

Aim: To determine induced sputum cell counts and interleukin-8 (IL-8), tumor necrosis factor alpha (TNF- α) and leukotriene B₄ (LTB₄) levels as markers of neutrophilic inflammation in moderate persistent asthma, and to evaluate the response to inhaled steroid therapy.

Methods: Forty-five moderate asthmatic patients and 10 non-smoker controls were included in this study. All patients received inhaled corticosteroid (800 μ g of budesonide) for 12 weeks. Before and after treatment pulmonary function tests were performed, and symptom scores were determined. Blood was drawn for analysis of serum inflammatory markers, and sputum was induced.

Results: Induced sputum cell counts and inflammatory markers were significantly higher in patients with asthma than in the control group. The induced sputum eosinophil counts of 12 patients (26%) were found to be less than 5%, the non-eosinophilic group, and sputum neutrophil counts, IL-8 and TNF- α levels were significantly higher than the eosinophilic group (neutrophil, $50 \pm 14\%$ versus $19 \pm 10\%$, $p < 0.01$). In both groups, there was a significant decrease in sputum total cell counts and serum and sputum IL-8, TNF- α and LTB₄ levels after the treatment. There was no change in sputum neutrophil counts. Although the sputum eosinophil count decreased only in the eosinophilic subjects, there was no significant difference in inflammatory markers between the groups. The symptom scores were significantly improved after treatment, while the improvement did not reach statistical significance on pulmonary function test parameters.

Conclusion: Notably, in chronic asthma there is a subgroup of patients whose predominant inflammatory cells are not eosinophils. Sputum neutrophil counts and neutrophilic inflammatory markers are significantly higher in these patients. In the non-eosinophilic group, inhaled steroid caused an important decrease in inflammatory markers; however, there was no change in the sputum eosinophil and neutrophil counts.

Key words: Asthma, Sputum eosinophilia, Neutrophilic inflammation, Interleukin-8, Tumor necrosis factor alpha

Introduction

Eosinophils, mast cells and T lymphocytes are involved in the pathogenesis of airway inflammation in asthma.¹ However, there is an increasing recognition of non-eosinophilic, especially neutrophilic, forms of airway inflammation in asthmatic patients.^{2,3} Prominent neutrophilic inflammation has been demonstrated in severe refractory asthma, fatal asthma, exacerbation periods, occupational asthma and nocturnal asthma.^{4–8}

Recently, heterogeneity of airway inflammation was reported in persistent asthmatics.⁹ Since corticosteroids are the cornerstone in asthma treatment, it

Inhaled corticosteroid effects both eosinophilic and non-eosinophilic inflammation in asthmatic patients

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is important to identify whether the presence of neutrophilic inflammation affects the response to inhaled steroids in asthmatic patients.

The pathogenetic mechanism of neutrophil recruitment in the airways is not well understood. It has not been clarified whether neutrophils are the main part of the inflammatory process, especially in chronic and severe asthmatics, or whether it is a consequence of the steroid treatment, since it is known that steroids inhibit the apoptosis of neutrophils and increase their survival in the airways.¹⁰

Several inflammatory markers have been investigated and found to be associated with chemotaxis and accumulation of neutrophils in the airways of

patients with asthma and chronic obstructive pulmonary disease (COPD). The most important and the most widely investigated markers include interleukin-8 (IL-8), tumor necrosis factor alpha (TNF- α) and leukotriene B₄ (LTB₄).

In this study, we speculated that the presence of non-eosinophilic inflammation in the asthmatic airways might be associated with IL-8, TNF- α , and LTB₄ levels, and it might affect the response to inhaled steroid. We used induced sputum as a novel method of evaluating airway inflammation and investigated the effect of inhaled steroid on induced sputum cell counts and IL-8, TNF- α , and LTB₄ in eosinophilic and non-eosinophilic asthmatics.

Materials and methods

Patients

Asthma was diagnosed by a history of recurrent wheezing and chest tightness, and was confirmed by reversible airway obstruction suggested by >12% or 200 ml of improvement in FEV₁ (forced expiratory volume in one second) after 200 μ g of salbutamol inhalation.¹ Forty-five patients classified as moderate persistent asthma according to GINA (Global Initiative for Asthma) guidelines volunteered to participate in the study. Ten age-matched non-smokers were used as the control group. The inclusion criteria for the study group were; FEV₁ or PEF (peak expiratory flow) >60% but <80% predicted, presence of daily asthma symptoms, non-smoker or ex-smoker for more than 5 years, no exacerbations in the past 3 months and no history of cardiopulmonary disease other than asthma. Patients were excluded from the study if they failed to meet the inclusion criteria, had inadequate sputum despite three induction procedures on separate days, had received systemic corticosteroids during the previous 6 weeks or had clinical evidence of a respiratory tract infection. The study was approved by the medical ethics committee of Kocaeli University School of Medicine and written informed consent was obtained from all participants.

Study design

All selected patients ceased using their current medications but was allowed to use an inhaled short-acting beta-2 agonist as needed during a 15-day run-in period. All patients received 800 μ g/day of inhaled budesonide (Pulmicort turbuhaler 2 \times 400 μ g; AstraZeneca, Lund, Sweden) for 12 weeks. Symptom scores were recorded, spirometry was performed, blood was drawn for measurement of serum inflammatory markers and sputum was induced by inhalation of hypertonic saline solution before and after the treatment period. Patients were classified into two

groups as eosinophilic (E) and non-eosinophilic (NE) according to their baseline sputum eosinophil counts. The effects of corticosteroid on the outcome measurements were compared between the groups. The study design is shown in Fig. 1.

Sputum induction

The sputum was induced as described by Pin *et al.*¹¹ All subjects were pretreated with 200 μ g of salbutamol administered by metered dose inhaler (Volumatic). For the induction process, a Pulmo-Aide (Brooklyn, NY, USA) ultrasonic nebulizer with an output of 0.35 ml/min and a particle size of 5 μ m was used and 3% hypertonic saline was nebulized. The nebulization time consisted of 5-min intervals until a maximum nebulization time of 30 min. PEF was measured after each period of inhalation. Subjects were asked to rinse their mouth and swallow the water and blow the nose to minimize contamination with saliva and postnasal drip. They were then encouraged to cough sputum into a sterile container. The procedure was continued until either a sufficient amount of sputum was obtained or the maximum nebulization time of 30 min was reached.¹²

Sputum processing

The sputum samples were processed within 2 h according to the validated protocol by Popov *et al.* with modifications.¹³ The volume of induced sputum was determined and mixed with an equal volume of 1% sputalysin (dithiothreitol; Sigma, Milan, Italy) freshly diluted to 0.1% by the addition of distilled water. The mixture was incubated at room

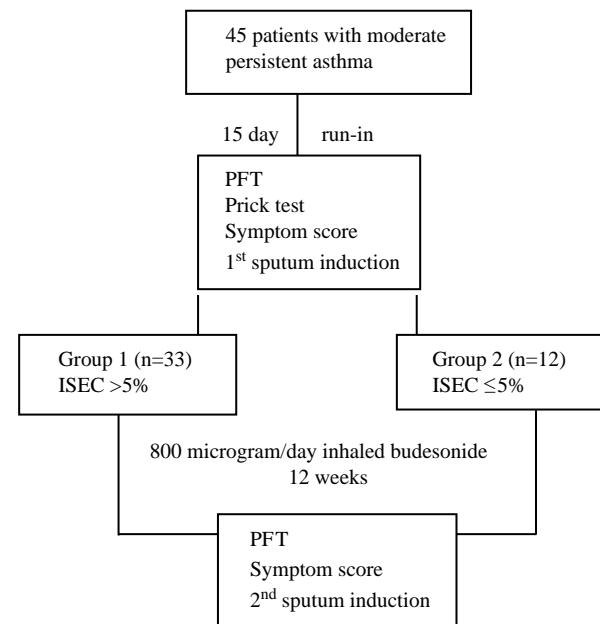


FIG. 1. Study design. PFT, pulmonary function test; ISEC, induced sputum eosinophil count.

temperature for 20 min, and during this time vortexed every 5 min to ensure homogenization and maximize cell dispersion. To stop the effect of dithiothreitol on the cell suspension, an equal volume of phosphate-buffered saline was added. The mixture was then centrifuged at 1500 rpm for 10 min. Supernatants were aspirated and stored at -70°C for later analysis of inflammatory markers. The cell pellets were resuspended with phosphate-buffered saline to obtain a final volume of 2–5 ml, then filtered thorough a gauze (pore size approximately 1 mm) to remove mucus and cell debris.

The total cell counts were performed in a hemocytometer (Thoma, Marienfeld GmbH, Baden-Württemberg, Germany). The cell suspension was adjusted to 1×10^6 cells/ml and cytopsin slides were prepared by using 50 μl of the cell suspension (Model 3 cytopsin; Shandon Scientific, Sewickley, PA, USA). Slides were air-dried and stained by May-Grünwald-Giemsa; 200–400 non-squamous cells were counted by the blinded investigator (cytopathologist). If >80% of the cells consisted of squamous cells, the quality of the sputum sample was judged to be unsatisfactory and excluded from the analysis.

Fluid phase measurements

The IL-8 and TNF- α levels in the supernatant of induced sputum were assessed by enzyme-linked immunosorbent assay (CytELISA; Cytimmune Sciences Inc., Rockville, MD, USA). The results were expressed as picograms per liter and adjusted for the dilution factor. For IL-8 the sensitivity was 25 pg/ml, the intra-assay precision was 0.6–2% and the inter-assay precision was 1.96–6.13%. For TNF- α the sensitivity was 4.8 pg/ml, the range of detection was 15.6–1000 pg/ml, the intra-assay variation was $\pm 8.3\%$ and the inter-assay variation was $\pm 10.8\%$. LTB₄ levels were also measured by enzyme immunoassay (DRG International Inc., Mountainside, NJ, USA) after prior purification of C18 columns (Altech, Los Altos, CA, USA). The sensitivity was 19.4 pg/ml,

the intra-assay precision was 5.9–6.8% and the inter-assay precision was 5–16.5%.

Blood was collected in tubes (Becton Dickinson, Franklin Lakes, NJ, USA, Vacutainer SST) for measurement of serum inflammatory markers and incubated at room temperature for 60–120 min. They were then centrifuged at $1300 \times g$ for 10 min. The serum was aspirated and stored at -20°C for later analysis of serum IL-8, TNF- α and LTB₄ levels.

Pulmonary function tests

Spirometry was performed using a Sensormedics (Yorba Linda, CA, USA) Vmax 20C. FEV₁, FVC, FEV₁/FVC (forced vital capacity) and VC (vital capacity) were measured. PEF was recorded using a peak flow meter before each sputum induction procedures.

Symptom score

The total symptom scores were recorded by using a questionnaire including day-night asthma symptoms, daily beta-2 agonist use and cough frequency.¹⁴

Statistical analysis

All statistical analysis was performed using the SPSS program. Data were expressed as means \pm standard deviation. Between-group-change outcome variables were determined by non-parametric Mann-Whitney U-test. Within-group-change outcome variables were compared with the Wilcoxon paired test. Symptom scores were determined by chi-squared analysis. $P < 0.05$ was considered significant.

Results

In total, 45 patients met the inclusion criteria and were enrolled. Three of them were excluded because of an exacerbation during the run-in period. The clinical characteristics of the study population are presented in Table 1.

Table 1. The clinical characteristics of the study population

	Eosinophilic	Non-eosinophilic	Control
<i>n</i>	33	12	10
Sex (male/female)	4/29	2/10	3/7
Age (years)	56.3 ± 15	54 ± 11.5	$45 \pm 7^*$
Disease duration (years)	10.3 ± 6	$19 \pm 10.4^*$	—
Atopy	14 (42%)	4 (33%)*	0 [†]
FEV ₁ (% predicted)	75.3 ± 22.4	73.4 ± 16.4	$95 \pm 12^{\dagger}$
FVC (% predicted)	84 ± 18.4	84 ± 11	$96 \pm 15^{\dagger}$
FEV ₁ /FVC (%)	67.3 ± 11	64 ± 8	$87 \pm 9^{\dagger}$
Ex-smokers (smoking pack-years)	5 (14 ± 7.5)	4 (18 ± 9)	0

* $p < 0.05$ eosinophilic versus non-eosinophilic subjects.

† $p < 0.05$ control versus asthmatic subjects.

Non-eosinophilic subjects were defined, as subjects with baseline sputum eosinophil counts less than 5%. Both clinical and functional features of E and NE patients were compared. Age, sex and pulmonary function tests were similar between the groups. Duration of the disease was found to be longer in the NE group than in the E group ($p < 0.05$) and NE patients were likely to be non-atopic. There were no current smokers in the study population and the prevalence of ex-smokers was similar between the groups (Table 1).

Sputum cell counts

When compared with normal subjects, there was an increase in baseline sputum total cell, eosinophil and neutrophil counts in asthmatic patients. Among asthmatic patients, sputum total cell counts and neutrophil counts were found to be higher in the NE subjects than in the E subjects (Table 2). Other cell types were similar between the groups.

After the treatment period the E subjects had a significant decrease in sputum eosinophil counts while there was no change in the NE subjects (Table 3 and Fig. 2). Also, no decrease was found with inhaled corticosteroid treatment on sputum neutrophil counts of either NE or E groups (Table 3, Fig. 2).

The intensity of the sputum cellularity, as reflected by the total cell counts were significantly decreased in both groups (Table 3).

Sputum and serum inflammatory markers

Both sputum and serum inflammatory markers were significantly higher in the subjects with asthma compared with the normal subjects (Table 2). Subjects with NE asthma had higher sputum and serum IL-8, TNF- α levels and higher sputum LTB₄ levels than those with E asthma.

Although inhaled corticosteroid caused a significant reduction in these inflammatory markers, this decrease did not reach statistical significance between the groups (Table 3).

Clinical and functional measurements

Baseline symptom scores and pulmonary functions were similar between the groups. After the treatment period, all patients experienced significant improvements in symptom scores. FEV₁ levels increased with treatment, although the increase was not statistically significant either in NE or in E asthma.

Tolerability

Sputum induction was well tolerated. Four patients required more than one induction procedure to obtain sufficient amount of sputum. None of the patients quit the treatment. Three patients were excluded from the study during the run-in period because of an exacerbation.

Discussion

In this study we have demonstrated that the non-eosinophilic form of a moderate persistent asthma is associated with neutrophilic inflammation and increase in IL-8, TNF- α and LTB₄ levels.

The non-eosinophilic form has been identified even in stable asthmatic patients. It is now accepted that there is at least two distinct patterns of airway inflammation in asthma. Jatakanon *et al.* have demonstrated increased neutrophil numbers and IL-8 levels in severe persistent asthmatics.⁴ Besides disease severity, Pavord *et al.* have found that non-eosinophilic inflammation was associated with smoking and atopy, since the NE patients in their study group were more likely to be non-atopic and current smokers.¹⁵ NE asthma was also shown to be associated with age and disease duration.⁹ In our study, we found that neutrophilic inflammation is related with disease duration and atopy. Together these data suggest that older, non-atopic patients with a more severe airway obstruction and longer disease duration tend to have non-eosinophilic airway inflammation.

Besides these clinical parameters several inflammatory markers have been thought to be associated with non-eosinophilic inflammation in asthma.

Table 2. Comparison of baseline induced sputum cell counts and inflammatory markers in asthmatics and controls

	Eosinophilic	Non-eosinophilic	Control
Total cell count ($\times 10^6/g$)	1.12 \pm 0.5*	1.75 \pm 0.7	0.3 \pm 0.2†
Eosinophil (%)	27.5 \pm 14*	4 \pm 2.3	1.4 \pm 1.3†
Neutrophil (%)	20 \pm 10.3*	50 \pm 15	27 \pm 14
Lymphocyte (%)	23 \pm 18	18 \pm 10.4	22 \pm 13.3
Macrophage (%)	30 \pm 17.2	28 \pm 13.4	49 \pm 17.4†
IL-8 (pg/ml)	573 \pm 173*	862 \pm 289	227 \pm 12†
TNF- α (pg/ml)	440 \pm 120*	624 \pm 128	219 \pm 49†
LTB ₄ (pg/ml)	13 \pm 9*	18 \pm 9	0.84 \pm 0.4†

* $p < 0.05$ eosinophilic versus non-eosinophilic subjects.

† $p < 0.05$ control versus asthmatic subjects.

Table 3. Comparison of before and after treatment-induced sputum cells and inflammatory marker levels in eosinophilic and non-eosinophilic subjects

	Eosinophilic			Non-eosinophilic		
	Before treatment	After treatment	p	Before treatment	After treatment	p
Total cell count ($\times 10^6/g$)	1.12 ± 0.5	0.5 ± 0.3	0.03*	1.75 ± 0.7	0.8 ± 0.4	0.01*
Eosinophil (%)	27.5 ± 14.2	16.4 ± 7	0.01*	4 ± 2.3	3.6 ± 2.5	0.70
Neutrophil (%)	20 ± 10.3	18.6 ± 11.2	0.06	50 ± 15	50 ± 13.5	0.20
Induced sputum						
IL-8	573 ± 173	466 ± 118	0.01*	862 ± 289	645 ± 174	0.01*
TNF- α	440 ± 120	357 ± 105	0.01*	624 ± 128	473 ± 117	0.02*
LTB ₄	13 ± 9.3	4.5 ± 3.2	0.00*	18 ± 9	6 ± 4	0.00*
Serum						
IL-8	292 ± 133	241 ± 141	0.05*	328 ± 129	237 ± 84	0.00*
TNF- α	294 ± 102	242 ± 89	0.00*	391 ± 110	271 ± 97	0.00*
LTB ₄	1.3 ± 0.5	0.6 ± 0.4	0.00*	1.3 ± 0.6	0.9 ± 0.5	0.00*

*p < 0.05, statistically significant.

Among these markers the most widely investigated is IL-8, which is a potent neutrophil chemoattractant and activator.¹⁶ It is likely to be involved in the recruitment of neutrophils in the airways of patients with COPD.^{17,18} It is also shown to correlate with the sputum neutrophil counts in patients with asthma.⁹ Chalmers *et al.* have demonstrated high sputum IL-8 levels in asthmatic smokers and suggested that neutrophilic inflammation in asthma is associated with smoking.¹⁹ However, no current smokers were included and the prevalence of ex-smokers was similar between the groups in our study. This finding suggests that only smoking history is not enough to explain the high levels of sputum IL-8 levels in some asthmatic patients.

TNF- α has been demonstrated both in COPD and asthma.¹⁸ It is derived from macrophages, mast cells or other inflammatory cells.²⁰ In asthma, TNF- α is known to upregulate the expression of cell adhesion molecules. We found that an increased level of TNF- α

is associated with neutrophil counts. Similarly previous studies have demonstrated the correlation between neutrophil counts and TNF- α levels, so it is possible to suggest that TNF- α is one of the important inflammatory markers, which are responsible for neutrophil recruitment in the airways.

LTB₄ is a lipid mediator that is thought to play an important role in the pathogenesis of COPD. Cysteinyl leukotrienes are found to increase in induced sputum samples of asthmatic patients.²¹ However, there are restricted data about the role of LTB₄ in airway inflammation in asthma. Recently, Montuschi and Barnes reported increased LTB₄ levels in the exhaled breath condensate of mild asthmatics compared with normal healthy controls.²² In our study, we found increased LTB₄ levels in all of the study population when compared with controls and the higher levels in NE asthma. Previous studies have demonstrated that the LTB₄ levels and neutrophil counts were associated in patients with COPD.^{16,23} Future studies are needed to determine the effects of LTB₄ in the pathogenesis of asthma.

Our results supported the previous data that reported heterogeneity in airway inflammation in asthma. Since we did not include the severe and mild persistent asthmatics, it is difficult to clarify the association between disease severity and neutrophilic inflammation. However, based on previous data it is possible to suggest that non-eosinophilic inflammation is more common than thought and it is even observed in mild asthmatics. The inflammatory markers discussed previously might be the source of neutrophil recruitment in the airways. However, it is still unclear why these markers are involved in airway inflammation in some asthmatics while others have characteristic features of eosinophilic inflammation.

Possible explanations for neutrophilic inflammation in asthma include the effects of corticosteroids, smoking, respiratory tract infection and atopy. Since the prevalence of ex-smokers was similar between the

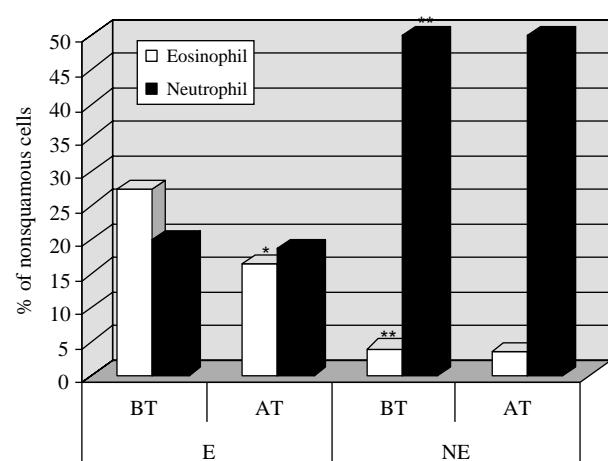


FIG. 2. Induced sputum eosinophil and neutrophil percentages, comparison of before treatment (BT) and after treatment (AT) levels in eosinophilic (E) and non-eosinophilic (NE) subjects. *p < 0.05 before versus after treatment, **p < 0.05 eosinophilic versus non-eosinophilic.

study groups, we thought that smoking was not an important explanation for our study population. Similarly, all inhaled corticosteroids withheld during the run-in period, we also thought that the neutrophil-predominated inflammation in our study is not due to corticosteroid treatment. Although we excluded the patients who had clinical evidence of a respiratory tract infection, we could not exclude the possibility of subclinical or viral infection of the lower respiratory tract; infection is less likely to be a possible explanation in this study. Similar with the previous reports, we found that neutrophilic inflammation was higher in non-atopic asthmatics than in the atopic subjects. Also the duration of the disease was different between the groups. Finally, we thought that non-atopic patients with chronic asthma were more likely to have non-eosinophilic inflammation.

Since corticosteroids are the main agents in asthma treatment and there is some evidence that neutrophilic inflammation is associated with a poor response to steroids, we also investigated the effects of inhaled steroids on non-eosinophilic airway inflammation.

Pavord *et al.* investigated the effects of inhaled steroids on spirometric measurements, symptom scores and induced sputum eosinophil counts in eosinophilic and non-eosinophilic asthma.¹⁵ They found that NE asthma is associated with a poor response to inhaled steroid and suggested that sputum eosinophil count is an important factor in determining the response to inhaled steroids in asthma. Green *et al.* have reported similar findings; they observed a significantly less improvement in VAS (visual analogue symptom) scores and FEV₁ levels in the neutrophilic subgroup compared with the eosinophilic subjects after 2 months treatment with inhaled budesonide.²⁴ On the other hand, in a recently published article Godon and coworkers suggested that sputum eosinophilia is not always a good indicator of a poor response to inhaled steroids.²⁵ They investigated the effects of fluticasone propionate on 51 mild uncontrolled, steroid-naïve asthmatics and found that symptoms, quality of life, FEV₁ and PC₂₀ (provocative concentration 20) were improved both in eosinophilic and non-eosinophilic asthmatics.

In our study, eosinophilic and non-eosinophilic subjects were balanced with respect to improvement in symptom scores and FEV₁ levels. Induced sputum total cell counts, IL-8, TNF- α and LTB₄ levels decreased significantly with the treatment and there was no significant difference between the groups.

Previous studies have reported a decrease in IL-8 levels after corticosteroid treatment. This finding is known to be associated with the suppression of IL-8 secretion from airway epithelial cells.²⁶ It was demonstrated that TNF- α plays an important role in determining the severity of airway hyperreactivity in

asthma and it was also shown to decrease with the treatment of inhaled steroids.^{27,28}

According to our knowledge this is the first study that demonstrates a significant decrease in sputum LTB₄ levels with steroid treatment. It is thought that steroid cannot inhibit the synthesis of lipid mediators.²³ However, we cannot identify the mechanism of the decrease in LTB₄ levels. It might be due to the inhibition of phospholipase enzyme activation by steroids.

In our study, we have found that response to inhaled steroids is similar both in non-eosinophilic and eosinophilic asthma. The most important insufficiency in our study is the lack of the parameters of eosinophil activity. Although we have shown that the sputum eosinophil count decreased significantly in the eosinophilic group, we could not determine whether eosinophilic inflammation parameters were similar between the groups. On the other hand, we have determined that IL-8, TNF- α and LTB₄ levels were associated with neutrophil recruitment in the asthmatic airways and it is possible to suppress their levels with steroid treatment.

In conclusion, we have suggested that there is a non-eosinophilic subgroup in moderate asthma, but their response to inhaled steroids is not significantly different from those who have eosinophilic inflammation.

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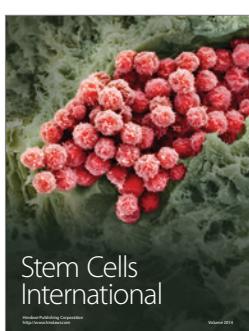
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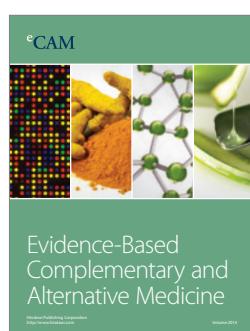
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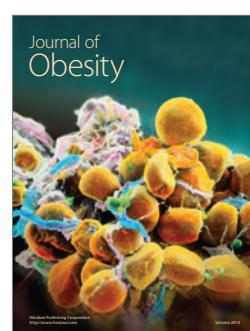
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