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The Possible Relationship Between Periodontitis and Alzheimer's Disease and the Beneficial Effects of Salvia Officinalis in an Experimental Rat Model: An Immunohistochemical Study

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Abstract

Objective

The present study aimed to investigate potential correlations between Alzheimer's disease (AD) and periodontitis in laboratory rat models with inflammation, the isoform of nitric oxide synthases (iNOS), sclerostin expression, and beneficial effects of *Salvia officinalis* (*S.officinalis*).

Material and Method

Eighty Wistar albino male rats were randomly divided into equal groups as controls (C), *S.officinalis* (S), periodontitis (P), and periodontitis+*S.officinalis* (PS), Alzheimer's disease (A), Alzheimer's disease+S. officinalis (AS), Alzheimer's disease+periodontitis (AP), and Alzheimer's disease+periodontitis+*S. officinalis* (APS) groups. While aluminum chloride (AlCl3) and d-galactose (D-gal) were intraperitoneally applied in an AD-like model, *S.officinalis* extract was administered by oral gavage. Sclerostin and iNOS expressions in periodontal tissues and amyloid-β (Aβ) in the hippocampus were evaluated together.

Results

Alveolar bone loss (ABL) was detected in Groups A and AS ($p<0.05$). There was no difference in ABL between Groups P and AP ($p>0.05$). iNOS and sclerostin expressions were detected in Group A. *S.officinalis* significantly decreased ABL, Aβ, and, in parallel, iNOS and sclerostin expressions in periodontal tissues in Groups PS and APS ($p<0.05$). The most marked sclerostin expression was observed in Group AP.

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Conclusion
Study findings revealed that periodontitis may increase Aβ in AD. Although ABL was not higher in Group AP than in Group P, the increase in iNOS and sclerostin expression in periodontal tissues in Group AP supports the pathogenetic association of AD with periodontitis. *S.officinalis* may be used as an immunomodulatory aromatic plant in the pathogenetic interaction of periodontal disease and AD.

Keywords: Alzheimer's disease, periodontitis, Salvia officinalis, iNOS, sclerostin.

Introduction

Bones play a vital role in the fundamental functions of the body (1). Bone emerges as a complex peripheral element able to communicate not only with peripheral organs but also with the brain, both indirectly through the peripheral nervous system and directly by releasing molecules that can cross the blood-brain barrier and act at the brain level (2). It has been reported that some proteins, such as osteocalcin, found in bone structure, cross the blood-brain barrier and have positive effects on anxiety, depression, and memory in Alzheimer's disease (AD) (3,4). Sclerostin, a glycoprotein, is released by osteocytes and inhibits osteoclastogenesis and bone formation by inhibiting the Wnt-β catenin pathway. Additionally, sclerostin keeps osteoclast-mediated bone resorption going by stimulating osteoblasts to produce the receptor activator of the NF-κB ligand (5). Sclerostin has pleiotropic effects, including the regulation of Wnt expression, synaptic plasticity, and memory, and is linked to the etiology of such neurodegenerative conditions as AD (1,6).

AD is a neurodegenerative health challenge leading to cognitive decline and dementia. Increased Amyloid -β (Aβ) and neurofibrillary tangle (NFY) levels are the main signs of AD (7). Inflammatory conditions are considered to contribute to the progression of AD. It was found that oxidative stress (OS) is an important factor initiating AD. It was reported that overexpression of reactive oxygen species (ROS) can induce the accumulation of Aβ, and there is an increase in the number of iNOS-positive neurons that accompany neuronal damage in AD patients (8). It was also pointed out that chronic inflammatory diseases, including periodontitis, could be of vital importance in the pathogenesis of AD (8).

Periodontitis, a long-term inflammatory condition, damages the alveolar bone (1). Dental plaque is a strong causative factor in the progression of the disease. The level of periodontal damage can be related to the produced inflammatory mediators, ROS, and nitrogen species (RNS), as well as environmental and genetic factors (9). The importance of OS in periodontal diseases, according to host and microbial stimulations, was revealed to activate ROS and RNS immediately (9). Nitric oxide (NO) plays a big role in the inflammatory reactions. (10). The nitric oxide synthase isoform (iNOS) is one of the variants of nitric oxide synthase (NOS). Studies revealed an increased level of iNOS in the inflamed periodontal tissues (10). Due to being a crucial negative regulator of bone formation, sclerostin prevents bone remodeling during periodontitis. Furthermore, an evidence-based study suggests that removing sclerostin appears to logically avoid resorption of the alveolar bone (11).

In recent years, several alternative treatment methods have been studied to inhibit OS, playing an important role in periodontitis and AD. *Salvia officinalis L.* (*S.officinalis*) is an aromatic plant known for its antioxidant, anti-inflammatory, and memory-enhancing effects with its high flavonoid and phenolic content (12). Based on the literature, pro-inflammatory cytokine and iNOS synthesis inhibition, anti-inflammatory, and antioxidant effects of various Salvia species have been determined (13). It has been shown that *S.officinalis* may provide therapeutic effects for periodontal infections (12). The present study, thus, aimed to evaluate the possible association between periodontitis and AD through its impact on periodontal tissues and hippocampus, and the beneficial effects of S.officinalis on the two diseases.

Material and Method

The present study was conducted with 80 Wistar albino rats randomly classified into eight groups: the controls (C), S.officinalis (S), periodontitis (P), periodontitis+S.officinalis (PS), Alzheimer's disease (A), Alzheimer's disease+S.officinalis (AS), Alzheimer's disease+periodontitis (AP), and Alzheimer's disease+periodontitis+*S.officinalis* (APS), in the Experimental Animal Production and Research Laboratory of Süleyman Demirel University, Isparta.

In the AD-like model, aluminum chloride (AlCl3) (Merck, Darmstadt, Germany) (10 mg/kg/day) and d-galactose (D-gal) (Sigma, Taufkirchen, Germany)

(150 mg/kg/day) intraperitoneally and *S.officinalis* extract (100 mg/kg/day) by oral gavage were administered for 21 days (14).

Periodontitis was induced by a 3.0 silk ligature (RT-ED, Shandong, China) to maxillary 2nd molar teeth in the last 14 days of the experiment. After the sacrification of the rats, Aβ deposition in the hippocampus, iNOS expression, and sclerostin in periodontal tissues were evaluated histomorphometrically and histopathologically.

S.officinalis extract was prepared in The Department of Pharmacognosy, Faculty of Pharmacy, Isparta Applied Sciences University, Isparta. Firstly, the leaves of the plant were separated and ground into powder. We kept it in ethyl alcohol (80%) in a shaker for 48 hours. Then we filtered the liquid part and evaporated the liquid until it dried with a rotavapor and put the remaining dry fraction in solution (0.5%) with carboxymethyl cellulose (Akbel Kimya, Bursa, Türkiye). *S.officinalis* extract (100 mg/kg/day) was administered through oral gavage for 21 days. The rats were euthanized by transcardial perfusion with cold phosphate-buffered saline pH 7.3 under deep anasthesia of xylazine (Rompun 2%, Bayer, İstanbul, Türkiye) 10mg/kg and ketamine HCl (Ketasol 10%, Richter, Pharma, Wels, Austria) 90 mg/kg body weight.

After the euthanasia, in addition to the brain, the maxilla was taken from each rat. The hippocampus was removed from the brain. The maxilla was separated into two halves along the sutura palatina.
Histomorphic method

The right maxillary halves were dissected from gingival tissue and incubated in 3% hydrogen peroxide (Natural H2O2, Istanbul, Türkiye) for 24 hours (15). To determine the cementoenamel junction (CEJ), 1% methylene blue (Noratex Chemistry, Istanbul, Türkiye) was kept for one minute, washed under running water, and dried. The alveolar bone level was measured at six points in the buccal and palatal areas between the CEJ and the alveolar crest (16). The samples were photographed with a digital camera (Leica DLUX3, Wetzlar, Germany), fixed to a stereomicroscope (Leica S4E Stereomicroscope, Wetzlar, Germany), and transferred to the computer environment (4X). The measurements were standardized using the ImageJ program (1.53f, Maryland, USA).

Histopathological Method
Histopathological and histomorphometric evaluations were made in the left maxillary halves and the hippocampus. The maxillary samples were decalcified with a 0.1 M EDTA solution for two weeks. After fixation in the 10% neutral formalin solution for two days, the samples of the tissues were then routinely processed and embedded in paraffin wax. The five-micron thickness sections were taken from the samples by a rotary microtome (Leica RM2155, Leica Microsystems, Wetzlar, Germany). Following drying the slides, the sections were placed on coverslip plates, stained with hematoxylin-eosin (HE), and examined under a light microscope. As a result, the histopathological alterations were blinded during grading.

Immunohistochemical Examinations
The tissue samples of the jaws, drowned on the polylysine slides, were immunostained with Aβ (beta Amyloid 1-42 antibody, bs-0076R, Bioss Antibodies Inc., Massachusetts, USA), iNOS (Rabbit anti-iNOS polyclonal antibody, bs2072R, Bioss Antibodies Inc., Massachusetts, USA), and sclerostin (anti-sclerostin antibody, (ab63097, Abcam, Cambridge, UK)) by the streptavidin-biotin technique.

The biotinylated secondary antibody and streptavidin–alkaline phosphatase conjugate were used for immunohistochemistry after the sections were incubated with the primary antibodies for 60 minutes. The secondary antibody was prepared using the EXPOSE Mouse and Rabbit Specific HRP/DAB detection IHC kits (Abcam®, Cambridge, MA, USA). The antigens were shown by using diaminobenzidine (DAB) as the chromogen. The primary antiserum was replaced with antibody dilution solutions for the negative controls. Blinded samples were used for each examination. Each slide was investigated for immunopositivity, and the percentage of immunopositive cells in each group was determined by counting 100 cells in 10 distinct fields for each section at a magnification of X40. The outcomes of the image analyzer were applied to the statistical analyses. The Database Manual Cell Sens Life Science Imaging Software System (Olympus Co., Tokyo, Japan) was used to carry out the morphometric analyses.

Statistical Analysis
Each variable was given as mean±standard deviation (SD). The ANOVA and Duncan tests were utilized to compare the histopathological and immunohistochemical scores among the groups. The Statistical Package for Social Sciences (SPSS) for Windows 15.0 program was used to perform the statistical analyses (SPSS Inc., Chicago, IL, USA). A p-value of <0.05 was accepted to be significant.

Results

Histomorphometric Findings

The histomorphometric findings are shown in Table 1. Alveolar bone loss (ABL) was seen in all the groups, except for Groups C and S, showing no statistical difference in histomorphometric measurements. It was noted that there was a statistical significance in terms of ABL in Groups AS and A, compared to Group C. The highest mean ABL was found in Groups P and AP. After adding S. officinalis extract to P and AP applications (Groups PS and APS), ABL was statistically significantly found to be decreased ($p<0.05$).

Histopathological Findings in Periodontal Tissues:

On the histopathological examination, normal gingival mucosae were observed at the interdental area in Groups C and S. Slight erosions were commonly observed at the interdental papilla in Group A. *S.officinalis* treatment was detected to decrease both the number and severity of the ulcers in Group AS. Periodontitis led to marked inflammation and a decrease in the height of the papilla in the periodontitis-induced group. The slight epithelization was also noticed in some rats' interdental areas in that group. The most marked inflammatory reaction was noticed in Group AP; however, a severe inflammatory reaction was also extended to the tooth roots in this group. The epithelization was generally very slight or absent in interdental areas in the group. *S.officinalis* treatment was seen to decrease the inflammatory reaction and induce the epithelization in both Group PS and Group APS (Figure 1).

Immunohistochemical Findings in the Hippocampus

In the immunohistochemical examination of hippocampal tissues for Aβ, while significant Aβ expression was noted in group A, the most severe Aβ expression was observed in the AP group. Aβ expression in the hippocampus was negative in the C and S groups. Mild Aβ expression was found in the hippocampus of 2 rats in group P. Aβ expression was not detected in any rat in the PS group. It was noted that Aβ expression decreased in the APS group compared to the AP group (Figure 2). Table 2 shows the findings of Aβ positive cells in the hippocampus in the groups. Immunohistochemical findings in the periodontal tissues

The immunohistochemical examination of sclerostin revealed negative-to-slight intracytoplasmic expressions in alveolar bone in Groups C and S. Slight-to-moderate expressions were also observed in

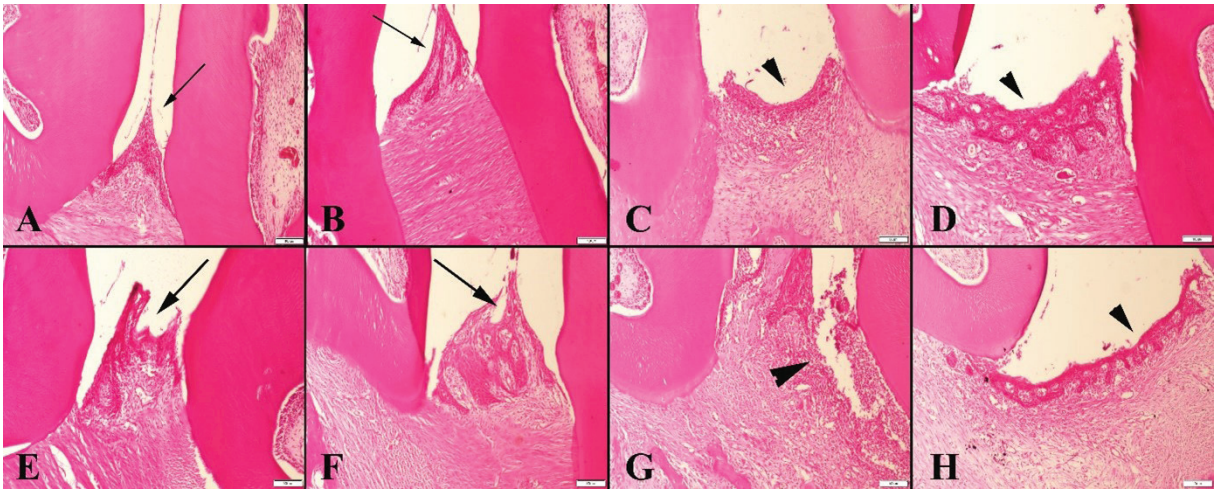

Figure 1

Representative histopathological appearances in the groups. (A) Normal interdental papilla (thin arrow) in Group Control. (B) Normal gingival mucosa in interdental papilla (thin arrow) in Group S. (C) Marked inflammatory reaction, ulcer, and slight epithelization at the interdental area (arrow head) in Group P. (D) Moderate amelioration, decreased inflammatory reaction, and increased epithelization (arrow) in Group PS. (E) Slight erosions at the interdental papilla (thick arrow) in Group A. (F) Decreased inflammation and amelioration in erosive reaction (thick arrow) in Group AS. (G) Very severe inflammatory reaction and severe ulcer (arrow head) in Group AP. (H) Decreased inflammation and increased epithelization in Group APS. HE, scale bars 100µm.

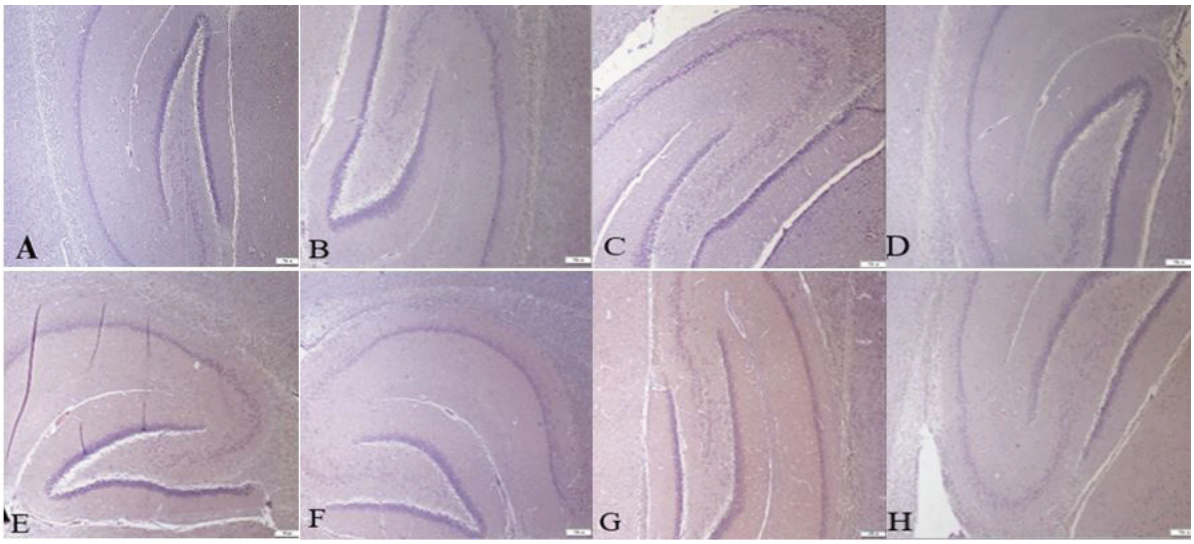

Figure 2

The Aβ expression in the hippocampus in the groups. (A) Negative Aβ expression in the hippocampus in Group C. (B) Aβ negative expression in hippocampus tissue in Group S. (C) Mild Aβ expression in hippocampus tissue in Group P. (D) Negative Aβ expression in hippocampus tissue in Group PS. (E) Increased Aβ expression in hippocampus tissue in Group A. (F) Negative Aβ expression in Group AS. (G) Significantly increased Aβ expression in hippocampus tissue in Group AP. (H) Decreased Aβ expression in the hippocampus tissue in Group APS. Streptavidin Biotin Peroxidase method, scale bars 200 µm.

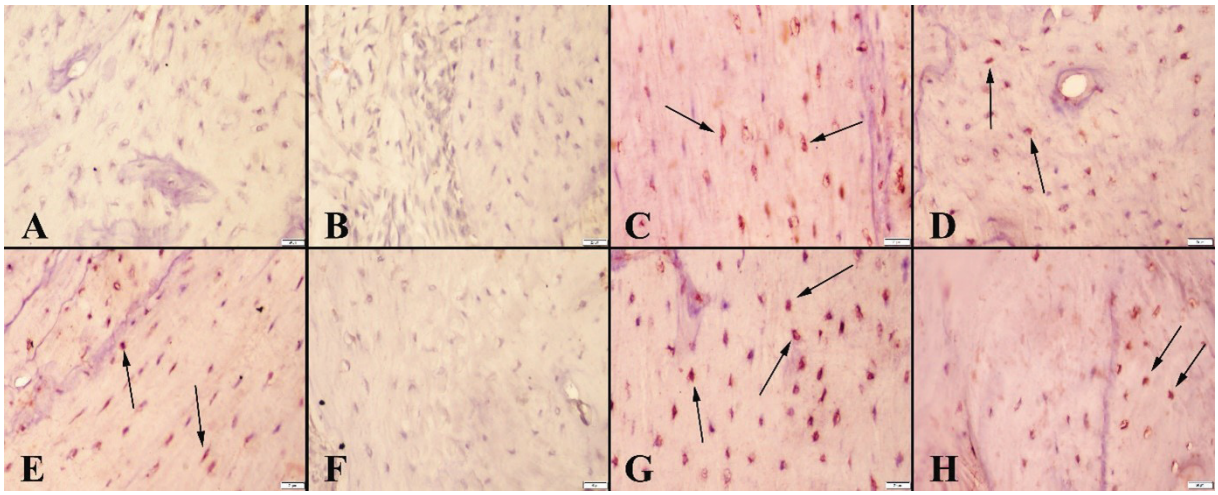

Figure 3

Sclerostin immunohistochemistry findings in the alveolar bone in the groups. (A) Negative expression in Group C. (B) No expression in Group S. (C) Increased immunoreaction (arrows) in Group P. (D) Decreased immunoexpression (arrows) in Group PS. (E) Slight expression (arrows) in Group A. (F) Decreased expression in Group AS. (G) Marked expression (arrows) in Group AP. (H) Decreased immunoreaction in Group APS. Streptavidin biotin peroxidase method, scale bars 20µm.

Table 1 ABL, iNOS, and sclerostin-positive cell examinations in alveolar bones

Groups	ABL	iNOS	Sclerostin
C (n=10)	0.179±0.006^{a}	5.70±0.42^{ef}	1.10±1.19^{k}
S (n=10)	0.187±0.014^{a}	4.90±0.50^{e}	0.80±0.78^{k}
P (n=10)	0.927±0.033^{d}	27.0±1.05^{i}	42.70±6.54^{l}
PS (n=9)	0.624±0.036^{c}	10.11±0.42^{g}	20.22±1.98^{m}
A (n=10)	0.340±0.027^{b}	19.20±0.93^{h}	11.80±2.74^{n}
AS (n=10)	0.318±0.015^{b}	9.80±0.42^{fg}	8.90±1.19^{n}
AP (n=9)	0.845±0.061^{d}	39.67±1.93^{j}	61.11±3.44^{o}
APS (n=10)	0.696±0.016^{c}	23.50±1.02^{i}	35.00±3.81^{p}

*Values are presented as mean±standard deviation. **Data with different superscripts indicate significant differences from each other (p>0.05). *** The same symbols used for different parameters differ from each other. A: Alzheimer's disease, ABL: Alveolar bone loss, AP: Alzheimer's disease+periodontitis, APS: Alzheimer's disease+periodontitis+ *S.officinalis*, AS: Alzheimer's disease+*S.officinalis*, C: Control, iNOS: Isoform of nitric oxide synthases, P: Periodontitis, PS: Periodontitis+*S.officinalis*, S: *S.officinalis*

Table 2 Aβ positive cell examinations in the hippocampus

		95% CI for Averages				
Groups	**n**	**Mean mm± SE**	**Lower Limit**	**Upper Limit**	**Minimum**	**Maximum**
C	10	0.00±0.00^{a}	0.00	0.00	0	0
S	10	0.00±0.00^{a}	0.00	0.00	0	0
P	10	1.20±0.36^{a}	0.39	2.01	0	3
PS	9	0.00±0.00^{a}	0.00	0.00	0	0
A	10	23.60±1.35^{d}	20.54	26.66	19	30
AS	10	7.20±0.81^{b}	5.36	9.04	2	10
AP	9	42.56±1. 63^{e}	38.81	46.30	38	53
APS	10	13.20±0.73^{c}	11.55	14.85	10	17

a, b, c, d: The averages of the group having the same letter are not different from each other (p>0.05). A: Alzheimer's disease, AP: Alzheimer's disease+periodontitis, APS: Alzheimer's disease+periodontitis+ S.officinalis, AS: Alzheimer's disease+*S. officinalis*, C: Control, iNOS: Isoform of nitric oxide synthases, P: Periodontitis, PS: Periodontitis+*S.officinalis*, S: *S.officinalis*

Group A. The induction of periodontitis brought about a marked increase in sclerostin expression in the P, AP, and APS groups. *S.officinalis* treatment decreased the expression in Group AS, compared to Group A. The most marked increase was observed in Group AP. The treatment of salvia was observed to decrease the expression in treated groups (Figure 2).

At the examination of the iNOS immunostained slides, mild-to-very slight expressions were noticed in Groups C and S; while increased expressions were observed in Groups P, A, and AP, a decrease in expression was seen in Groups PS, AS, and APS (Figure 3). iNOS and sclerostin-positive cells were immunohistochemically examined in each group, and

the findings are demonstrated in Table 1.

Discussion

Periodontitis induction by ligature is a suitable model for investigating the host responses during the development of periodontitis (17). Toker et al. (18) observed that in the model where periodontitis was induced by the ligature, ABL reached the highest level on the 11th day. Additionally, Molon et al. (19) found a significant increase in ABL and proinflammatory cytokine expression within the first 14-day period in terms of the measurements on the 1st, 3rd, 5th, 7th, 14th, and 21st days, and recorded no significant alterations in ABL and inflammatory process between the findings on those days. In our study, the induction of periodontitis was performed for 14 days with 3-0 silk sutures in the maxillary second molar teeth of the rats (20). Periodontitis developed in all rats undergoing the induction, and the distance between CEJ and ABL was evaluated histomorphometrically.

Studies revealed that cholinergic system disruptions, OS, and memory problems similar to AD occur with the use of combined AlCl3 and D-gal (21,22). The combined use of AlCl3 and D-gal increases Aβ levels in the cortex and hippocampus (21). Al+3 cholinergic in the CNS is known to lead to dysfunction (21). In various animal studies, aging-like changes, including learning and memory problems, were stated to increase the production of ROS while decreasing the activities of antioxidant enzymes after the administration of D-gal (21,22). In our study, AlCl3 (10 mg/kg) and D-gal (150 mg/kg) were intraperitoneally applied for 21 days to create an AD-like model (14). The gold standard is the neuropathological evaluation of Aβ and NFYs (23,24). In our study, Aβ was examined in the hippocampal tissue to determine the pathological characteristics of AD. No memory or behavioral analysis tests were performed on the rats. In all AD-induced groups, increased Aβ expression was detected in the hippocampus.

Periodontitis may have vital importance in the neuroinflammatory process through the direct invasion of periodontal pathogens, bacterial products activating microglia, or pro-inflammatory cytokines produced by the host response (25). It was reported that chronic periodontitis was likely to increase the development of AD (26). Kamer et al. (27) reported the relationship between the load of Aβ detected through the positron emission tomography scanning and the severity of increased periodontitis in cognitively normal elderly individuals. The accumulation of Aβ increased in the hippocampus, in mice with transgenic AD when periodontitis induction was added, (28,29). However, in the study by Holmer et al. (8), ABL was detected further in individuals with poor cognitive performance, compared to the healthy controls. Ishida et al. (29) also found ABL further in periodontitis and higher levels of interleukin-1β and tumor necrosis factor-α in brain tissues in transgenic AD mice, compared to the controls. In the study by Kantarcı et al. (28), ABL was reported although periodontitis was not induced in transgenic AD mice; however, the authors pointed out that the rate of periodontitis induction in transgenic AD mice was not greater than that of ABL in only periodontitis-induced mice (28).

The detection of Aβ expression in two rats in Group P demonstrated that the chronic inflammatory nature of periodontitis may have contributed to neuroinflammation, although AD was not induced in our study, and thus, periodontitis may have caused the accumulation of Aβ. Additionally, compared to Group C, the histomorphometric examination of the maxillary halves showed a significant rate of ABL, supported by the increased hyperemia and inflammation in Group A. Even though no difference was detected between the mean ABL in Groups P and AP, more severe inflammation findings were found in the histopathological evaluation, consistent with those detected by Kantarcı et al. (28), which may be due to the impact of AD on periodontitis.

S.officinalis has memory-enhancing effects, as well as antimicrobial, antioxidant, anti-inflammatory, antinociceptive, and antimutagenic effects (30). It is also known that, as well as its high flavonoid and phenolic content, *S.officinalis* repairs DNA damage caused by free radicals and prevents the reactions leading to lipid peroxidation (31). In the study by Kolac et al. (32), S.officinalis was reported to decrease the parameters of OS.

In the light of the literature, however, we encountered no study exactly examining the relationship between *S.officinalis* and periodontitis. Even so, various reports have investigated that the other members of the salvia family, such as *S.sclarea*, *S.officinalis*, and S.miltiorrhiza, showed a decreasing effect on ABL in periodontitis (33,34). In our study, it was histopathologically determined that hyperemia was significantly reduced in *S.officinalis* in the hippocampus of the rats. The accumulation of Aβ detected in Group A was seen as a negative expression in Group AS. However, the extract of *S.officinalis* was applied along with the induction of AD in Group APS, and periodontitis was found to decrease the expression of Aβ. Our findings were consistent with those defined in the literature.

Synthesized via iNOS as an oxidant, NO is of crucial importance in host defense and homeostasis. Leitao et al. (35) revealed that ABL was prevented in rats with the inhibitor of NO synthesis in periodontitis.

Nevertheless, Pan et al. (36) stated that the degree of periodontal disease in periodontal tissues was correlated with an increase in iNOS expression. Increased levels of iNOS in periodontal tissues in those with periodontitis cause an increase in NO levels (37). Despite its neuroprotective mechanism at low concentrations, higher concentrations of NO are potentially neurotoxic to brain cells (38). It is believed that iNOS may be connected to the pathophysiology of AD and that patients with AD have a marked increase in the number of iNOS-positive neurons in their brains (38). Haas et al. (39) found that iNOS increased the expression of mRNA in brain tissues in AD. The close location of iNOS-positive microglial cells with the accumulation sites of Aβ suggests that Aβ is a contributor to the activation of microglial cells (39).

In our study, iNOS expression was determined to be increased in periodontal tissues with both AD and periodontitis induction. Based on our findings, while AD could modulate the inflammatory response in periodontal tissues, the higher rate of iNOS-positive inflammatory cells in periodontal tissues in both diseases reflected the increased rate of OS. In different studies, Salvia strains are stated to be able to reduce iNOS expression (33,40). However, there was no study in the literature evaluating the expression of iNOS with *S.officinalis*. Our findings revealed that *S.officinalis* significantly reduced the expression of iNOS in periodontal tissues in periodontitis and induction of AD, a similar finding to the antioxidant effects of other Salvia species.

Sclerostin is a critical factor with its negative regulator impacts on bone formation, thereby inhibiting bone remodeling in the development of periodontitis. Furthermore, data suggest that removing sclerostin appears to logically prevent the resorption of the alveolar bone (12). In most studies, it was revealed that the monoclonal antibody treatment with sclerostin can contribute to bone strength by promoting bone formation and reducing the resorption of the bone (41). In addition, sclerostin antibodies show a good performance in implants by enhancing the osseointegration and bone regeneration around the bone implants and dental implants, suggesting a possible therapeutic strategy to shorten patients' healing periods by accelerating bone regeneration after the placement of implants (11). The suppression of Wnt appears to play a critical role in the neurodegeneration development due to the progression of AD (4). Therefore, it is not surprising that sclerostin, an antagonist to Wnt signaling, was negatively associated with cognitive changes, almost exclusively expressed in the skeleton. In our study, the most marked increase in the expression of sclerostin was observed in Group AP, and *S.officinalis* treatment decreased the expression of sclerostin. Particularly, the regulation of the sclerostin expression is a novel therapeutic strategy for regulating bones for oral health and is likely to be a positive contributor to future research for general health.

In conclusion, we consider that our findings will contribute to future studies in which the pathogenetic interaction of periodontal disease and AD, and different aromatherapy approaches will be evaluated, and so *S.officinalis* will be benefited as an immunomodulatory aromatic plant.

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Conflict of Interest Statement
The authors declare no conflicts of interest.

Ethical Approval
The present study was carried out under the ethical standards of the research committee of the institution by the 1964 Helsinki Declaration and its later amendments or comparable ethical standards, after the decision of the Süleyman Demirel University Animal Experiments Local Ethics Committee of the university (Approval number: 24/10/2019, 12/03).

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Availability of Data and Materials
The data of the study can be obtained through the corresponding author upon reasonable request.

Artificial Intelligence Statement
The authors declare that no type of artificial intelligence program has been used in writing of this manuscript, nor for the creation of figures, graphics or tables.

Authors Contributions
İY: Conceptualization; Project administration; Formal analysis; Investigation; Validation; Writing-original draft.
UY: Conceptualization; Project administration; Methodology; Data curation; Validation; Visualization;

Supervision; Writing-original draft; Writing-review and editing.

FYK: Conceptualization; Project administration; Methodology; Data curation; Validation; Visualization; Supervision; Writing-original draft; Writing-review and editing.

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OTA: Conceptualization; Project administration; Methodology; Writing-original draft.

SD: Conceptualization; Methodology; Supervision; Writing-original draft.

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