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ORIGINAL INVESTIGATION
ÖZGÜN ARAŞTIRMA

A New Marker for Early Diagnosis in Neonatal Sepsis: Polymorphonuclear Leucocyte Elastase Levels

Yenidoğan Sepsisinin Erken Tanisinda Yeni Bir Yöntem: Polimorfonükleer Lökosit Elastaz Düzeyleri

Müge Özay Payaslı[1], Ayşe Ayaz Özkul[2], Selime Ayaz[3], Emel Ataoğlu[4], Murat Elevli[4]

ABSTRACT
ÖZET

Objective: The aim of this study was to evaluate the importance of polymorphonuclear leucocyte (PMN) elastase as an early indicator and follow-up parameter in neonatal sepsis.

Material and Methods: The study group consisted of forty patients with the diagnosis of sepsis and the control group included twenty newborn. Inclusion criteria were formerly sought in our subjects who were diagnosed using the Töllner scoring system and based on the clinical observations and laboratory findings. The results of white blood cell and platelet counts, immature/total neutrophil ratio, CRP and PMN elastase values were evaluated in both groups. Enzyme linked immunoassay methods were used to determine the PMN elastase levels.

Results: The mean PMN elastase level was found to be 145.1±34.6 ng/mL in patients with neonatal sepsis and 75.5±9.8 ng/mL in healthy subjects (p<0.001). When the plasma PMN elastase levels were compared between both groups, the specificity was %96.3, sensitivity was %94.6, negative estimation value was %93.5 and positive estimation value was %95.1.

Conclusion: These findings indicate that PMN elastase level is a major indicator for the early diagnosis of newborn sepsis.

Key words: Early diagnosis, PMN elastase, C-reactive protein, sepsis

Amaç: Bu çalışmanın amacı yenidoğan sepsisinin erken tanısı ve takibinde polimorfonükleer lökosit elastaz (PMN) düzeylerinin yerini değerlendirmektir.

Gereç ve Yöntemler: Sepsis tanısı konulan 40 hasta ve kontrol grubu olarak alınan 20 yenidoğan çalışma grubunu oluşturdu. Çalışmaya alınma kriterleri önceden saptanan bu olgularda klinik ve laboratuvar yöntemleri ve Töllner skorlama sistemi kullanılarak tanıya gidildi. Her iki grubun kültür sonuçları, lökosit ve trombosit sayıları, immatür/total nötrofil oranları, CRP ve polimorfonükleer lökosit elastaz değerleri incelendi. polimorfonükleer lökosit elastaz düzeyleri ELISA yöntemi ile ölçüldü.

Bulgular: Ortalama polimorfonükleer lökosit elastaz düzeyleri neonatal sepsis grubunda 145,1±34,6 ng/mL, kontrol grubunda 75,5±9,80 ng/mL olarak saptandı (p<0,001). Her iki grup karşılaştırıldığında plazma polimorfonükleer lökosit elastaz düzeyinin özgünlüğü %96,3, duyarlılığı %94,6, negatif tahmin değeri %93,5, pozitif tahmin değeri %95,1 olarak hesaplandı.

Sonuç: Yenidoğan sepsisinin erken tanısında ve tedaviye cevabın izlenmesinde PMN elastaz düzeylerinin önemli bir gösterge olduğu belirlendi.

Anahtar kelimeler: Erken tanı, PMN elastaz, C-reaktif protein, sepsis

[1]Department of Pediatrics, Bakırköy Maternity and Children Diseases Training and Research Hospital, İstanbul, Turkey

[2]Department of Pediatrics, Başkent University Hospital, İstanbul, Turkey

[3]Department of Hematology, Yüksek İhtisas Hospital, İstanbul, Turkey

[4]Department of Pediatrics, Haseki Research and Training Hospital, İstanbul, Turkey

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Correspondance/Yazışma
Dr. Ayşe Ayaz Özkul, Başkent University, Faculty of medicine, İstanbul Health Research and Application Center, Kısıklı Cad. Oymacı Sok. No:7, 34662 Altunizade, İstanbul, Turkey
Phone: +90 216 554 15 00
e.mail: drayseayaz@hotmail.com

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Introduction

Neonatal sepsis, characterized by systemic signs of infection in the first month of life, remains an important clinical syndrome (1). Despite the advances in perinatal and neonatal care and use of newer potent antibiotics, the incidence of neonatal sepsis remains high and the outcome is still severe (2).

Early diagnosis before obvious clinical signs of illness, is an important goal but is difficult for various reasons: Firstly, early signs and symptoms are nonspecific then there is the difficulty of distinguishing the clinical picture of neonatal sepsis from non-infectious causes (1-3). Abnormal hematological counts, acute-phase reactants, and inflammatory cytokines are neither sensitive nor specific, especially at the onset of illness. Further, microbiological culture results are not usually available until at least 48-72 hours after the specimen reaches the laboratory, and high false-negative rates of culture results may occur (3-5). Thus rapid diagnostic tests that differentiate infected from non-infected infants, have the potential to make a significant impact on neonatal care.

Polymorphonuclear (PMN) granulocytes play an important role as primary defence in the inflammatory reactions. This includes the release of cytokines, activation of PMN, and activation of plasma protein cascade systems such as the complement and contact phase systems (6-8). There is evidence suggesting that PMN are involved in the pathogenesis of sepsis and multiple organ dysfunction syndromes (9-12). Release of interleukin-8 (IL-8), which is strongly chemotactic for PMN and induces the expression of adhesion molecules on endothelial cells, may encourage activated PMN to adhere to endothelial cells, thereby inducing endothelial damage (6, 13).

Polymorphonuclear granulocytes use proteinases to digest these agents and tissue debris. One of these proteinases is PMN elastase, which is localized in the azurophilic granules of the polymorphonuclear granulocytes (14-17). Elastase is considered to be one of the most potent proteolytic enzymes present in azurophilic granules of neutrophils. It plays an important physiological function in degrading phagocytosed substances and facilitating cell migration through vascular walls. Excessive amounts of free elastase, unbound to natural inhibitors present in systemic fluids, may result in the enzyme becoming hazardous for the surrounding tissues. Elastase degrades mainly the essential elements of the interstitium (elastin, collagen, proteoglycans), but it also, as a non specific enzyme, causes decomposition of plasma transport proteins, proteinase inhibitors, blood coagulation factors, immunoglobulins and the basal membrane of renal glomeruli. A particular role is played by elastase in the pathogenesis of chronic respiratory diseases, as it damages the ciliary epithelium and enhances mucus production (13-18).

The aim of the study was determine the sensitivity and specificity of PMN elastase levels for the diagnosis of neonatal sepsis and investigate its role in determining the efficacy of treatment and the prognosis of the disease.

Material and Methods

This prospective study was performed on newborns who were hospitalized for neonatal sepsis at the Neonatal Intensive Care Unit, Pediatric Department, Haseki Training and Research Hospital, Istanbul, Turkey. The protocol was approved by the local ethics committee. Informed written consent was obtained from parents of all the patients.

Forty newborns were diagnosed as suspected clinical sepsis based on their Hematological Score and Töllner Score (19) results (Hematological Score ≥3 and Töllner Score ≥10). Clinical signs of sepsis were defined as the presence of three or more of the following categories of clinical signs: apnea, tachypnea (>60/min), respiratory distress, hypotonia, bradycardia (<100/min), tachycardia (>180/min), seizures, change in skin colour and perfusion, irritability, and lethargy. Two or more abnormal values of the sepsis screen were considered as supportive for diagnosis of infection. The control group included twenty neonates who were admitted to the hospital with non-infectious diseases, such as hypoglycemia, indirect hyperbiluribinemia, intrauterine growth retardation, transient tachypnea, without clinical findings of infection.

Before starting antibiotic therapy, blood samples for blood culture, routine biochemistry, whole blood count, peripheral blood smear, immature neutrophil: total neutrophil (I/T) ratio, C-reactive protein (CRP) and PMN elastase were taken. Chest radiograph, cerebrospinal fluid culture and urine culture were done whenever clinically indicated. Antibiotics were commenced after the blood specimens were collected. Second blood samples for sepsis markers were obtained from these patients on the 4th day of treatment.

Complete blood counts were carried out with an automatic counter. Leukocytosis was considered if the white blood cell (WBC) count was more than 25000/mm^3 and leukopenia less than 5000/mm^3, and thrombocytopenia less than 100000/mm^3. By examining peripheral blood smears prepared with Giemsa stain, band forms, myelocytes and metamyelocytes in leukocyte formula evaluated as immature neutrophils and I/T ratio were calculated. Pathologic I/T ratios were greater than 0.2. Specimens of blood were obtained from each infant by a sterile technique and were inoculated into commercially-prepared BD Bactec Peds Aerobic/F vials (Peti-Bact blood culture, Organon Technica). Positive cultures were detected by chemical sensors sensitive to increases in carbon dioxide produced by growth of the organisms. The organisms were then identified based on gram staining and growth on agar media.

Blood samples were obtained on the 1st and 4th days, their serum extracted and CRP investigation was made immediately, remaining serum was preserved for PMN elastase evaluation. Blood samples that were obtained from the control group and from neonates with sepsis were centrifuged at 2500 x g for 15 min and the serum portion was preserved by freezing at -20°C for PMN elastase studies.

C-reactive protein level was measured by the Behring Nephelometer 100 Analyzer BN II (NY, USA). CRP levels ≤6 mg/L were accepted as normal. Blood analyses were carried out in the bacteriology and biochemistry laboratories of Haseki Training and Research Hospital (Istanbul, Turkey).

Polymorphonuclear elastase levels were measured with an enzyme-linked immunosorbent assay (ELISA) with PMN elastase study kits (BMS 269-Austria). The samples were studied in the hematology laboratory of Türkiye Yüksek İhtisas Education and Research Hospital (Ankara, Turkey).

Statistical analysis

The Statistical Package for Social Sciences for Windows version 11.0 (Sigma Stat; SAS Institute Inc.) was used for statistical analysis. The results were given as mean±standard deviation. Comparisons between the two groups were analyzed by the unpaired Student's t-test for normally distributed (parametric) data and Mann-Whitney U test for non-parametric data. P value less than 0.05 was considered to be statistically significant. To determine a diagnostic value for PMN elastase and CRP in newborns, a ROC curve was constructed for each sampling point. Sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) were calculated at different selected cut-off values for each marker and for combinations of markers. The Wilcoxon test was used for the interpretation of the difference between at time of diagnosis and after therapy.

Results

In this study, 40 neonates with sepsis (Sepsis Group) and 20 healthy neonates (Control Group) were investigated. Characteristics of the participants are shown in Table 1 and Table 2. There were no significant differences in gender and birth place between the two groups (chi square test; p>0.05). In addition, there were no significant differences between the groups with respect to mean gestational age, age and birth weight (Student's t-test; p>0.05). The age of onset of sepsis ranged from day 2 to day 30. Except for one infant, all others presented at more than 72 hours of age. Therefore, ü the distinction of early and late sepsis has not been made.

Fourteen patients had positive blood culture in the neonatal sepsis group. Staphylococcus epidermidis (n=5, 12.5%), Klebsiella oxitoca (n=4, 10%), Klebsiella pneumoniae (n=2, 5%), Staphylococcus

Table 1. Characteristics of the sepsis group and control group

		Neonatal Sepsis Group		Control Group		χ^2	p
		n	%*	n	%*		
Gender						2.12	>0.05
	Male	26	65	13	65		
	Female	14	35	7	35		
Place of birth						2.12	>0.05
	Home	10	25	5	25		
	Hospital	30	75	15	75		

*percentage of rows

Table 2. Demographic characteristics of the sepsis group and control group

	Sepsis group		Control group		t*	p
	Mean	SD	mean	SD		
Gestational age (week)	39.5	1.2	39.8	0.8	1.045	>0.05
Birth weight (gr)	3147.0	423.3	3149.0	456.0	0.865	>0.05
Age (day)	5.98	4.33	7.00	5.30	3.757	>0.05

*Student's t-test

Table 3. Causative organisms of neonatal sepsis

	n	%*
Gram positive bacteria	8	20
Staphylococcus epidermidis	5	12.5
Staphylococcus hemolyticus	2	5
Group B streptococcus	1	2.5
Gram negative bacteria	6	15
Klebsiella oxytoca	4	10
Klebsiella pneumoniae	2	5
Total	14	35

*percentage of rows

Table 4. Comparison of PMN elastase and CRP levels between culture-proven and culture negative sepsis

	Culture-proven sepsis (n=14)		Culture-negative sepsis (n=26)		t	p
	Mean	SD	Mean	SD		
PMN elastase	153.89	39.02	137.93	15.77	1.29	0.219
CRP	38.85	34.31	29.00	23.28	1.00	0.335

PMN: Polymorphonuclear, CRP: C-reactive protein

hemolyticus (n=2, 5%), Group B streptococcus (n=1, 2.5%) were isolated from blood culture (Table 3). Two patients had meningitis; no pathogen was isolated from the cerebrospinal fluid (CSF), however, Klebsiella oxitoca and, the other coagulase-negative Staphylococci were present in their blood samples (Table 3). There was no significant difference in PMN elastase or CRP levels between proven sepsis and culture-negative sepsis (Student's t-test; p>0.05) (Table 4).

Polymorphonuclear elastase levels, CRP levels, I/T ratio, white blood cell count and platelet count in the Sepsis and Control Groups are shown in Table 5. We found that mean serum PMN elastase levels, CRP levels and I/T ratio were significantly higher in the Sepsis Group than those in the Control Group (Student's t-test; p<0.001). No significant difference was found in WBC count or platelet count between the groups (Student's t-test; p>0.05).

The specificity, sensitivity, negative predictive value (NPV) and positive predictive value (PPV) were identified using ROC analysis for the first day. As shown from Table 6, the highest specificity (96.3%), sensivity (90.3), NPV (93.5) and PPV (95.1%) were found for PMN elastase levels in prediction of sepsis. The sensitivity of CRP in detecting sepsis was 90.3%, its specificity was 91.2%, its positive predictive value was 89.7% and its negative predictive value was 89.7%. IT ratio had a high specificity and PPV, but lower sensitivity and NPV. In determining sepsis, platelet count was found to be the lowest sensivity and NPV.

Serum values of CRP and PMN elastase before treatment and at the 4th day of treatment are shown in Table 7. Before treatment, serum PMN elastase and CRP levels were significantly higher than levels at the fourth day of antibiotic treatment, showing a significant recovery with antibiotic in the Sepsis Group

In 7 patients having normal CRP levels of the Sepsis Group (<5 mg/dL), PMN elastase levels were higher than the control group (157.42±23.78, t=17.51, p=0.000). In the control group, none of PMN elastase levels was high, while ten newborns had raised CRP levels (≥5 mg/dL).

Discussion

In this study, 40 septic neonates and 20 healthy neonates were investigated to evaluate the value of serum PMN elastase and CRP in determining early diagnosis and prognosis of neonatal sepsis. It was established that mean serum PMN elastase and CRP values before treatment were significantly higher in septic neonates compared with healthyones, and PMN elastase had high sensitivity and specificity in the early diagnosis of neonatal sepsis. PMN elastase detection was particularly valuable in determining early diagnosis and the prognosis of the disease.

Neonatal sepsis is still a leading cause of mortality in neonatal intensive care units all over the world. Early diagnosis and treatment of the newborn infant with suspected sepsis are essential to prevent severe and life threatening complications (3). Since the symptoms and findings are nonspecific, neonatal sepsis diagnosis is quite difficult. There is a great need for new diagnostic laboratory methods for the early diagnosis of the disease and the evaluation of prognosis and treatment efficacy.

Clinical manifestations are nonspecific and laboratory parameters such as WBC count or I/T ratio are of limited value in identifying infected newborns (20). Blood culture is the gold standard laboratory technique for the diagnosis of infection, but culture results may

Table 5. Comparison of the laboratory findings in the sepsis (n=40) and control groups (n=20)

	Sepsis Group		Control Group		t	p
	Mean	SD	Mean	SD		
PMN elastase (ng/mL)	145.07	34.67	75.5	9.81	8.754	0.000
CRP (mg/L)	26.95	26.83	6.20	2.67	3.436	0.000
I/T neutrophil (%)	0.26	0.12	0.09	0.05	6.338	0.000
WBC (/mm^3)	16642	7455	13970	7556	1.303	0.101
Platelets (/mm^3)	230975	139479	196430	108331	0.970	0.428

PMN: Polymorphonuclear, CRP: C-reactive protein, WBC: White blood cell

Table 6. Specificity, sensitivity, negative predictive value (NPV) and positive predictive value (PPV) for PMN elastase, CRP, I/T Neutrophil, white blood cell, and platelet count

	Specificity (%)	Sensitivity (%)	NPV (%)	PPV (%)
PMN elastase (ng/mL)	96.3	94.6	93.5	95.1
CRP (mg/L)	90.3	91.2	91.1	89.7
I/T neutrophil (%)	93.2	80.5	82.7	93.0
WBC (/mm^3)	88.2	60.3	64.8	89.7
Platelets (/mm^3)	87.7	58.0	59.5	87.0

WBC: White blood cell, NPV: Negative predictive value, PPV: Positive predictive value

Table 7. PMN elastase and CRP levels of the sepsis group before treatment and at 4th day of antibiotic therapy

	Before treatment	4th day	t	p*
PMN elastase (ng/mL)	145.07±34.67	99.15±25.45	9.34	0.000
CRP (mg/L)	26.95±26.82	22.88±19.95	6.35	0.008

*Wilcoxon test, PMN: Polymorphonuclear, CRP: C-reactive protein

take 48-72 hour, and culture positivity rates range from 8% to 73% (21). False negative culture may also occur (3). As a consequence, appropriate diagnosis and therapy could be delayed, worsening the prognosis of the patient (21).

For years, investigators have searched for a test or panel of tests able to identify septic neonates accurately and rapidly while awaiting culture results, in order to obtain an early diagnosis and develop a specific effective treatment for a successful outcome. When high mortality of neonatal sepsis is taken into consideration, it is desirable for the ideal diagnostic tests with maximal sensitivity and negative predictive value. To minimize the unnecessary use of antibiotics in false-positive cases, a diagnostic marker also needs to have reasonably high specificity and a good PPV (22).

Hematological parameters have been evaluated in previous studies. WBC count, total neutrophil count, immature neutrophil count, I/T ratios, platelet count are the indices most commonly used. These hematological counts and ratios showed a limited accuracy with wide range of sensitivity (17-90%) and specificity (31-100%), due to the relatively long period necessary to become positive and the significant influence of non-specific factors. However, I/T ratio of >0.2 may reach a sensitivity of 90% and negative predictive value of 98% (22). In this study, WBC and platelet count showed low detection sensitivity, specifity, NPV and PPV in neonatal infection ($p>0.05$), and I/T ratio failed to reach an appropriate sensitivity and NPV in neonatal sepsis.

Since the end of the 1980s, CRP has been routinely investigated and used for the diagnosis of neonatal sepsis. CRP is an acute phase protein synthesized by the liver within 6-8 hr in response to inflammatory cytokines, peaks at 24-48 hr, and then diminishes rapidly after elimination of the source (23). Elevated levels of CRP are present during bacterial, viral and other infections, and in non-infectious inflammatory diseases and malignancies (22). In variable studies using CRP ≥1 mg/dL as the cut-off value, the range of reported statistical outcomes is as follows: sensitivity 70% to 93%; specificity 41% to 98%; positive predictive accuracy 6% to 83%; and negative predictive accuracy 97% to 99% (23). On the other hand, quantitative CRP values, particularly when repeated, are highly specific and have good sensitivity. In addition, serial measurements can be helpful in monitoring the response to treatment. In spite of the reduced early sensitivity, CRP still remains the preferred index in most neonatal intensive care units (22). In this study CRP levels on the first day were significantly higher than control groups ($p<0.001$). After antibiotic treatment CRP levels were decreased by the fourth day ($p<0.01$). However, in seven infants, PMN elastase levels were measured high while CRP levels were normal. The increase in the serum levels of CRP is rather slow during the first 12-24 hours of infection, and this may cause false negative results. In addition, increase in CRP levels in non-infected clinical conditions (trauma, surgical intervention, recent vaccination, prolonged rupture of membranes, fetal distress, perinatal asphyxia, intraventricular hemorrhage, meconium aspiration, burns and malignancies) can cause a false positive test. In our study, in the control group PMN elastase levels were normal in all of the cases. On the contrary, ten newborn in the control group also had raised CRP levels. This increase in CRP concentrations in the control group is thought to affect the specificity of the test. Our findings suggest that PMN elastase is more sensitive and specific than CRP.

In the past few decades, acute phase proteins, cytokines, adhesion molecules, chemokines, cell surface markers, complement system components, and combinations of these were investigated for the early and reliable diagnosis of the neonatal sepsis. Results of different published studies in relation to IL-1β *and* TNF-α are contradictory. Publishing data regarding TNF-α is alsocontradictory. Some studies found the diagnostic utility of this cytokine, while others demonstrated similar or even lower levels in infected newborns compared to healthy newborns (24-27).

Polymorphonuclear elastase is a serine protease stored in the azurophilic granules of neutrophils and secreted by neutrophils during inflammation (13, 15, 17). The exact role PMN elastase in early detection of neonatal sepsis was not well investigated. Investigations, performed mainly in adults, are still rare in pediatric patients. There are numerous studies indicating that neutrophils play an important

role in the pathogenesis of neonatal sepsis and multiple organ failure (28-31).

Various published studies have shown PMN elastase to be a useful marker of early infection in the newborn. Tsaka et al. (28) showed that septic newborns had significantly increased PMN elastase levels at the time of recognition of infection. Jensen et al. (29) and Wojsky et al. (30) found that PMN elastase is higher in septic than in nonseptic newborns. In this study, it was detected that PMN elastase levels of newborns with sepsis were significantly higher than controls ($p<0.001$). Lawskoska et al. (31) also found that in full-term neonates cord blood neutrophil elastase is a good marker of infection.

Wojsky et al. (30) reported that the sensitivity and a specifity of serum PMN elastase in the early diagnosis of neonatal sepsis were 76%, 81%, respectively. In our study, the sensitivity of PMN elastase in the diagnosis of neonatal sepsis was 91.2%, specificity was 96.3%, PPV was 95.1% and NPV was 93.5%. CRP results for sepsis were 91.2% sensitivity, 90.3% specifity, 89.7% PPV and 91.1% NPV. The results of this study showed that the sensitivity, specifity, PPV and NPV of the PMN elastase were high for early diagnosis of neonatal sepsis. Our findings suggest that PMN elastase is an almost perfect marker and more sensitive and specific than CRP in the diagnosis of neonatal sepsis. However, lack of correction for reference ranges for neonatal PMN elastase values may influence the outcome of PMN elastase as a marker for bacterial infection. In addition, methodological difficulties in detecting PMN elastase and the absence of their routine usage in all centers have limited its use in daily practice.

Another characteristic of the markers that are used in the diagnosis of neonatal sepsis is that it gives information about the prognosis of the disease and helps in coming to a decision as to whether to stop or continue antibiotic treatment. In this study, it is found that PMN elastase levels were statistically decreased in newborns after 4^{th} day of therapy compared to newborns at the time of diagnosis ($p<0.001$). Similar results have been obtained by Tsaka et al. (28). When blood samples obtained before treatment and on the 4^{th} day of treatment both from patients recovered and from those who died were compared, it was observed that mean serum PMN elastase values were significantly decreased in recovered patients during the treatment. On the other hand, mean serum PMN elastase levels were detected to significantly increase in the deceased patients. With these findings, it is observed that PMN elastase is a useful marker in determining the prognosis of the disease and treatment efficacy.

Conclusion

Delay in commencing antibiotic therapy in an infant with sepsis could result in serious consequences. Over-treatment of neonates with antibiotics based on false positive results will promote the emergence of multi-resistant bacteria in the neonatal intensive care unit. We conclude that PMN elastase is an important marker in the diagnosis of neonatal sepsis, and this marker is also valuable in following the effectiveness of treatment and determining the prognosis of the disease. When used with CRP and clinical signs, it will be a useful tool for helping doctors to decide whether antibiotics should be started or withheld at the onset of symptoms in newborns, while awaiting results of the blood culture. However, it is thought that these findings need to be confirmed by means of future studies examining different categories of infections and larger number of neonates.

Conflict of interest
No conflict of interest was declared by the authors.

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Authors' contributions: Conceived and designed the experiments or case: AAO, MOP, ME. Performed the experiments or case: MOP, AAO, SA, EA, ME. Analysed the data: AAO. Wrote the paper: AAO, MOP. All authors have read and approved the final manuscript.

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Yazarlar herhangi bir çıkar çatışması bildirmemişlerdir.

Hakem değerlendirmesi: Bağımsız hakemlerce değerlendirilmiştir.

Yazar katkıları: Çalışma fikrinin tasarlanması: AAO, MOP, ME. Deneylerin uygulanması: MOP, AAO, SA, EA, ME. Verilerin analizi: AAO. Yazının hazırlanması: AAO, MOP. Tüm yazarlar yazının son halini okumuş ve onaylamıştır.

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