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Evaluation of the effect of metformin on gingivitis caused by experimental Alzheimer's disease in male rats

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ABSTRACT

Background: Periodontitis is associated with several comorbidities, including Alzheimer's disease (AD). Metformin (MET) has been shown to affect AD positively.

Objectives: In the present study, the effectiveness of MET in gingivitis caused by experimental AD was investigated in male rats.

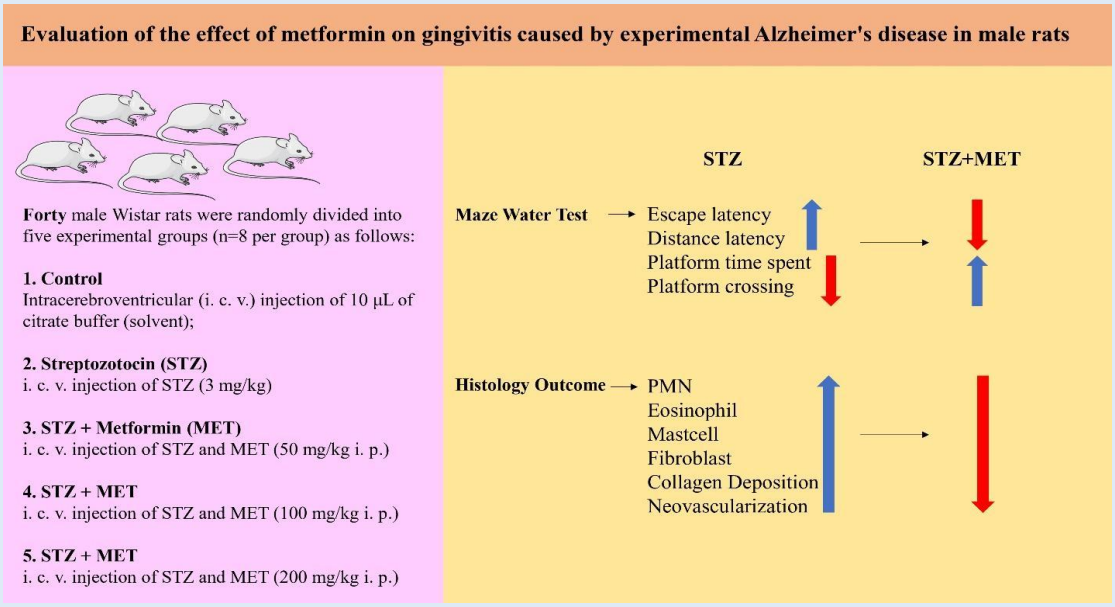
Methods: Forty male Wistar rats were randomly divided into five experimental groups (n=8 per group) as follows: control, intracerebroventricular (ICV) injection of 10 µL of citrate buffer (solvent); streptozotocin (STZ), ICV injection of (3 mg/kg) STZ; and three groups of STZ + MET, ICV injection of STZ and different dosages of MET (50, 100, 200 mg/kg i. p.). 24 hours after the last injection, the Morris water maze test was administered to each animal. The mice were then sacrificed under deep anesthesia, and sampling of the papilla around the two central incisor teeth was performed. The number of inflammatory cells, angiogenesis, fibroblasts, and collagen deposition were evaluated. The results were analyzed using ANOVA and Tukey's test.

Results: The results showed that the STZ injection significantly impaired learning and spatial memory. Moreover, STZ significantly increased periodontal inflammatory cells, angiogenesis, fibroblasts, and collagen deposition in the gingiva of rats compared with the control group. Additionally, MET improved learning and spatial memory and reduced histopathologic parameters in the gingiva of experimental Alzheimer's model rats.

Novelty of Study: This study fills a significant gap in evaluating the effect of MET on gingival inflammation linked to Alzheimer's disease (GAD) using a STZ-induced rat model. While previous research has explored metformin's neuroprotective and anti-inflammatory roles separately, this study uniquely combines behavioral assessments and histological analysis to show that metformin improves cognitive performance and reduces gingival inflammation. This study is the first to demonstrate that MET reduces gingival inflammation associated with experimental AD while simultaneously improving cognitive function, suggesting a dual therapeutic role.

Conclusions: Based on these findings, AD may cause or aggravate gingivitis. Moreover, MET has the potential to alleviate AD symptoms and experimental gingivitis caused by AD in rats.

Keywords: Alzheimer's disease, metformin, inflammatory cells, periodontal diseases, rats, gingival inflammation, dual-action effect, novel therapy



Graphical Abstract: Evaluation of the effect of metformin on gingivitis caused by experimental Alzheimer's disease in male rats

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INTRODUCTION

Alzheimer's disease (AD), the prevalent form of dementia, is a gradual neurodegenerative condition that predominantly impacts memory and cognitive abilities with potential effects on behavior, speech, motor functions, and visuospatial orientation [1]. Senile plaques composed of amyloid beta (Aβ) that accumulate extracellularly, and neurofibrillary tangles composed of hyperphosphorylated tau (Tp) that deposit intracellularly, causing the loss of synapses between

neurons, are two characteristics of this disease [2]. AD was ranked fifth among Americans aged 65 years and older and sixth among the causes of death in the US in 2019 [3]. Several community-based follow-up studies have reported a two-to-five-fold increased risk of death associated with AD [4]. Multiple studies have suggested that neuroinflammation plays a significant role in the progression of AD, along with tau and Aβ aggregation. Inflammatory mediators such as cytokines and chemokines, brain cells such as microglia and astrocytes, and amyloid beta are all involved in inflammation [5].

The term 'periodontic diseases' describes conditions affecting the tissues surrounding the teeth. These conditions can be caused by inflammatory, traumatic, developmental, neoplastic, genetic, or metabolic abnormalities. When discussing periodontal disease, we refer to the common inflammatory conditions gingivitis and periodontitis, which are caused by pathogenic microflora found in dental plaque or biofilm that continuously forms around teeth. The term 'periodontitis' refers to an inflammatory process that deeply affects tissues, leading to the loss of connective tissue and alveolar bone. Adults are more susceptible to periodontitis [6-7]. An increasing amount of data indicates that periodontitis is associated with several comorbidities, such as osteoporosis, rheumatoid arthritis, Parkinson's disease, AD, respiratory infections, and type 2 diabetes (T2DM) [8]. There may be a link between periodontal disease and AD due to the migration of inflammatory or infectious substances from the oral cavity to the brain [9]. Diabetes and periodontitis are closely related conditions that have well-established bidirectional effects. This means that individuals with diabetes are more likely to develop periodontitis and tooth loss, and individuals with periodontitis are more likely to develop systemic inflammation and diabetes [10].

Metformin (MET) is a commonly prescribed antidiabetic medication [11-12]. MET can lower chronic inflammation by improving metabolic markers like insulin resistance, atherogenic dyslipidemia, and hyperglycemia. Furthermore, research indicates that MET may directly reduce inflammation by inhibiting nuclear factor κB (NFκB) through both independent and dependent pathways of AMP-activated protein kinase (AMPK) [13]. The potential disease-modifying effects of MET on various aspects of AD pathophysiology, including tau phosphorylation, neuroinflammation, and Aβ production and clearance, have been widely established due to its pleiotropic properties [14].

STZ is a glucosamine-nitrosourea compound originally discovered as an antibiotic. It is frequently used to cause experimental diabetes in animals because it is toxic to pancreatic β-cells. The ICV injection of STZ can mimic the pathology of AD, making it an excellent tool for investigating the underlying pathophysiological mechanism of the disease in its early stages, when interventions may be able to stop the disease's neurodegenerative processes [15-17]. Bioactive compounds in functional foods have been used for chronic disease and health [18-19]. Although MET has been investigated for AD and separately for periodontitis, its effect on gingival inflammation caused by AD has not been explored. This study addresses this gap by simultaneously assessing behavioral and histological outcomes in an experimental AD model.

MATERIALS AND METHODS

Subjects: Adult male Wistar rats were purchased from the Pasteur Institute, Karaj, Iran. They were kept in a 12/12 h light-dark cycle at 22 ± 2 °C and 55–65% humidity and weighed between 220 and 260 g. One week before surgery, rats were acclimated to the laboratory setting and had unrestricted access to food and water. Given the presumption that the time frame from 9:00 to 14:00 does not significantly influence nocturnal learning and memory, the experiments were carried out during the daylight phase [20]. During the period of adaptation, the animals were handled for five minutes during the day to

reduce their stress levels. The experimental procedures were approved by the Ethical Committee of the Qazvin University of Medical Sciences, Qazvin, Iran (IR.QUMS.REC.1402.005).

Chemicals: Streptozotocin was purchased from Santa Cruz Company (USA). Metformin was obtained from the Osvah Pharmaceutical Company (Iran). Sodium citrate buffer (CB, pH 4.5) was used as the STZ solvent and prepared ex tempore.

Experimental design: In the following manner, forty male Wistar rats were split into five experimental groups at random (n = 8 per group): control, intracerebroventricular (ICV) injection of 10 µL of citrate buffer (solvent); STZ, ICV injection of STZ (3 mg/kg); STZ + MET, ICV injection of STZ and MET (50 mg/kg i. p.); STZ + MET, ICV injection of STZ and MET (100 mg/kg i. p.); STZ + MET, ICV injection of STZ and MET (200 mg/kg i. p.). According to findings from article searches, where the predominant MET doses ranged from 50 to 200 mg/kg, specific MET dosages (50, 100, and 200 mg/kg) were selected [21]. MET was injected once a day for 2 weeks starting a week after the ICV administration of STZ. Next, every animal was subjected to the Morris water maze test for five days.

Intracerebroventricular injection of streptozotocin: To produce anesthesia, rats received an intraperitoneal injection of a mixture of xylazine (10 mg/kg) and ketamine (100 mg/kg). Solvent or streptozotocin was administered to each lateral ventricle following stereotaxic surgery based on Bregma: AP= -0.5, ML= ±1.2, DV= -3.2. Animals were secured in the stereotaxic apparatus, and with the animal stabilized, an incision was made at the midline of the back of the head. After cleansing the skull's surface and identifying the bregma point as a reference, the intended injection site was marked on both sides of the head using the Paxinos atlas. After marking the target points on the skull surface, two holes were drilled using a dental drill, and the drugs were gradually injected bilaterally into the lateral ventricles using a Hamilton syringe. A dose of 3 mg/kg and a volume of 5 µL each ventricle (a total of 10 µL in both ventricles) were used for the injection. Citric buffer was used to dissolve STZ. STZ was dissolved in citrate buffer. Equal amounts of citrate buffer were injected into the control group.

Morris water maze test: An MWM apparatus was used to evaluate spatial learning and memory [22]. The device was a black circular pool that was separated into four equal quadrants and measured 136 cm in diameter by 60 cm in height. Every rat was submerged in water with its back to the pool wall. Water (21 ± 1 °C) was poured into the tank until it was 25 cm deep. The center of one of the quadrants was a circular escape platform made of plexiglass, measuring 10 cm in diameter and situated 1-2 cm below the water's surface. The location of each rat was monitored using a digital TV system, and the Any-Maze tracking system was used to analyze the data. The animals were allowed to swim in the pool for 60 s without the platform the day before the experiment began to help them become accustomed to it. Both the acquisition (learning) and probe (memory) tests were included in the experiment [23].

Four consecutive days with four trials each and a five-minute break in between were part of the acquisition test procedure. On each acquisition day, each animal was placed in one of four quadrants (four trials). In each trial, the rats were given up to 60 s to swim in search of a hidden platform. The animal was allowed to remain on the platform for 30 seconds after it was discovered on its own. If a rat was unable to locate the platform in 60 s, the researcher led it and let it be there for 30 s.

A probe trial was conducted 24 h after the acquisition test. The rats were placed in the quadrant opposite the target quadrant after the hidden platform was removed from the pool. They had 60 s to swim

around freely. To evaluate spatial memory retention, the amount of time and visits to the target quadrant were recorded and analyzed [23].

Histological Examination: The rats were sacrificed under deep anesthesia, and sampling was performed from the papilla around the two central incisor gingival areas fixed in 10% formalin solution. The samples were embedded in paraffin and paraffin blocks were prepared. Serial 5-micron sections were then made using a microtome. Staining and examination of inflammatory cells (eosinophils, neutrophils, and mast cells), fibroblasts, and angiogenesis were performed using hematoxylin and eosin staining techniques. Mast cell counts and evaluations were performed using toluidine blue staining. Using a 400x objective lens, eosinophil, neutrophil, mast cell, and fibroblast counts were made. Using a 100x objective lens, areas with a high density of newly formed vessels were located first. These fields were chosen, and the process of counting angiogenesis was carried out at 400x magnification. Angiogenesis scoring was carried out histologically, as indicated in Table 1 [24].

Table 1. Histomorphologic classifying of angiogenesis

Classification	0	1	2	3	4
Angiogenesis	No evidence of blood vessels	4-8 vascular channels	12-15 vascular channels	15-20 vascular channels	More than 20 vascular channels

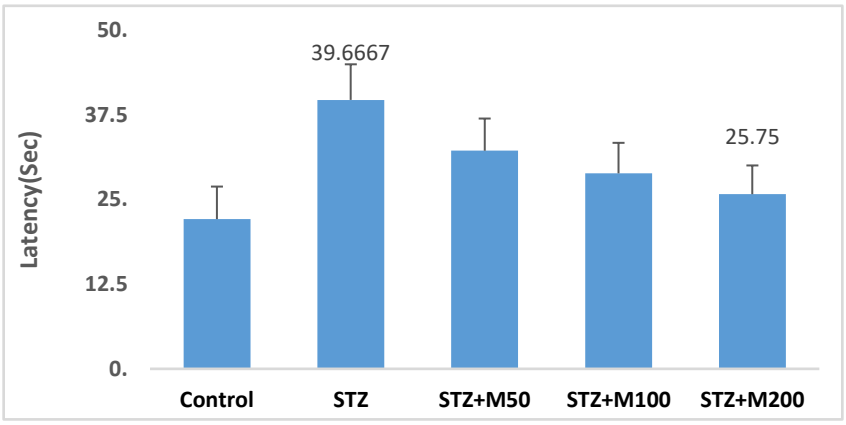
Additionally, Talas' trichrome staining method was used to score the density of collagen fibers in stained tissues, and Van Gieson staining was used to demonstrate collagen deposition [25].

Statistical analysis: The data were analyzed using SPSS 21 software one-way ANOVA followed by Tukey's post-hoc test, and the raw data of each experimental group were entered sequentially, and the standard deviation was determined. The difference between the groups was determined separately by Tukey's post-hoc test, and $p < 0.05$ was proposed as an indicator of significance.

RESULTS

Maze Water Test: The findings of the acquisition study showed that STZ injection significantly increased the average time spent finding the escape platform (escape latency) and the average distance traveled to find the rescue platform (distance latency) compared to the control group ($p<0.01$). These results show that STZ significantly impaired spatial learning in mice and induced AD. MET administration improved these parameters in a dose-dependent manner, and this change was significant at a dose of 200 mg/kg ($p<0.05$). (Figures 1A,B)

A



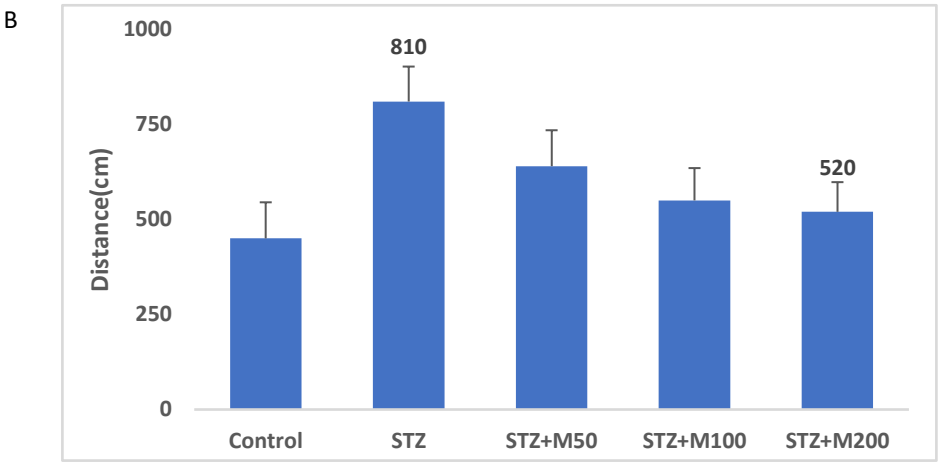


Figure. 1 Effect of STZ and STZ + MET (50, 100, 200 mg/kg) on (A) average duration of the delay in reaching the platform and (B) average distance in reaching the platform.

Each column represents Mean+SD (n = 8). *p < 0.05 and ** p < 0.01 compared with control group, whereas # p < 0.01 compared with STZ group of animals. STZ, streptozotocin. MET, Metformin.

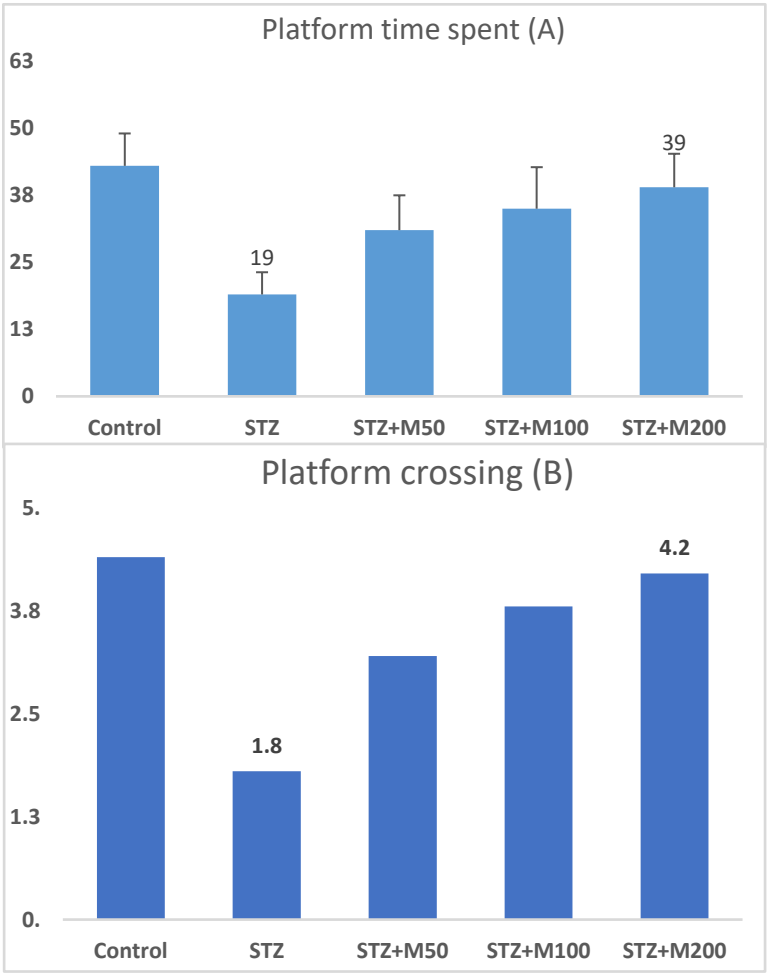


Fig. 2 Effect of STZ and STZ + MET (50, 100, 200 mg/kg) on (A) average duration of presence in the target quarter and (B) average number of crossing the target quadrant.

Each column represents Mean+SD (n = 8). *p < 0.05 and **p < 0.01 compared with control group, whereas #p < 0.01 compared with STZ group of animals. STZ, streptozotocin. MET, Metformin.

Analysis of the probe trial results showed that the STZ group performed significantly worse than the control group in terms of the amount of time spent in the target quadrant ($p < 0.01$) and the number of times it crossed the target quadrant (the previous location of the platform) ($p < 0.05$), all of which are signs of impaired spatial memory in mice, and confirmed the occurrence of AD. However, in the group receiving STZ and MET (200 mg/kg) groups, these parameters increased and approached those of the control group ($p < 0.05$) (Figures 2A,B).

Histology Outcomes: There was a significant increase between all groups and the control group in the amount of PMN ($p<0.0001$). Moreover, there was a significant increase in the number of eosinophils in the STZ ($p<0.001$) and STZ+MET50 mg/kg ($p<0.01$) groups compared to that in the control group. Administration of MET in all concentrations significantly reduced eosinophil counts compared with the STZ group. There was a significant increase in the number of mast cells between all groups and the control group, except for the group STZ+MET200. MET 200 significantly decreased the number of mast cells compared to the STZ group ($p<0.05$) (Table 2) (Figure 3).

Overall, the results showed that the number of PMNs, eosinophils, and mast cells in the STZ group increased significantly compared to that in the control group, which evokes this acute inflammatory condition in the gingival tissue. However, these increases in the MET-treated groups were lower than those in the STZ alone.

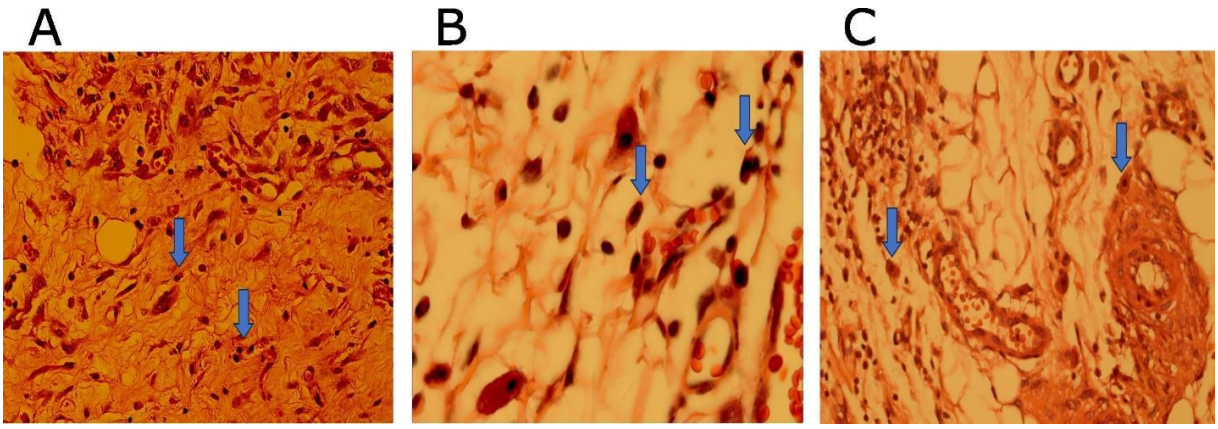


Figure. 3 The gingival biopsies have shown scar tissue infiltrated by (A) PMNs leukocytes, (B) eosinophils, and (C) mast cells. (400×, Hematoxylin & Eosin).

Changes in the number of fibroblasts, collagen deposition, and neovascularization in the mice were evaluated. There was a significant increase in the number of fibroblasts in the control group compared to the STZ group ($p<0.001$), STZ+MET 50 ($p<0.01$), and STZ+MET100 ($p<0.01$) groups. Administration of MET 200 ($p<0.05$) significantly reduced the number of fibroblasts compared with that in the STZ group. There was a significant increase in collagen deposition in the control group compared to that in the STZ ($p<0.05$), STZ+MET 50 ($p<0.01$), and STZ+MET100 ($p<0.05$) groups. There was a significant increase in neovascularization in the control group compared to the STZ group and all STZ+MET groups. Although a higher concentration of MET decreased the number of collagen deposits and neovascularization in a dose-dependent manner, the results were not significant (Table 2).

The results confirmed that STZ-induced AD in rats increased the number of PMN, eosinophils, mast cells, fibroblasts, new blood vessels, and collagen deposition in the gingival tissue, confirming inflammation of the gingiva in rats. These changes were significantly lower in the MET-receiving groups.

Table 2. Effect of STZ and STZ+MET on histological parameters in gingival tissue

	Control	STZ	STZ+MET50	STZ+MET100	STZ+MET200
PMN	0.84 ± 4.25	13.125 ± 0.63***	0.94*** ±12.37	± 11.62 0.56***	10.62 ± 0.62***
Eosinophil	3.5 ± 0.83	10.75 ± 0.92***	7.5 ± 0.42**###	4.37 ±0.32###	4.87 ± 0.51###
Mastcell	0.47 ± 2.75	5.5 ± 0.21**	7.5 ± 0.18***	5.12 ± 0.29**	3.85 ± 0.66#
Fibroblast	18.12 ± 0.56	± 36.621.61***	31.125 ±2.59**	30.62±2.29**	27.37±2.80#
Collagen Deposition	1.62 ± 0.52	± 2.50.28*	3.12 ± 0.22**	2.87± 0.22*	2.5 ± 0.26
Neovascularization	0.64± 1.87	4.87 ± 0.35***	5.25 ± 0.67***	5.125±0.44***	3.87 ± 0.29*

Abbreviations: MET, metformin; STZ, streptozotocin; SD, standard deviation. All values are expressed as mean ± SEM (n = 8). * $p < 0.05$, ** $P < 0.01$, *** $p < 0.001$ compared with control group of animals.

DISCUSSION

While MET's neuroprotective and anti-inflammatory effects have been reported separately, our study is the first to demonstrate that MET simultaneously improves cognitive deficits and gingival inflammation in an AD rat model. The results of this study showed that ICV STZ injection worsened spatial learning and memory. In addition, there was a significant increase in inflammatory parameters, fibroblasts, collagen deposition, and neovascularization in the gingival tissue of STZ-induced AD rats compared with the control group. Daily injection of MET improved spatial learning and memory deficits, and attenuated inflammatory parameters, fibroblasts, collagen deposition, and neovascularization impairment in a dose-dependent manner. MET, especially at a dose of 200 mg, produced the best results in improving inflammatory and histological indicators of GAD in rat models. Therefore, it may be useful in the treatment of GAD.

Diabetes and AD share several pathophysiological mechanisms, including insulin sensitivity, brain glucose metabolism, and cognitive function [26]. MET is one of the most popular anti-insulin resistance medications used as first-line treatment for type 2 diabetes. It has been shown that MET has positive effects on cognitive function in both diabetes and AD [27,28]. MET induces anti-AD effects via different mechanisms. MET can reduce reactive astrocytes and inflammatory cells as well as dying neurons in the cortex and hippocampal regions and enhance cognitive function in AD [29]. c-Jun N-terminal kinase (JNK) is a tau kinase, and its activated/phosphorylated level will be increased in the hippocampus of diabetic rats. Metformin can downregulate JNK activation and attenuate the increase in total tau and phospho-tau levels [30]. According to additional laboratory research, MET can decrease tau phosphorylation via mTOR/PP2A (protein phosphatase 2A) signaling, which can lessen the molecular pathologies associated with AD [31].

In mouse models, it has been discovered that MET lowers Aβ levels via the insulin-degrading enzyme (IDE) pathway [32]. β-secretase BACE1 (β-site amyloid precursor protein cleaving enzyme 1) and the γ-secretase complex sequentially cleave the membrane protein amyloid precursor protein (APP) to produce the amyloid plaque component Aβ. MET can lower the levels of BACE1 protein in neurons and thus lower the level of Aβ [33]. Neuroinflammation, type 2 diabetes, and insulin resistance are all associated with AMPK dysregulation. As AMPK has been demonstrated to regulate both tau phosphorylation and Aβ generation, AMPK signaling plays a significant role in the progression of AD. AMPK activation is necessary for the inhibition of tau phosphorylation and Aβ production in neuronal culture. MET administration can activate AMPK. The AMPK pathway is activated when MET inhibits complex I in the

electron transport chain, which is necessary for mitochondrial respiration [34].

This study aimed to investigate the effects of MET on gingival inflammation. Based on these results, the number of inflammatory cells, fibroblasts, collagen deposition, and neovascularization was significantly higher in the STZ-induced AD rats than in the control group. According to these results, AD, which is associated with dementia and cognitive decline, increases inflammation of gingiva and is also associated with periodontitis. Moreover, Met can reduce these impairments, especially at a dose of 200 mg.

A retrospective cohort study demonstrated that individuals with chronic periodontitis had a higher risk of developing AD and general dementia than those without chronic periodontitis. Additionally, there appears to be a trend toward an elevated risk of vascular dementia in individuals with chronic periodontitis [35]. In a different study, periodontitis at baseline was linked to a six-fold increase in the rate of cognitive decline over a six-month follow-up period. Throughout the six-month follow-up period, a relative increase in the proinflammatory state was linked to baseline periodontitis. These findings demonstrated that regardless of the initial cognitive state, periodontitis is linked to a higher rate of cognitive decline in Alzheimer's disease. This association may be mediated by systemic inflammation [36]. Oral microbiota may affect AD risk through circulatory or neural access, and treatment of the oral microbiota may be helpful for the treatment of AD. In this regard, COR388, a bacterial protease inhibitor that targets P. gingivalis, is currently being tested in a double-blind, placebo-controlled Phase II/III study involving 573 mild-to-moderate Alzheimer's disease patients (NCT03823404, GAIN Trial) [37,38]. The two mechanisms that link these two illnesses are an invasion of the brain by bacteria found in the dental plaque biofilm and the host response, which raises the body's levels of proinflammatory cytokines [39]. The pathological degradation of the extracellular matrix (ECM) in periodontal tissues, destructive periodontal diseases, and inflammation of periodontal tissues are attributed to matrix metalloproteinases (MMPs) [40]. MMPs change the bioactivity and increase the bioavailability of cytokines and chemokines by releasing them from the extracellular matrix. However, MMPs can also cleave chemokines and cytokines, producing shortened products that have the potential to function as competitive antagonists. Inflammatory destruction of periodontal tissues is determined by the relative abundance of chemokines and cytokines that favor the infiltration of neutrophils, macrophages, and Th1/Th17 lymphocytes [41]. Reactive oxygen species (ROS) activate MMPs in periodontal tissues [42].

The effects of metformin on periodontal disease have been investigated in several studies. A retrospective cohort study showed that metformin uses over approximately 2 years or at a cumulative dose of 670 mg was linked to a significantly lower risk of GPD [43]. The results of a systematic review and meta-analysis showed that when MET is used as an adjuvant for periodontal therapy, it may lessen the need for further interventions and lower the amount of inflammation that patients experience; moreover, no side effects have been reported [44]. MET can regulate the expression of NF-κB p65, AMPK, and Hmgb1 genes, which lowers the levels of malondialdehyde, IL-1β, and TNF-α and controls bone loss. Finally, MET demonstrated antioxidant and anti-inflammatory properties as well as reduced bone loss [45]. Periodontitis and cognitive decline are directly and reciprocally correlated. Therefore, treatment of periodontitis can improve cognitive impairment in patients with AD [46].

Recent studies on bioactive food compounds support our findings that anti-inflammatory interventions can improve both metabolic and neuro-oral health. For example, polysaccharides from the split-gill mushroom Schizophyllum commune (a functional food) significantly lowered blood glucose and

inflammatory markers (e.g. TNF-α, IL-1β) and restored insulin-signaling proteins (GLUT4/GLP-1R) in diabetic rats [47]. Similarly, plant-derived menthol (a bioactive compound found in mint oils) demonstrated neuroprotective and antidiabetic effects. It improved cognitive deficits, reduced hyperglycemia and oxidative stress, and activated antioxidant (Nrf2/ARE) signaling in diabetic encephalopathy models [48]. Other functional-food extracts also show parallel benefits. For instance, a Thai soup powder extract (Namya Kanom Jeen) lowered blood glucose, insulin levels, and oxidative stress markers (MDA, HOMA-IR) in diabetic rats [49]. These examples illustrate that dietary bioactives can modulate key metabolic and inflammatory pathways similarly to metformin [47,48]. Together, this suggests that incorporating functional foods or their active compounds might complement pharmacologic therapy, potentially helping to mitigate inflammation in conditions linked to diabetes and AD including gingivities.

Scientific Innovation and Practical Implications: This study demonstrates that MET can reduce GAD in a rat model, even without local periodontal treatment. By improving cognitive function and decreasing inflammatory markers in gingival tissue, MET shows potential as a dual-action therapy for both neurodegeneration and oral inflammation. These findings highlight a novel application of MET beyond diabetes and AD management, suggesting its use in addressing oral-systemic complications in neurodegenerative conditions. Future research may explore the role of MET as an adjunct in periodontal care for patients with cognitive decline.

CONCLUSION

AD and gingivitis are associated. AD may cause or aggravate gingivitis by increasing the inflammatory parameters, fibroblasts, collagen deposition, and neovascularization in the gingiva of rats with STZ-induced AD. This study provides the first evidence that MET can simultaneously alleviate cognitive deficits and gingival inflammation in experimental AD, suggesting a novel therapeutic avenue for managing oral-systemic complications in neurodegenerative disorders. Further studies are needed to determine the mechanisms underlying the effects of AD on periodontal disease and other associated diseases.

Conflicts of Interest: None declared

Ethical Approval: The experimental procedures were approved by the Ethical Committee of the Qazvin University of Medical Sciences, Qazvin, Iran (IR.QUMS.REC.1402.005).

Authors' Contributions: Conceptualization: J.H., M.S., MH.E.; methodology: O.A., M.S. E.B.; data analysis: M.S., N.KA.; writing and original draft preparation: F.S., E.B.; review and editing: F.S., E.B; visualization: F.S.; supervision: F.S., M.S.; project administration: M.S. Manuscript has been read and approved by all the authors.

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