxygen saturation levels, plasma CRP levels, and blood WBC, neutrophil and lymphocyte counts, respectively in the BPD group; and correlation analyses between visfatin and MMP-9 and Nf-κB respectively in the BPD group were performed using the Spearman correlation test. P values <0.05 were considered statistically significant.

Results

The demographic data and clinical characteristics of patients and control subjects are presented in Table I.

Our findings showed that the plasma levels of visfatin were significantly higher in the BPD group (7.05±4.07 ng/mL) compared to the control group (2.13±1.66 ng/mL), ($p<0.0001$). On the other hand, nesfatin-1 levels did not differ significantly between BPD and control groups (27.09±10.92 ng/mL vs. 22.83±8.34 ng/mL, respectively, Table II). The levels of IL 4 in BPD and control groups were determined as 18.18±6.86 pg/mL and 15.73±2.68 pg/mL, respectively, while the IL-10 levels were 139.62±83.65 pg/mL and 133.32±83.16 pg/mL, respectively and no significant difference was calculated between the groups. The plasma MMP-9 levels were significantly higher in the BPD group (2.34±0.94 ng/mL) compared to the

Table I. Demographic and clinical characteristics of BPD and control groups.

Variable	BPD (n=30)	Control (n=30)
Birth week (mean±SD)	28.53± 2.9	37.85±1.97
Birth weight (gram) (mean±SD)	1188.96±489.2	3152.21±310
Current age (month) (mean±SD)	32.12±6.58	34.00±2.12
Female/Male	1/2	1.2/1.8

BPD: bronchopulmonary dysplasia.

Table II. The comparison of plasma levels of visfatin, nesfatin-1, interleukin-4, interleukin-10, matrix metalloproteinase-9 and nuclear factor kappa B in bronchopulmonary dysplasia patients and healthy controls.

	Control (n=30) mean±SD	BPD (n=30) mean±SD	p value
Visfatin (ng/mL)	2.13±1.66	7.05±4.07	<0.0001
Nesfatin-1 (ng/mL)	22.83±8.34	27.09±10.92	0.3180
IL-4 (pg/mL)	15.73±2.68	18.18±6.86	0.2527
IL-10 (pg/mL)	133.32±83.16	139.62±83.65	0.1902
MMP-9 (ng/mL)	1.46±0.64	2.34±0.94	<0.0001
Nf-κB (ng/mL)	0.81±0.58	4.51±3.86	<0.0001

BPD: bronchopulmonary dysplasia; IL-4: interleukin-4; IL-10: interleukin-10; MMP-9: matrix metalloproteinase-9; Nf-κB: nuclear factor kappa B. P < 0.05 is considered as statistically significant.

control group (1.46±0.64 ng/mL, p<0.0001). Similarly, a significant increase was observed in plasma Nf-κB levels in the BPD group (4.51±3.86 ng/mL) compared to the control group (0.81±0.58 ng/mL, p<0.0001, Table II).

According to the results of PCR experiments, mRNA expression levels of visfatin, nesfatin-1 and MMP-9 increased 1.36±0.12, 1.31±0.06 and 0.75±0.11 times in the BPD group compared to the control group, respectively (p< 0.05, Table III).

In the present study, the correlation analysis was performed between plasma visfatin levels and increased cytokines, MMP-9 and Nf-κB in the BPD group and no significant correlation was found between these cytokines (r values were 0.390 and 0.533 and p values were 0.073 and 0.074, respectively). The relationship between cytokines and various clinical variables in BPD patients was also examined by correlation analysis and no significant correlation was found (Table IV). When the relationship between cytokines and medication parameters was examined, a significant increase in MMP-9 levels was found in the group supplemented with oxygen at home compared to the group not receiving it (Table IV).

Table III. Fold change on mRNA expression levels of visfatin, nesfatin-1 and MMP-9 detected by RT-PCR in BPD group.

Age	Fold change mean±SD	p value
Visfatin	1.36±0.12	0.0121
Nesfatin-1	1.31±0.06	0.0187
MMP-9	0.75±0.11	0.0385

BPD: bronchopulmonary dysplasia; MMP-9: matrix metalloproteinase-9; RT-PCR: real-time polymerase chain reaction.
P < 0.05 is considered as statistically significant.

Discussion

Adipokines, through their involvement in inflammatory processes, may play a significant role in the pathogenesis of BPD by influencing lung inflammation and tissue damage in preterm infants. The roles of visfatin and nesfatin-1 in the pathogenesis of BPD remain largely obscure, with limited research exploring their potential contributions to lung inflammation and disease progression.

Table IV. Correlation analyses between plasma cytokine levels and oxygen saturation levels, plasma CRP levels, blood WBC, neutrophil and lymphocyte counts[a]; and comparisons of plasma cytokine levels of BPD patients grouped according to inhaled steroid treatment, oxygen supplementation and chest radiography status[b].

Parameters[a]		Visfatin		MMP-9		Nf-κB	
		r	p-value	r	p-value	r	p-value
Oxygen saturation (%)		-0.058	0.799	0.105	0.579	0.043	0.872
CRP (mg/L)		0.012	0.957	0.238	0.206	0.457	0.076
WBC (mm^3)		-0.051	0.820	-0.096	0.614	-0.021	0.937
Neutrophil (mm^3)		-0.032	0.887	0.238	0.204	0.172	0.520
Lymphocyte (mm^3)		-0.001	0.998	-0.327	0.078	-0.146	0.584
Parameters[b]		**Visfatin**		**MMP-9**		**Nf-κB**	
		Mean±SD	p-value	Mean±SD	p-value	Mean±SD	p-value
Inhaled steroid treatment	+ (n=18)	7.61±1.27	0.495	0.23±0.01	0.841	0.47±0.04	0.342
	- (n=12)	6.38±1.18		0.23±0.03		0.41±0.04	
Oxygen supplement at home	+ (n=5)	8.41±1.69	0.511	0.31±0.06	0.03	0.52±0.02	0.257
	- (n=25)	6.63±1.05		0.21±0.01		0.42±0.03	
Pathological finding on chest radiography	+ (n=16)	7.51±1.48	0.634	0.24±0.02	0.479	0.45±0.04	0.910
	- (n=14)	6.66±1.04		0.22±0.02		0.44±0.05	

CRP: C-reactive protein, MMP-9: matrix metalloproteinase-9, Nf-κB: nuclear factor kappa B, WBC: white blood cells.

Visfatin is a pro-inflammatory cytokine involved in inflammatory and innate immune responses[7,15] and it was found to be associated with acute lung injury developing in the lungs.[7] In addition, inhibition of visfatin synthesis has been shown to increase inflammation and apoptosis associated with severe viral infection in the lung endothelium.[16] One study has reported that serum visfatin were elevated in stable chronic obstructive pulmonary disease and its acute exacerbations.[15] Nesfatin-1 is a recently discovered protein and it plays role in the inflammatory responses.[17,18] The studies have shown that nesfatin-1 plasma levels were found to be significantly higher in patients with lung cancer related to changes in fat mass[11,19] and in patients with cystic fibrosis with low fat mass.[20] In the present study, we reported significantly higher plasma levels of visfatin in BPD patients compared to healthy controls. Although plasma nesfatin-1 levels were higher in the BPD group compared to healthy controls, this difference was not statistically significant. Besides, mRNA expression of both visfatin and nesfatin-1 were higher in BPD patients. These results support the possible role of these novel adipocytokines on the inflammatory status of BPD patients.

Recent studies suggest that other cellular lines, such as mast cells accumulating in lung tissue and reactive T cells in peripheral blood, may influence the development of BPD.[21] The Nf-κB family of transcription factors are ubiquitously expressed, and function to regulate different cellular processes such as proliferation, differentiation, survival, and immunity.[22] There is limited data linking Nf-κB and BPD. A study on this subject, reported increased NfKB activation in tracheal lavage fluid of premature infants who develop BPD.[23] In the present study, we reported the increased plasma Nf-κB levels in BPD patients supporting the inflammatory status in BPD patients.

Matrix metalloproteinases are a family of zinc-dependent endopeptidases, and MMP-2 and -9 are gelatinases that are associated with inflammatory lung injury.[24] MMP-9 is synthesized and stored in neutrophil and eosinophil granules in the bone marrow. The production of MMP-9 is also induced by IL-1β, macrophages, Clara cells, alveolar type II cells, smooth muscle cells, fibroblasts and bronchial epithelial cells.[24] In our study, the MMP-9 plasma levels as well as mRNA expression

were found to be significantly increased in BPD patients, but no significant correlation were observed between MMP-9 and visfatin levels. It has been shown that increased MMP-9 activity in the lungs is associated with the development of BPD in newborn infants and animal models.[25] In contrast, one study reported that MMP-9 activity in the newborn lung might be a host-defense mechanism, which protects the lung against inflammatory injury, instead of playing a pathogenetic role in the development of BPD as previously suggested.[26]

In the present study, increased plasma visfatin levels were observed compared to controls, along with elevated levels of MMP-9 and NF-κB, both of which are key markers of inflammation and tissue remodeling.[27,28] Based on these findings, a correlation analysis was conducted to explore potential relationships between visfatin and other increased cytokines MMP-9 and Nf-κB, as well as some clinical variables. No significant correlation was found between visfatin and other cytokines. The lack of meaningful correlation may suggest that, while visfatin, MMP-9, and Nf-κB are individually elevated in BPD, their roles in disease progression involve different, non-interdependent pathways or mechanisms of action, rather than direct interaction. It is suggested that the complexity of the inflammatory processes in BPD involves multiple mediators contributing to lung damage through distinct but overlapping pathways. Additionally, the absence of a correlation may indicate that larger sample sizes or more targeted experimental conditions are required to better understand the interplay between these factors.

Given that receiving inhaled steroid treatment or home oxygen support may impact inflammation levels, our study compared the plasma cytokine levels of BPD patients who received these treatments with those who did not. We found that there was a significant increase in plasma MMP-9 levels only in those receiving oxygen support at home.

In conclusion, visfatin, one of the new adipocytokines, was investigated for the first time in BPD patients in the present study, and it was found that there was a significant increase in its levels. Therefore, the significant change in visfatin, MMP-9 and Nf-κB levels in BPD patients may be associated with the risk of developing inflammation in BPD. This finding is important in terms of revealing the existence of a new therapeutic target in the follow-up and treatment of the disease in question. In this context, the findings of the study will guide the development of new treatment strategies in the field of health.

Ethical approval

The study was approved by Ankara University Ethics Committee (date: 08.04.2019, number: 07-533-19).

Author contribution

The authors confirm contribution to the paper as follows: Study conception and design: BH, FBA; data collection: GO, NC, ST; analysis and interpretation of results: BH, NC, FBA; draft manuscript preparation: BH, NC, FBA. All authors reviewed the results and approved the final version of the article.

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Conflict of interest

The authors declare that there is no conflict of interest.

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