MMDJ
MEANDROS MEDICAL AND DENTAL JOURNAL

March 2026 27(1):58-68

Meandros Medical and Dental Journal
doi: 10.69601/meandrosmdj.1662094

Research Article

THE EFFECTS OF FOLIC ACID SUPPLEMENTATION ON NOVEL BIOMARKERS OF THE ENDOTHELIAL FUNCTION IN RATS WITH EXPERIMENTAL PERIODONTITIS

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Received: *21 March 2025*
Revised: *14 July 2025*
Accepted: *23 September2025*
Published: *24 March 2026*

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ABSTRACT

Objective: The aim of this study is to investigate the effect of folic acid (FA) supplementation on endothelial damage caused by periodontitis.

Materials and Methods: 48 rats were divided randomly into five groups: healthy control, sham control, lipopolysaccharide-induced periodontitis (LPS), lipopolysaccharide-induced periodontitis with low-dose FA (4 mg/kg) (LPS+4), and high-dose FA (12 mg/kg) (LPS+12) administered groups. Rats were euthanized after 15 days of the first lipopolysaccharide injection. Circulating progenitor cells taken from each rat's blood samples were counted using flow cytometry. ADMA, CD34+, and CD133+ expression in periodontal and cardiac tissues were labeled by using immunoperoxidase method.

Results: There were no differences among the groups regarding ADMA expression in periodontal tissues. However, the LPS group exhibited the highest expression levels of CD34+ and CD133+, which were significantly elevated compared to those in the healthy group. Statistically significant differences in all biomarkers were observed among the groups in the cardiac tissue. Flow cytometry analysis results did not differ in ADMA expression level, but CD34+ and CD133+ expression levels were statistically significant between groups.

Conclusion: The results of our study suggest that periodontitis induces an increase in the expression of endothelial biomarkers, while folic acid supplementation exerts a beneficial effect on periodontitis-associated endothelial dysfunction.

Keywords: Cardiovascular disease, folic acid, experimental periodontitis

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INTRODUCTION

Periodontitis is a chronic inflammatory disorder that has been reported to be independently correlated with arteriovascular disease. This association has been attributed to several biological mechanisms, including endothelial dysfunction, recurrent low-level bacteremia originating from periodontal tissues, and a sustained systemic inflammatory response. Together, these mechanisms are thought to contribute to vascular impairment by exposing the endothelium to inflammatory mediators and microbial products over time (1).

Several clinical and experimental studies have demonstrated that periodontal treatment leads to improvements in endothelial function, supporting the concept that periodontal inflammation may exert reversible effects on the vascular endothelium (2). Endothelial dysfunction represents an early and critical event in the development of atherosclerosis and cardiovascular disease and has traditionally been assessed using biomarkers such as intercellular adhesion molecule (ICAM), components of the nitric oxide–endothelial nitric oxide synthase (NO–eNOS) system, reactive oxygen radicals, tissue plasminogen activator, plasminogen activator inhibitors, and selectins. Although these markers provide valuable information regarding vascular inflammation and thrombogenic activity, they remain insufficient to fully explain the complex processes involved in endothelial damage, repair, and ongoing recovery (3, 4).

In recent years, increasing attention has been directed toward novel circulating biomarkers that more specifically reflect endothelial injury and regenerative capacity. Circulating progenitor cells (CPC), asymmetric dimethylarginine (ADMA), endothelial microparticles (MPs), circulating endothelial cells (CECs), and endothelial progenitor cells (EPCs) have been identified as sensitive indicators of endothelial dysfunction and vascular homeostasis (5). These progenitor and endothelial cell populations play a crucial role in vascular repair mechanisms and are actively involved in responses to endothelial and endocardial injury through systemic circulation (6). Accordingly, the evaluation of these biomarkers may provide more valid and reliable information regarding the pathogenesis of periodontitis and its systemic complications, particularly in distant vascular tissues, when compared with traditional endothelial markers (7).

Folic acid (FA) is an essential vitamin involved in numerous physiological processes, including DNA synthesis, methylation reactions, and cellular regeneration. Within periodontal and other tissues, FA contributes to functional events that are critical for tissue integrity and repair (8). Clinical studies have demonstrated that dietary FA supplementation improves endothelial function in patients with coronary artery disease, indicating a potential protective role against vascular dysfunction (9). However, the precise mechanisms underlying the beneficial effects of FA on the vascular endothelium remain unclear. Moreover, there are currently no studies evaluating the effects of FA supplementation on the newly described endothelial biomarkers, nor its potential role in reducing systemic and cardiovascular complications associated with periodontitis.

Therefore, the present study aimed to investigate whether FA administration exerts a positive effect on endothelial damage and cardiovascular system complications in experimental periodontitis by assessing newly identified and more specific endothelial biomarkers.

MATERIALS AND METHODS

The experimental protocol employed in this study was reviewed and approved by the Ethics Committee of XXX University (Report no =12/02-12/33). All procedures adhered to the Guide for the Care and Use of Laboratory Animals and ARRIVE guidelines.

The sample size calculation was based on the assumption of a significant difference exceeding 1.5 times the standard deviation (SD) in the alveolar bone loss parameter, with Type I (α) and Type II (β) error rates set at 5% and 20%, respectively. As a result, the minimum required sample size was determined to be six animals per group. The experiment was conducted with forty-eight 6-month-old male albino Wistar rats weighing 230–250 g, who were fed a selected solid diet and received water ad libitum over a 12-h light/dark cycle at temperatures between 22°C and 24°C. One cage was set for each group. Periodontitis was created by injecting lipopolysaccharide (LPS) into the gingiva in LPS+4, LPS+12, and LPS groups (EO). The rats were distributed into five groups:

- LPS+4 Group (n=12): Periodontitis+FA low-dose (4 mg/kg);
- LPS+12 Group (n=12): Periodontitis+FA high-dose (12 mg/kg);
- LPS Group (n=12): Periodontitis;
- Healthy Control (HC) Group (n=6): No treatment was given, and the same care and nutrition were provided;
- Sham Control (SC) Group (n=6): Gingiva was injected with sterile phosphate-buffered saline (PBS) solution (10µl).

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In groups LPS+4 and LPS+12, each rat was gavaged with FA orally and daily for 14 days (NE). Experimental periodontitis was induced by injecting a total of 10 μL (1 mg/mL) of LPS derived from Escherichia coli (dissolved in PBS solution, 1 mg/mL, strain 055:B5; Sigma Co., St. Louis, MO, USA) into the palatal gingiva between the first and second maxillary molars, and into the labial and oral gingiva of the central incisors in both the maxilla and mandible, using a microsyringe. Injections were performed under inhalation anesthesia three times per week for a period of 15 days. In the SC group, the same protocol was followed, except that sterile PBS was injected instead of LPS. The HC group received no injections but was maintained under identical care and nutrition conditions.

Blood samples were obtained on the 15th day following the first LPS injection. Rats were euthanized by 10 mg pentobarbital injection intramuscularly per the ARRIVE guidelines, and systemic necropsy was performed (HTA). 2.1 The collection of tissue, tissue fixation, and processing After systemic necropsy, gingival tissue samples and heart were collected. Tissues were fixed in 10% buffered formalin for 48-76 hours. Following the routine tissue processing, all tissues were embedded in paraffin, and 4-5 μm thick sections were collected. Sections were stained with hematoxylin and eosin and examined histopathologically under a trinocular light microscope (Olympus BX51 and DP25 digital camera, Shinjuku, Tokyo, Japan).

Immunoperoxidase examinations

The commercial indirect immunoperoxidase streptavidin/biotin immunoperoxidase technique was used to demonstrate the immunopositivity of ADMA, CD34, and CD133 antigens in tissues. Sections were deparaffinized with xylene and rehydrated using absolute alcohol, 95% and 70% alcohol, and water, respectively, for 10 minutes. Endogenous peroxidase activity was removed in 3% hydrogen peroxide for 5 minutes, boiling with citrate buffer (pH 6.0) for 20 minutes, or with 0.1% proteinase-K at 37°C, digestion was applied, and was treated with protein blocking serum for 5 minutes. # Then, sections were incubated with monoclonal anti-ADMA, anti-CD34, and anti-CD133 primary antibodies at room temperature for 50 min, after, 10 min secondary antibody step and 10 min streptavidin step were done. An aminoethyl carbazole chromogen/substrate set (Labvisio Corp., Fremont, CA, USA) was used for 15 minutes for the color reaction. Mayer's hematoxylin was used for counterstain, and sufficient aqueous mounting medium was applied to cover the section. In each test, mouse kidney, liver, and skin tissues were used as positive control for ADMA, CD34, and CD133 immunohistochemistry. Also, the mice's non-immunized serum was used as an isotype antibody control.

Flow cytometry analysis

2 ml of blood samples were collected from the jugular vein of each of the rats under deep anesthesia. Leucocytes were separated from the erythrocytes for counting CD34+ and CD133+ cells and incubated with related primary antibodies for one hour. Then, the origin of the species of primary antibodies was marked with a specific FITC-labeled anti-antibody and taken into a fluorescence-activated cell Sorting solution. Counts were made in a flow cytometry instrument (BDFACS AriaII, San Jose, California, USA).

Quantitative analysis of immunopositive cells and statistics

The quantitative analysis of immunopositive cells was used as the primary outcome, and flow cytometry analysis was used as the secondary outcome measurement. The tissue distribution of antigens in immunoperoxidase tests was quantitatively determined as positive-stained immunopositive cells. At high magnification, images were obtained from five representative fields, and sequential microscopic fields at 20× objective magnification were captured using the Olympus DPW-DS2 image analysis software.

Statistical analysis was conducted by calculating the mean and standard deviation (mean ± SD) to describe the immunopositive staining area percentage. The proportion of stained area (% pixels) relative to the total field was calculated, and the average staining area for each slide was determined based on percentage pixels. Statistical analysis of ADMA, CD34+, and CD133+ immunohistochemical results were compared between groups using the Kruskal-Wallis test. Pair-wise comparisons were performed by comparison of the Mann-Whitney U test after the analysis was performed with assumptions of normality and homogeneity of variance. All statistical analyses were performed using SPSS Statistics 22 software (SPSS IBM, Turkey).

RESULTS

Experimental periodontitis model

The experimental periodontitis model was successfully established in the LPS, LPS+4, and LPS+12 groups via repeated gingival LPS injections. However, two rats in the LPS+4 and LPS groups and one rat in the LPS+12 group

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failed to recover from deep anesthesia and were excluded from the study. Consequently, a total of 45 rats were included in the final analysis, and their blood and tissue specimens were collected for further evaluation.

Histopathological findings in the gingival tissue

Histopathological examination of the LPS group revealed marked vascular dilatation and increased vascular permeability within the submucosal blood vessels. The perivascular connective tissue displayed a loosely organized architecture with interstitial separation due to edema and signs of hyalinization. In the periodontal region, prominent inflammatory infiltrates composed predominantly of neutrophilic granulocytes and macrophages were observed along the gingival sulcus. Multifocal aggregates of inflammatory cells were also detected in the soft palatal mucosa and alveolar bone, indicative of widespread inflammation. Notably, dense neutrophilic infiltration was observed extending toward the cemento-enamel junction and into the gingival epithelium, accompanied by areas of epithelial necrosis.

In the LPS+4 group, marked vasodilation extending into the periodontal tissues and palatal mucosa was evident, particularly around inflammatory foci, where capillaries were engorged with numerous erythrocytes. Compared to the LPS group, this group exhibited reduced inflammatory cell infiltration; however, intense neutrophilic accumulation and clusters of bacteria progressing toward the mucosal surface, oral cavity, and gingival sulcus were observed in two specimens. In these regions, the gingival tissue adjacent to the enamel margin presented with a distinct pit-like morphological alteration.

In the LPS+12 group, advanced hyperemia and vascular edema were noted, accompanied by a relatively milder inflammatory response compared to the LPS and LPS+4 groups. The extent of inflammatory cell infiltration was notably less severe in this group.

No significant pathological alterations were observed in the HC group, aside from mild hyperemia and occasional inflammatory cells in a few specimens. In the SC group, localized hyperemia and focal hemorrhages were detected at the PBS injection sites.

Immunoperoxidase test

Each antigen was evaluated by histomorphometric analysis, and the percentage of the immunopositive area was quantified for each specimen. Immunoreactivity was predominantly observed in regions of intense inflammation, within the vascular walls, and along the surfaces of endothelial and epithelial cells. Representative microscopic images of periodontal tissue sections stained for CD34+, CD133+, and ADMA are presented in Figures 1–3. The mean values of these markers across the experimental groups are summarized in Table 1.

Statistical analysis revealed a significant difference in CD34+ expression among the groups in periodontal tissues ($p < 0.05$). The highest CD34+ expression was observed in the LPS group, while the lowest was noted in the healthy control group. CD34+ levels in both the LPS+4 and LPS+12 groups were significantly reduced compared to the LPS group ($p < 0.05$). No statistically significant differences were found between the SC group and either the LPS+4 or LPS+12 groups. However, CD34+ expression in the LPS+12 group was significantly lower than that in the LPS+4 group ($p < 0.05$) (Figure 1).

Table 1. Statistical distribution of $CD34^+$, $CD133^+$, and ADMA markers among study groups in periodontal tissue, heart tissue, and flow cytometry analyses. Data are presented as mean ± standard deviation (SD).

		Healthy Control	Sham Control	LPS	LPS+4	LPS+12
Periodontal Tissues	**CD34+**	4,99± 0,86	8,43 ± 0,81*	16,85 ± 8,28*,†	9,71 ± 2,44*,‡	6,91± 2,33*,‡,§
	CD133+	6,86± 3,71	8,08± 0,98	12,26 ± 4,38*,†	11,12 ± 2,95*,†	7,80± 3,11‡,§
	ADMA	4,39 ± 1,08	6,91 ± 2,18	6,83 ± 2,18	5,91 ± 1,55	5,33± 1,86
Heart Tissues	**CD34+**	6.29± 0.51	4.75 ± 1.22*	8.44 ± 1.35*,†	7.42 ± 1.15*,†	7.49 ± 0.99*,†
	CD133+	5.44 ± 1.12	6.20 ± 1.20	8.12 ± 1.85*	7.49 ± 1.36*	4.81 ± 0.85†,‡,§
	ADMA	7.84 ± 1.36	5.87 ± 1.24	6.23 ± 1.04	7.66 ± 1.08†,‡	4.88 ± 1.14*,‡,§
Flow Cytometry	**CD34+**	41,24 ± 3,87	51,98 ± 3,87*	50,55 ± 3,57*	38,17 ± 6,01†,‡	45,78 ± 13,2§
	CD133+	37,38 ± 2,99	45,92 ± 4,15*	43,28 ± 3,82*	30,82 ± 5,64*,†,‡	43,79 ± 6,53§
	ADMA	27,26 ± 1,18	31,5 ± 8,80	28,26 ± 4,33	24,52 ± 3,05	29,72 ± 3,42

*p< 0.05 Statistically significant difference with healthy control group., †p< 0.05 Statistically significant difference with the sham control group, ‡p< 0.05 Statistically significant difference with the LPS group, §p< 0.05 Statistically significant difference with LPS+4

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doi: 10.69601/meandrosmdj.1662094

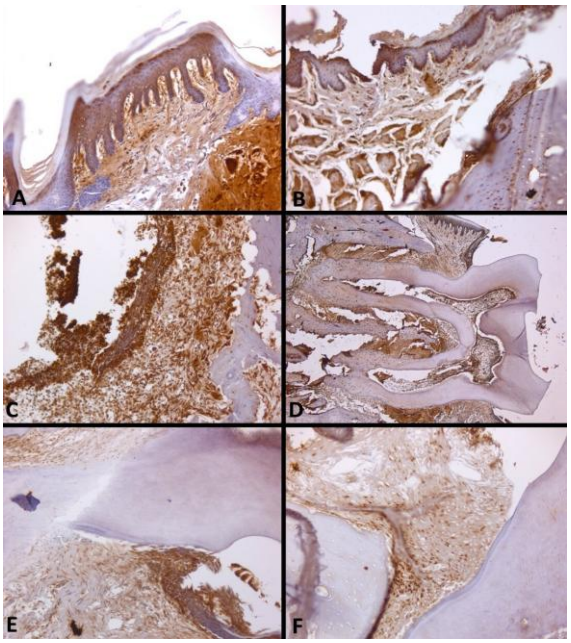


Figure 1. Microscopic images of periodontal tissue sections stained with CD133+ antibody. A: Sham Control Group, B: Healthy Control Group, C, D: LPS Group E: LPS+4 Group, F: LPS+12 Group.

When CD133+ expression was evaluated in the periodontal tissues, the highest levels were observed in the LPS group. This increase was statistically significant compared to all other groups, except for the LPS+4 group ($p < 0.05$). CD133+ levels in the LPS+12 group were lower than those in the SC, LPS, and LPS+4 groups, yet higher than in the HC group. While significant differences were detected between the LPS and LPS+4 groups, the difference between the SC and HC groups was not statistically significant ($p < 0.05$) (Figure 2).

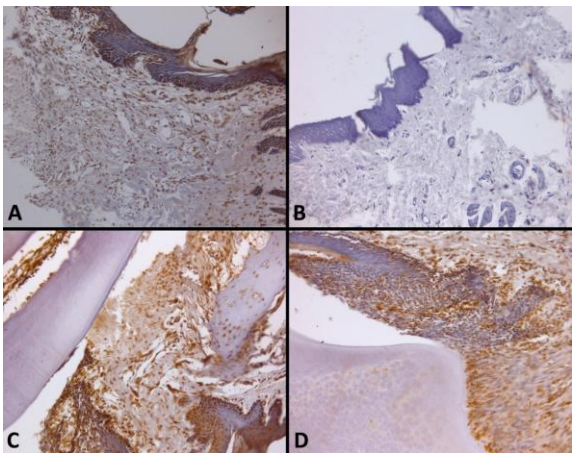


Figure 3. Microscopic images of periodontal tissue sections stained with CD34+ antibody. A: Sham Control Group, B: Healthy Control Group, C: LPS Group, D: LPS+4 Group.

Regarding ADMA expression in periodontal tissues, no statistically significant differences were noted among the experimental groups ($p > 0.05$). However, numerically, the highest ADMA values were recorded in the SC, LPS, LPS+4, LPS+12, and HC groups, respectively (Figure 3).

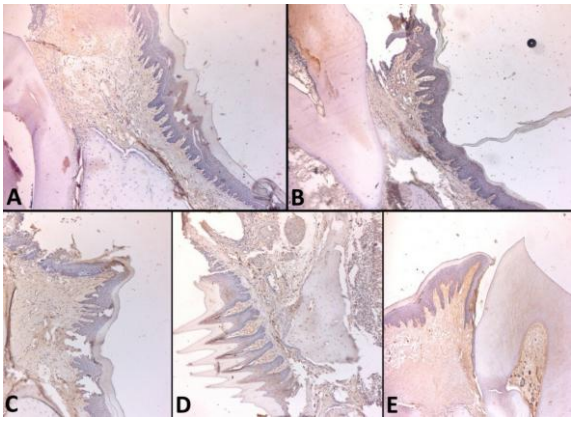


Figure 2. Microscopic images of periodontal tissue sections stained with ADMA antibodies. A: Sham Control Group, B: Healthy Control Group, C: LPS+4 Group, D: LPS Group, E: LPS+12 Group.

The comparative levels of CD34+, CD133+, and ADMA markers in heart tissues are summarized in Table 1. Representative immunohistochemical images of heart tissue sections stained with anti-CD34, anti-CD133 antibodies and ADMA antibody are presented in Figure 4.

Upon evaluation of CD133+, CD34+, and ADMA—biomarkers indicative of endothelial dysfunction—in heart tissues, a statistically significant difference in CD34+ expression was found between the HC and SC groups ($p < 0.05$). Additionally, both CD34+ and CD133+ levels were significantly higher in the LPS group compared to the HC group ($p < 0.05$). Although all markers, except ADMA, exhibited peak levels in the LPS group, no statistically significant differences were observed between the HC and LPS groups for ADMA ($p < 0.05$) (Figure 4) (Table 1). Furthermore, CD34+ and CD133+ levels in the HC group were significantly lower than those observed in the LPS+4 group ($p < 0.05$). In the LPS+12 group, CD34+ levels were significantly elevated compared to the HC group, while ADMA levels were significantly reduced ($p < 0.05$). Notably, CD34+ expression in the LPS group was also significantly higher than in the SC group ($p < 0.05$) (Table 1).

In the LPS+4 group, both CD34+ and ADMA levels were significantly elevated compared to the SC group ($p < 0.05$). In the LPS+12 group, CD34+ levels were significantly

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higher, whereas CD133+ levels were significantly lower than those in the SC group ($p < 0.05$) (Table 1).

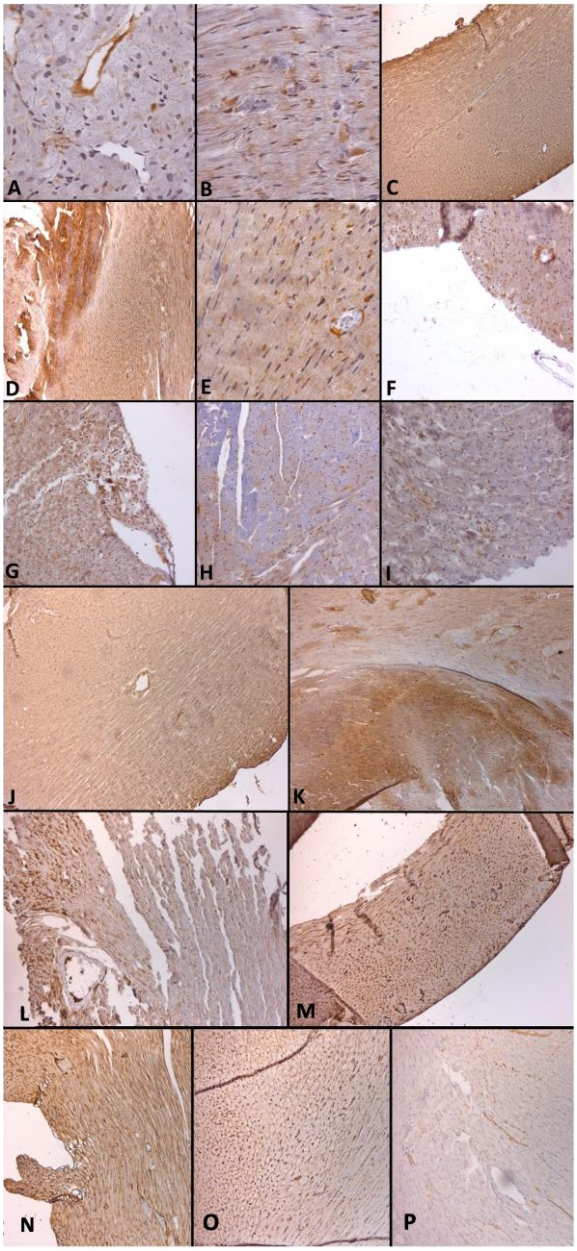


Figure 4. Microscopic images of the heart tissue sections stained with $CD34^+$, $CD133^+$ and ADMA antibodies. A: healthy control group $CD34^+$, B: healthy control group, $CD133^+$, C: Sham control group $CD34^+$, D: Sham control group $CD133^+$, E: LPS group $CD34^+$, F: LPS group,$CD133^+$, G: LPS+4 group $CD34^+$, H: LPS+4 group $CD133^+$, I: LPS+12 group $CD34^+$, J: LPS+12 group $CD133^+$, K: LPS+12group $CD133^+$, L: Healthy control group ADMA, M: Sham control group ADMA, N: LPS group ADMA, O: LPS+4 group ADMA, P: LPS+12 group ADMA.

When compared with the LPS group, the LPS+12 group exhibited significantly reduced levels of CD133+ and ADMA ($p < 0.05$). Conversely, ADMA levels in the LPS+4 group were significantly higher than those in the LPS group ($p < 0.05$). Additionally, both CD133+ and ADMA levels were significantly lower in the LPS+12 group than in the LPS+4 group ($p < 0.05$) (Table 1).

Flow cytometry analysis

In each flow cytometric analysis, 10,000 events were recorded, and the proportion of positively stained cells was expressed as a percentage of total events.

Statistical data regarding the expression of the three antigens are presented in Table 1. CD34+ expression was significantly higher in the LPS group compared to the HC group ($p < 0.05$). Although the difference between the SC and LPS groups was not statistically significant, CD34+ levels in the SC group were higher than those in the HC group. The LPS+4 group demonstrated significantly lower CD34+ expression compared to all other groups, except the HC group ($p < 0.05$). A statistically significant difference was also noted between the LPS+4 and LPS+12 groups, with higher CD34+ levels observed in the LPS+12 group ($p < 0.05$). No significant differences were found between the LPS and LPS+12 groups; however, CD34+ levels in the LPS+12 group were numerically lower than those in the LPS group (Table 1).

Analysis of CD133+ expression revealed that the lowest levels were observed in the LPS+4 group. Statistically significant differences were detected between the LPS+4 group and all other groups ($p < 0.05$). CD133+ levels were significantly elevated in the LPS group compared to the HC group ($p < 0.05$). Similarly, the SC group exhibited higher CD133+ expression than the HC group. The LPS+12 group showed significantly higher CD133+ levels compared to the LPS+4 group ($p < 0.05$); however, no significant differences were found between the LPS+12 group and the remaining groups (Table 1).

Regarding ADMA expression, no statistically significant differences were noted among the experimental groups ($p > 0.05$) (Table 1).

DISCUSSION

The objective of the study was to investigate the effect of FA supplementation on the endothelial function of an experimental periodontitis model created in rats compared with the control groups. Our study hypothesized that protective FA treatment against

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systemic inflammation caused by periodontitis positively affects endothelial biomarkers. As a result, it has been observed that FA supplementation positively affects periodontal tissues and increased CD34+ and CD133+ levels in circulation due to periodontitis.

As is known, it has been proven with epidemiological studies that there is a strong association between periodontitis and cardiovascular disease. This relationship may be explained by many different potential biological mechanisms (4). Increased inflammatory activity plays the most critical role in the atherogenic process and endothelial dysfunction, leading to cardiovascular disease (10).

Briefly, periodontitis, a chronic oral infection, causes bacteria (or products) to enter the bloodstream. The bacteria activate host responses by several different mechanisms, and as a result, host immune responses may cause atheroma formation, maturation, and aggravation (11). In our study, under this information, in 3 cases of experimental periodontitis groups, histopathological changes have been found in heart tissue.

Also, in our study, it has been proven once again that periodontitis leads to systemic inflammation by increasing levels of circulating endothelial biomarkers CD133+ and CD34+ in the LPS group compared to the HC group and caused endothelial damage. Periodontal infection affect macro- and micro-endothelial cells by up-regulating levels of systemic inflammation and contributing to endothelial dysfunction, and as a result, may cause the initiation of atherosclerosis (12).

ADMA, CPCs, EPCs, CECs, and MPs are shown as new endothelial biomarkers (5), and they are believed to be more reliable in endothelial function assessment (3, 4). ADMA has a relationship with periodontal disease in a dose-dependent and is a serological marker of endothelial function (13). It exerts an inhibitory influence on the NO-eNOS system and plays a role in various regulatory mechanisms involved in vascular homeostasis. Additionally, it is strongly linked to endothelial dysfunction and cardiovascular events (14). In our study, we could not obtain a significant difference in immunohistochemistry and flow cytometry results associated with ADMA levels between LPS and the HC groups. This finding contrasts with other studies. A clinical investigation has demonstrated that oxidative stress and pro-inflammatory cytokines elevate ADMA levels, thereby contributing to endothelial dysfunction (15). Interestingly, despite ADMA being widely recognized as a biomarker of endothelial dysfunction, our study did not reveal statistically significant differences in ADMA levels across the experimental groups in either blood or tissue samples. This may be attributed to several biological and methodological factors. Notably, there is currently no published study directly examining ADMA levels in rats with experimental periodontitis. However, Nijvelt et al. reported significantly reduced ADMA concentrations in rats following intravenous LPS injection, attributing this to increased metabolic turnover of ADMA during endotoxemia (16). Their findings support the hypothesis that the enzyme dimethylarginine dimethylaminohydrolase (DDAH), responsible for metabolizing over 70% of ADMA, may be upregulated under inflammatory conditions (17). Indeed, in IL-1-stimulated vascular smooth muscle cells, inducible nitric oxide synthase (iNOS) expression has been shown to enhance DDAH activity, leading to decreased ADMA levels (18). Moreover, Isola et al. demonstrated that periodontitis alone did not significantly elevate serum or salivary ADMA levels in the absence of systemic comorbidities such as coronary heart disease (CHD) (19). These findings collectively suggest that in otherwise healthy animals, moderate inflammation associated with experimental periodontitis may not be sufficient to induce a measurable increase in ADMA, potentially due to compensatory degradation pathways or the need for a systemic disease background to amplify ADMA expression.

It is considered the other two markers evaluated in this study, CD34+ and CD133+, are commonly used cell surface markers to determine EPC, and CD34+ cells are more closely related to cardiovascular disease risk than those with biomarkers such as CRP (20). The number and function of circulating EPCs are associated with cardiovascular diseases, and this relationship reflects the repair capacity of damaged vascular tissue in the host. The number of EPCs is considered an indicator of endothelial dysfunction and cumulative cardiovascular risk (21). The flow cytometry results of our study are consistent with data obtained in other studies in terms of CD34+ and CD133+. Accordingly, systemic CD34+ and CD133+ levels were higher in the LPS group than in the HC group. Chronic periodontitis has been shown to be associated with elevated levels of EPCs in circulation in a similar study (22). In another study, periodontal treatment has been shown to significantly reduce the number of CD34+ cells in the circulation (7). In our study, the histopathological examination of CD34+ and CD133+ markers in periodontal tissues was different between groups. Both markers were also detected as the highest in

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the LPS group. These values, as expected, were higher in the heart tissue of the LPS group than those in the HC group, which confirms that periodontitis-induced systemic inflammation may cause injury to cardiovascular tissue.

FA treatment has been shown to positively affect endothelial function in many studies conducted so far (9). Our study observed that the levels of endothelial markers CD34+ and CD133+ were decreased in periodontal tissue in a dose-dependent manner in the LPS+4 and LPS+12 groups than in the LPS groups. Similarly, both high- and low-dose FA therapies have reduced CD34+ and CD133+ values in heart tissue compared to the LPS group. This difference for CD133+ was significant between the LPS and LPS+12 groups but not between the LPS and LPS+4 groups. These results indicate that FA supplementation can be a treatment option for preventing endothelial tissue damage induced by periodontitis. The impact of FA on vascular function, such as reducing vascular and thrombotic events, is formed by the reduction of elevated plasma homocysteine concentration, a risk factor for cardiovascular disease (9). Homocysteine can reduce the presence/availability of NO, the master regulator of endothelial homeostasis, by inducing the formation of ADMA, an enzymatic nitric oxide synthase inhibitör (23). It can be considered that FA reduces ADMA levels indirectly by reducing homocysteine levels. However, there was no difference among the groups regarding circulating ADMA levels in our study. Likewise, FA cannot change the ADMA levels in histopathological examination of the heart and the periodontal tissues. Only in the heart tissue ADMA levels have been shown to significantly reduce in the LPS+12 group compared to the HC and the LPS groups. Our study differs from a study that found FA and vitamin B12 supplementation have been shown to significantly decrease ADMA and homocysteine levels in patients with acute ischemic shock (24). Again, it was stated that FA and vitamin B12 supplementation reduced the plasma ADMA and homocysteine levels in patients with hypertension (25). In another study, FA treatment was applied to patients with coronary artery disease, and FA has been shown not to change the levels of ADMA (26). Existing different responses to treatment in different systemic situations or markers showing variability, such as EPCs and ADMA (22). Accordingly, the host generates more EPCs during atherosclerosis to compensate dysfunction of EPCs or contribute to potential endothelial repair in healthy individuals; however, this compensation cannot occur in patients with palpitation or coronary artery disease, and the EPC levels remain low. Initially, the beneficial effects of FA were considered to result from a reduction in plasma homocysteine concentrations. However, findings have shown FA to have many beneficial effects on vascular endothelial function, independent of changes in plasma homocysteine levels (9).

FA plays a role as an antioxidant, is a cofactor for eNOS, and stimulates eNOS directly (27). In many studies, FA supplements have been shown to reduce the risk of cardiovascular disease by improving endothelial function (9). Although folic acid supplementation led to a dose-dependent reduction in CD34+ and CD133+ expression in both periodontal and cardiac tissues, this relationship was not consistently reflected in peripheral blood levels. In our study, the circulating CD34+ and CD133+ levels have been shown to be significantly lower in the LPS+4 group compared with other groups. Low-dose FA supplementation has been shown to reduce systemic CD34+ and CD133+ levels in the LPS group compared to the HC group and has a beneficial role in repairing vascular damage. However, the same effect was not seen in rats given high doses of FA supplements. One possible explanation is that low-dose FA may cause modest reductions in certain circulating markers without producing a meaningful improvement in endothelial function. This phenomenon has been observed in clinical studies as well. For instance, Pullin et al. reported that low-dose FA (400 μg/day), despite reducing serum homocysteine levels, did not improve endothelial function in healthy adults, as measured by flow-mediated dilation (FMD) (28). In contrast, high-dose FA (⩾5 mg/day) was associated with significant improvements in FMD, even independently of homocysteine reduction (29). These findings suggest that certain biomarker changes may not fully reflect functional vascular outcomes, especially at lower doses. Therefore, future experimental studies should consider incorporating endothelial function tests such as FMD, in addition to molecular markers, to better assess the true physiological impact of folic acid supplementation.

Interestingly, CD34+ expression was significantly higher in the sham control group compared to the healthy control, despite the absence of any inflammatory stimulus. This may be attributed to mechanical stress induced by repeated gingival injections, which can lead to local microtrauma. CD34 is known to be a sensitive indicator of early endothelial activity and tissue remodeling (20). No such increase was observed in CD133+ or ADMA expression, supporting the specificity of this CD34+ response. Future studies are warranted to explore the relationship between CD34 expression and non-

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Meandros Medical and Dental Journal
doi: 10.69601/meandrosmdj.1662094

inflammatory mechanical factors, particularly in the context of experimental protocols involving repeated tissue interventions.

It is important to note that the biomarkers evaluated in this study—CD34+, CD133+, and ADMA—were assessed in distinct biological compartments, namely periodontal tissues, cardiac tissues, and peripheral blood. These tissues differ considerably in terms of cellular composition, vascular architecture, and immunologic environment. Such physiological differences may have influenced the magnitude and pattern of biomarker expression. Therefore, while the findings provide insight into the systemic impact of periodontitis and the potential modulatory effect of folic acid, direct quantitative comparisons between tissue types should be interpreted with caution.

One limitation of the present study is the absence of pre- and post-intervention measurements of serum or tissue folic acid levels. Without these assessments, it is not possible to determine the actual systemic folate status of the animals, which limits our ability to confirm the bioavailability of the administered folic acid and to establish a clear dose-response relationship. Additionally, all animals in the periodontitis and sham control groups received intraperitoneal injections of LPS or PBS, respectively, under anesthesia. The use of anesthesia may have influenced cardiac function and, consequently, altered systemic physiological responses, potentially confounding the interpretation of cardiovascular or inflammatory outcomes. Future studies should consider including serum folate level assessments to better correlate supplementation doses with physiological effects and to ensure accurate interpretation of folic acid–related outcome.

CONCLUSION

For the first time, the effect of FA on periodontitis-induced endothelial dysfunction has been evaluated in this study. As a result, FA has been shown to reduce the levels of CD34+ and CD133+ endothelial damage markers in both the heart and periodontal tissues and have beneficial effects on the modulation of endothelial function. In light of this knowledge, this vitamin may be recommended as an adjunct to periodontal therapy, especially for individuals at risk for cardiovascular disease. Periodontitis, independent of well-known risk factors for cardiovascular disease, increases the risk of future cardiovascular disease and is now very well known today . Therefore, in light of the data obtained in our study, FA supplementation is recommended as a protective agent for individuals with periodontal disease without cardiovascular disease.

In addition to the results obtained from our study, it has been further supported that CD34+ or CD133+ markers are reliable indicators showing periodontitis-induced endothelial damage. Consequently, our study's results have shown that periodontitis increases endothelial markers in periodontal tissue, heart tissue, and circulation, and FA supplements have a positive effect on the endothelial dysfunction induced by periodontitis.

Acknowledgments
The authors would like to express their sincere gratitude to all participants who contributed their time to this study and whose voluntary participation made this research possible. The authors acknowledge that a preliminary version of this study was presented as an E-Poster| Research Presentation at the EuroPerio 9 Congress (Amsterdam, 20–23 June 2018), and the abstract was published in the Journal of Clinical Periodontology, Volume 45, Issue S19 (Special Issue: Abstracts of EuroPerio 9), under the code PR074.

Authorship contributions
Concept: EO, OK, HTA Design: EO, OK, HTA Data Collection or Processing: EO, HTA, NE, TA Analysis or Interpretation: EO, HTA, NE, TA Literature Search: EO, OK, NE Writing: EO, HTA, NE

Data availibity statement
The authors report no conflicts of interest related to this study.

Declaration of competing interest
The authors report no conflicts of interest related to this study.

Ethics
The experimental protocol of the present study was approved by the Ethics Committee at Kirikkale University (Report no =12/02-12/33).

Funding
This study was supported by the grant TUBITAK (1001), Project Number:112S159

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MEANDROS MEDICAL AND DENTAL JOURNAL
March 2026 27(1):58-68
Meandros Medical and Dental Journal
doi: 10.69601/meandrosmdj.1662094

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Meandros Medical and Dental Journal
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