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Minocycline Reduces Spontaneous Hemorrhage in Mouse Models of Cerebral Amyloid Angiopathy

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Abstract

Background and Purpose—Cerebral Amyloid Angiopathy (CAA) is a common cause of recurrent intracerebral hemorrhage (ICH) in the elderly. Previous studies have shown that CAA induces inflammation and expression of matrix metalloproteinase-2 and -9 (gelatinases) in amyloid-laden vessels. Here, we inhibited both using minocycline in CAA mouse models to determine if spontaneous ICH could be reduced.

Methods—Tg2576 (n=16) and 5×FAD/ApoE4 knock-in mice (n=16), aged to 17 and 12 months, respectively, were treated with minocycline (50 mg/kg, i.p.) or saline every other day for two months. Brains were extracted and stained with X-34 (to quantify amyloid), Perl's blue (to quantify hemorrhage), and immunostained to examine Aβ load, gliosis (GFAP, Iba-1), and vascular markers of blood-brain-barrier integrity (ZO-1 and collagen IV). Brain extracts were used to quantify mRNA for a variety of inflammatory genes.

Results—Minocycline treatment significantly reduced hemorrhage frequency in the brains of Tg2576 and 5×FAD/ApoE4 mice relative to the saline-treated mice, without affecting CAA load. Gliosis (GFAP and Iba-1 immunostaining), gelatinase activity, and expression of a variety of inflammatory genes (MMP-9, Nox4, CD45, S-100b, Iba-1) were also significantly reduced. Higher levels of microvascular tight junction and basal lamina proteins were found in the brains of minocycline-treated Tg2576 mice relative to saline-treated controls.

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Authorship Experiments were conceived and designed by P.Y., S.M.G., D.M.H., and J.-M.L. Experiments were performed by P.Y., A.Z., Q. X., A. K., E.G., and R.P. Data were analyzed by P.Y., A.Z., and J.-M.L. The manuscript was written by P.Y., A.Z., and J.-M.L. with revisions from all others.

Potential Conflicts of Interest D.M.H. is a co-founder of C2N Diagnostics LLC, is on the scientific advisory boards of AstraZeneca, Genentech, and C2N Diagnostics, and consults for Eli Lilly. Washington University receives grants from the Tau Consortium, Cure Alzheimer's Fund, the JPB Foundation, Eli Lilly, Janssen, and C2N Diagnostics.

Conclusions—Minocycline reduced gliosis, inflammatory gene expression, gelatinase activity, and spontaneous hemorrhage in two different mouse models of CAA, supporting the importance of MMP-related and inflammatory pathways in ICH pathogenesis. As an FDA-approved drug, minocycline might be considered for clinical trials to test efficacy in preventing CAA-related ICH.

Keywords

APOE; Cerebral amyloid angiopathy; gliosis; Intracerebral hemorrhage; matrix metalloproteinase; minocycline

Introduction

Although ischemic stroke is the most common stroke subtype, intracerebral hemorrhage (ICH) results in greater morbidity and mortality¹. The most common cause of ICH in the elderly is cerebral amyloid angiopathy (CAA)², a disorder caused by the deposition of β -amyloid peptide (A β) in small cerebral vessels—most prominently, penetrating arterioles of the cortex^{3,4}. While CAA is commonly found in the brains of AD patients, it is also present in the elderly without AD⁵. ICH is a recurrent complication of CAA⁶; however, the pathogenesis of CAA-induced ICH has not been fully established. A better understanding of the underlying pathophysiology of CAA-induced ICH may result in the identification of potential targets for intervention. Further, the frequent recurrence of ICH in patients with CAA provides opportunity for prevention in this high-risk population.

The recognition that several mouse models of Alzheimer's disease (AD), including Tg2576 and APP23, also develop CAA and microvascular hemorrhage^{7, 8} has provided animal models to study CAA-induced ICH. These mice develop age-dependent accumulations of amyloid plaques and amyloid angiopathy, and at a later stage of pathogenesis spontaneously develop hemorrhage^{9, 10}. In addition to providing a model to study the cellular and molecular pathogenesis of CAA-related ICH, these mice provide a preclinical model to test the efficacy of interventions to prevent recurrent ICH.

Earlier studies demonstrated that exogenous A β could induce expression and activity of matrix metalloproteinase-9 (MMP-9) and MMP-2, gelatinases implicated in vascular remodeling and hemorrhage, in cerebral endothelial cells (CECs) and vascular smooth muscle cells, in vitro^{11,12,13}. Isolated rat microvessels exposed to exogenous A β demonstrated increased gelatinase expression and decreased endothelial tight junction proteins, claudin-1 and -5, suggesting a mechanism for blood-brain-barrier (BBB) breakdown¹⁴. Moreover, gelatinase expression was found to be increased in amyloid-laden vessel and especially prominent in vessels with evidence of prior hemorrhage in Tg2576 mice¹⁵. Pharmacological inhibition of MMP reduced not only CAA-associated gelatinase activity but microvascular oxidative stress in Tg2576 mice¹⁶. In another microvascular CAA model lacking spontaneous ICH (Tg-SwDI mice), minocycline reduced microglial activation and improved behavioral deficits¹⁷. These studies raised the possibility that enhanced gelatinase activity in CAA vessels might play a role in CAA-related ICH. In this study, we examined the efficacy of minocycline, an FDA-approved antibiotic known to inhibit

gelatinase activity and inflammation, in reducing spontaneous hemorrhage in two different mouse models of CAA.

Methods

Animals

Tg2576 (a.k.a. APPsw)¹⁸ mice of both genders, expressing human APP₆₉₅ with Swedish mutations at positions 670/671 under control of the prion promoter, were used in this study. 5×FAD/apoE4-knockin mice were produced by crossing 5×FAD transgenic mice (Tg7031 line)¹⁹ containing five familial AD mutations (APP K670N/M671L + I716V + V717I and PS1 M146L + L286V) with apoE4-targeted replacement mice (endogenous murine *ApoE* gene was replaced with the *APOE4* gene)²⁰. All experimental protocols were approved by the Animal Studies Committee at Washington University, and all studies were conducted in accordance with the US Public Health Service's Policy on Humane Care and Use of Laboratory Animals.

Minocycline Treatment

Mice were treated minocycline (50 mg/kg, i.p., Sigma-Aldrich, St. Louis, Mo., USA) or vehicle (saline) every other day for 8 weeks. Age- and sex-matched Tg2576 mice (17 ± 2 months old) were treated with minocycline. Eight mice were used in each group (5 females and 3 males). In addition, male 5×FAD/ApoE4 mice (12 ± 1 months old) were randomly assigned to treatment with minocycline (n=8) or saline (n=8). A power analysis was performed based on hemorrhage frequency (6.2 hemorrhages per hemisphere) and standard deviation (2.77) in 12 m Tg2576 mice²¹ (and unpublished data). A sample of size of 8 mice per group was found to provide at least 85% statistical power at a significance level of 5% to detect a 50% reduction in the mean number of hemorrhages due to the treatment. Total body weight was measured weekly and injection volumes were modified accordingly. Mice were sacrifice 6 h after the last injection.

Brain extraction and preparation

Mice were deeply anesthetized with isoflurane and transcardially perfused with 0.01M PBS. The brains were removed, and hemispheres were separated. Left hemispheres were immediately dissected, snap-frozen on dry ice, and stored at -80°C for biochemical analysis. Right hemispheres were fixed in 4% paraformaldehyde for 24 h and transferred to 30% sucrose in 0.1M PB. Coronal sections, 50 μm thick, were made with a sliding microtome and stored in 0.1M PB, 30% sucrose, and 30% ethylene glycol at -20°C until further analysis.

Histology

Right hemispheres were sectioned and stained for hemorrhage using Perl's Blue. Hemorrhages were counted by an assessor who was blinded to treatment assignments; and graded based on size (grade 1: 1-3 blue puncta; grade 2: 4-10 puncta; grade 3: >10 puncta)⁷ in 25-30 brain sections (50 μm thick) from each mouse, spaced 300 μm apart. To quantify CAA and amyloid plaque load, 4 regularly-spaced brain sections from rostral anterior commissure to caudal hippocampus from each mouse was stained with Congo Red or X-34. Area stained with the amyloid dye were quantified and expressed as mean fraction of section

area from each mouse²². Groups were compared using Student's t-test or Mann-Whitney Rank Sum Test.

Immunohistochemistry

For A β immunohistochemistry, brain sections were incubated with mouse anti-A β antibody (HJ3.4, 1:1000)²³ overnight at 4°C. Vectastain ABC (1:400) followed by 0.025% 3, 3'-diaminobenzidine tetrachloride in 0.25% NiCl₂ and 0.05% H₂O₂ was used to develop staining. Immunofluorescent double labeling was performed as follows: a mixture of primary antibodies was incubated with the brain sections overnight at 4°C. Fluorescently labeled secondary antibodies were then incubated at room temperature for 1 hr. Primary antibody pairs: mouse anti-GFAP monoclonal antibody (1:1000; sigma, St Louis, MO, USA) and rabbit anti-Iba1 antibody (1:1000; Wako Pure Chemicals Industries, Tokyo, Japan); rabbit anti-ZO-1 antibody (1:250, Invitrogen, Camarillo, CA, USA) and biotin-conjugated tomato lectin (1:400; Vector Laboratories, Inc., Burlingame, CA, USA); mouse anti-collagen IV antibody (1:200, Sigma) and tomato lectin. Secondary antibodies: Cy3-conjugated donkey anti-rabbit IgG antibody (1:800; Jackson ImmunoResearch); AlexaFluoro 488-conjugated donkey anti-mouse IgG antibody; AlexaFluoro 488-conjugated streptavidin (1:100; Molecular Probes).

A β ELISA

To measure A β , dissected cortices were homogenized in PBS and then in 5M guanidine in TBS, pH 8.0. After each homogenization step, samples were centrifuged at 12,000 rpm for 20 min, and supernatants were collected for sandwich ELISA²³. A β_{x-40} and A β_{x-42} were assessed using mouse monoclonal capture antibodies HJ2 (anti-A β_{35-40}) and HJ7.4 (anti-A β_{37-42})²⁴, respectively, and a biotinylated central domain antibody, HJ5.1 (anti-A β_{13-28}) was used as the detecting antibody followed by streptavidin-poly-HRP-40 (Fitzgerald Industries). All ELISAs were developed using Super Slow ELISA 3, 3',5,5'-tetramethylbenzidine (Sigma), and absorbance was read on a Bio-Tek Epoch plate reader (Winooski, VT) at 650 nm. Standard curves were generated from synthetic human A β_{1-40} or A β_{1-42} peptides (American Peptide, Sunnyvale, CA, USA).

RT-PCR

Messenger RNA was extracted from the left hemisphere and reverse transcribed with the cDNA Reverse Transcription kit. qPCR was performed using the ABI 7500 in the default thermal cycling mode with Power SYBR. Mouse β -actin was used as a normalization reference. Relative mRNA levels were calculated using the comparative C_t method, and expressed as a percentage of control. Please see <http://stroke.ahajournals.org> for table I primer sequences used in RT-PCR.

Gelatin-substrate zymography

Some of the left cortices were homogenized in 500 μ l of lysis buffer and supernatant was collected after centrifugation. The brain supernatant was incubated with 50 μ l of gelatin-Sepharose 4B (Pharmacia Biotech, Uppsala, Sweden) for 1 h with constant shaking. After centrifugation, the pellet was then incubated for 30 min with 50 μ l of elution buffer

consisting of lysis buffer plus 10% dimethylsulfoxide (DMSO). Each 4 μ l of eluted sample from the left cortex was mixed with 25 μ l of 4 $\times$ nonreducing sample buffer loaded onto 7.5% SDS-PAGE containing 0.1% gelatin, and electrophoresed at 180V. The gel was incubated for 20h at 37°C in buffer for 20 min and was stained for 1 h with 0.1% Coomassie Blue ²⁵.

Statistical Analysis

All data were expressed as mean $\pm$ SD. Differences between groups were determined using two-tailed Student's t test for two-group comparisons, and one-way ANOVA followed by post hoc Tukey's test for comparison among multiple groups. Differences were deemed statistically significant at * $p < 0.05$ and ** $p < 0.01$.

Results

Minocycline reduced hemorrhage frequency in Tg2576 mice

Mice tolerated minocycline treatment without adverse effects: average weights before, during, and after treatment did not differ between groups (Supplemental Fig 1A). One mouse died after 4 weeks of minocycline treatment, and was subsequently replaced by another age- and sex-matched mouse.

After two months of treatment with minocycline (50 mg /kg, ip, q.o.d.) or saline, brains sections were stained with Congo Red to assess amyloid load and Perl's Blue to quantify hemorrhage frequency. The Tg2576 mice treated with minocycline had almost half the number of hemorrhages compared to those treated with saline (Fig 1B). Hemorrhages were graded by size, based on the methods of Jucker (Fig 1A)⁷. Grade 1 hemorrhages were most frequent, regardless of treatment. Though minocycline treatment had fewer microhemorrhages for all grades, only grade 2 hemorrhages had statistically significantly fewer microhemorrhages in the minocycline-treated group compared to saline treatment (Fig 1C). Five of the eight mice were females, and when compared separately, showed a trend toward fewer microhemorrhages (12 vs. 7.4 hemorrhages/hemisphere, $p = 0.059$).

CAA load was quantified by measuring the cross-sectional area of Congo Red stained vessels and expressed as % area of the total section. There was no significant difference in CAA load between minocycline-treated vs. saline-treated mice (Fig 1D). In addition, minocycline treatment appeared to have little or no effect on amyloid plaque load. Plaque load, determined using anti-A β antibody (HJ3.4) and amyloid dye (X-34), was unchanged after chronic minocycline treatment (Fig 2B). These findings were corroborated by brain tissue A β levels measured by ELISA. Soluble A β was unchanged by minocycline treatment; however, insoluble A β_{x-40} was modestly increased by minocycline (Fig 2C). These findings are consistent with previous studies which have found variable effects of minocycline on amyloid accumulation in a variety of APP transgenic mouse models^{17,26-28}.

Minocycline reduced gliosis and gelatinase activity

A large body of literature demonstrates that chronic administration of minocycline inhibits inflammation and gliosis in numerous different models of CNS disease (for review, see ²⁹). In Tg2576 mice, minocycline treatment markedly decreased astrocytosis (GFAP

immunohistochemistry) and microgliosis (Iba-1 immunohistochemistry), in both cortex and hippocampus (Fig 3A-D). Real-time qPCR confirmed the reduction in gliosis with reduced expression of Iba-1, CD-45, and S-100b mRNA (Fig 4A). In addition, several other genes associated with vascular inflammation were reduced by chronic minocycline treatment, including NOX4 and MMP-9. NOX4 expression has been reported to be selectively increased in other APP transgenic models³⁰. To confirm that decreased MMP mRNA resulted in a reduced MMP activity, we performed gelatin zymography on brain extracts from minocycline-treated mice. Minocycline significantly reduced both MMP-9 and MMP-2 activity compared to saline-treated controls (Fig 4B, C).

Minocycline enhances expression of BBB proteins

Previous studies have demonstrated that vascular MMP-9 expression can lead to degradation of basement membrane proteins (including collagen IV) and endothelial tight junction proteins (ZO-1, occluding, and claudin 5), leading to compromise of the BBB³¹. To determine if minocycline reduces degradation of proteins involved in neurovascular integrity, we examined the expression of the tight junction protein, ZO-1, and the basement membrane protein collagen IV (ColIV). Focusing on vascular expression, we co-stained all sections with biotin-conjugated tomato lectin to visualize capillaries and small arterioles (Fig 5A, B). We quantified expression of these antigens (ZO-1 and Col IV) within vessels stained with lectin (normalized to the lectin-positive vascular footprint), and expressed as % capillary area. Minocycline treatment increased the expression of vascular ZO-1 and Col IV by more than 2-fold (Fig 5C, D). Staining of vessels with tomato lectin was unchanged by minocycline treatment (data not shown), indicating that minocycline increased expression of ZO-1 and Col IV without altering vessel density.

Minocycline reduces microhemorrhage in 5x FAD ApoE4 mice

Preclinical stroke studies over the past three decades have demonstrated the importance of replication using different animal models (STAIR criteria)^{32, 33}. To confirm the efficacy of minocycline in reducing spontaneous hemorrhage, we repeated the minocycline study using a novel mouse model of CAA-related spontaneous hemorrhage. 5x FAD mice (line 7031)¹⁹ with targeted replacement of mouse Apolipoprotein E with human Apolipoprotein E ε4 (5x FAD/ApoE4) develop amyloid plaques and CAA by 5-6 months of age, and hemorrhages appear spontaneously beginning at 7 months of age (unpublished observation). Minocycline treatment (50 mg /kg, ip, qOD) was initiated at 12 months of age (when significant CAA and hemorrhages were already present) and treated for 2 months. Brain sections were stained with Perl's Blue to quantify hemorrhage frequency. Minocycline treatment reduced the number of hemorrhages by greater than 50%. Both grade 2 and grade 3 hemorrhages were significantly reduced by minocycline (Fig 6A). Similar to the study in Tg2576 mice, 2 months of minocycline treatment did not alter CAA or plaque load (Fig 6 B-D), nor did it affect body weight over this period of time (Supplemental Fig IB).

Discussion

CAA is the leading cause of ICH in the elderly and recurrence is common; yet, there are no treatments for the prevention of CAA-related ICH. In the present study, we examined the

efficacy of chronic minocycline treatment on hemorrhage frequency in two different mouse models of CAA. We found that minocycline reduced hemorrhage frequency in both CAA models without affecting amyloid load. Minocycline also inhibited MMP-2 and -9 activity, reduced gliosis and expression of select neuroinflammatory genes in brains of Tg2576 mice. These changes occurred in parallel with increases in vascular tight junction and basal lamina proteins, suggesting that minocycline preserved structural integrity of cerebral vessels, leading to reduction in hemorrhage frequency. In both models, treatment was initiated during advanced stages of disease, when microhemorrhage was already present. Therefore, the reduced frequency of microhemorrhage likely reflects a reduction in the accumulation of microhemorrhage during the 2-month treatment period.

Minocycline, a semi-synthetic second generation tetracycline with FDA-approval for the treatment of acne vulgaris, has a large literature supporting its neuroprotective properties³⁴. The tetracyclines were first described to have anti-inflammatory, anti-apoptotic, and anti-gelatinase activities limiting angiogenesis and tumor metastasis³⁵⁻³⁸. Minocycline became the tetracycline of choice for central nervous system (CNS) delivery, owing to its lipophilic nature allowing it to pass through the blood-brain barrier³⁹. Indeed, minocycline has emerged as the most effective neuroprotectant among the tetracyclines, demonstrating activities in experimental models of cerebral ischemia^{40, 41}, traumatic brain injury⁴², and several neurodegenerative diseases such as Parkinson's disease^{43, 44}, Huntington's disease^{45, 46}, and amyotrophic lateral sclerosis⁴⁷. In AD mouse models, minocycline has been shown to improve behavioral performance, but have little effect on amyloid plaque load^{17,26,28}. Consistent with these earlier reports, our findings also demonstrate little effect on plaque accumulation.

Double-blind randomized clinical trials examining the efficacy of minocycline in a variety of neurodegenerative disease, including Parkinson disease^{48, 49}, Amyotrophic lateral sclerosis (ALS)^{50, 51}, and Huntington's Disease^{52, 53} have not demonstrated definitive efficacy in attenuating disease progression. In these clinical trials, the precise target of minocycline's mechanism of action was not identified, and therefore target engagement was not confirmed. In all trials except for one, safety after chronic administration of minocycline in these aged populations was clearly demonstrated. In the phase III trial in ALS patients, neurological deterioration occurred more rapidly in the minocycline group compared to the placebo group⁵¹; however, a higher dose of minocycline was used in this trial compared to all others.

The dose of minocycline used in the current study (50 mg/kg, ip) produces peak plasma concentrations extrapolated to be equivalent to that following 1000 mg, po in humans⁵⁴, according to published pharmacokinetic studies in rodents⁵⁵. Given every other day, this is approximately 2.5 times the usual daily dose of minocycline for treatment of bacterial infections. Minocycline is a known sclerosing agent and has been reported to induce pleuritis in rodents when injected intrapleurally⁵⁶. In our study, we administered minocycline via intraperitoneal injection every other day to minimize its sclerosing effects. Although one mouse in the minocycline-treated group died after 1 month of dosing; the remaining animals appeared healthy (maintaining constant body weight) without overt evidence of peritonitis.

Our studies demonstrate that minocycline inhibits gliosis, certain vascular inflammatory mediators, mediators of oxidative stress, and gelatinases, consistent with findings in other studies. All of these mediators have been implicated in CAA-related pathology. Chronic minocycline treatment of transgenic mice expressing the vasculotropic Dutch/Iowa (E693Q/D694N) mutant APP did not alter CAA load but reduced microgliosis and inflammation in the brains of these mice. Moreover, cognitive deficits were improved with minocycline treatment¹⁷. Of note, this model of microvascular CAA does not result in spontaneous hemorrhage. In another study, CAA-induced free radical formation was inhibited by chronic treatment with minocycline and other more specific MMP-9 inhibitors in Tg2576 mice¹⁶. While these inhibitors did not have a direct antioxidant effect, it is hypothesized that minocycline's antioxidant effect was indirectly mediated via its MMP-9 inhibitory activity. Therefore, the effect of minocycline in attenuating CAA-related pathology has been confirmed in several animal models and by several independent laboratories.

Our findings are the first to demonstrate that chronic minocycline treatment reduced spontaneous hemorrhage in an established CAA model (Tg2576 mice). In addition, we have confirmed these findings in another newly developed CAA mouse model, the 5×FAD/ApoE4 mouse (Fig 6). This mouse model is highly relevant to human disease, because ApoE4 genotype has been independently associated with severe CAA, CAA-related vasculopathy, and intracerebral hemorrhage^{57, 58}. Even in the absence of CAA pathology, recent studies suggest that the ApoE4 genotype alone may induce neurovascular injury with resultant BBB leak. A recent study utilizing targeted replacement of human ApoE genes in mice demonstrated that ApoE4 specifically induced a pro-inflammatory pathway in pericytes resulting in the induction of MMP-9, degradation of BBB proteins (ZO-1 and collagen IV), and BBB leak³¹. In parallel with its MMP-9 and -2 inhibitory activity, we have found that minocycline preserved elevated expression of the tight junction protein, ZO-1, and the basement membrane protein, collagen IV, in brain microvessels (identified with tomato lectin, Fig 5). Both of these proteins are known substrates of the gelatinases^{59, 60}.

A limitation of this study, and other minocycline treatment studies, is the absence of a definitive target that is responsible for its mechanism of action. Circumstantial evidence from CAA animal models suggests that the gelatinases may be the relevant target. MMP-9 is expressed in CAA vessels, especially those with evidence of prior hemorrhage¹⁵. Inhibition of MMP's with non-specific inhibitors (such as minocycline) reduced spontaneous hemorrhage (see above) and neurobehavioral outcomes¹⁷. Further, more specific MMP-9 inhibitors reduced other downstream consequences of CAA, including CAA-induced oxidative stress¹⁶.

Conclusion

The current study demonstrates the efficacy of minocycline in reducing spontaneous hemorrhage in two different mouse models of CAA, during advanced stages of disease. Minocycline reduced gliosis, inhibited the expression of inflammatory mediators and gelatinase activity, resulting in the preserved expression of the BBB proteins, ZO-1 and collagen IV. Because minocycline is already FDA-approved and known to be safe in elderly

populations, we propose that minocycline be considered as a candidate treatment in clinical trials for the prevention of ICH in CAA patients.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Chronic minocycline treatment reduced hemorrhage frequency without altering CAA load in Tg2576 mice. **A.** Representative photomicrographs of Tg2576 brain sections stained with Perl's Blue and Congo Red, to demonstrate microhemorrhage size (grade 1, 1-3 puncta; grade 2, 3-10 puncta; grade 3, >10 puncta). Bar=50 μ m. **B.** The number of hemorrhages was significantly lower in the minocycline-treated mice compared to controls (n=8 mice/group; 30 brain slices per animal). **C.** Comparison of hemorrhage frequency by grade. **D.** CAA, stained with Congo Red, was quantified and expressed as percent area (4 equally spaced brain sections from each animal). No significant difference was found between treatment groups. Values are mean $\pm$ SD. * p < 0.05.

Figure 2.

Minocycline had little effect on amyloid plaque load in Tg2576 mice. Plaques stained with HJ3.4 immunohistochemistry (**A**, bar=1mm) or X-34 were quantified (n=8 mice/group), and showed no significant difference between treatment groups (**B**). PBS-soluble and insoluble Aβ were serially extracted from cortex and hippocampus, and quantified with ELISA (**C**). No difference in Aβ_{x-40} and Aβ_{x-42} levels were found between groups, except in PBS-insoluble Aβ_{x-40} levels (**p*=0.045). Values are mean ± SD.

Figure 3.

Minocycline reduced gliosis in Tg2576 mice. Representative brain sections from Tg2576 mice show dramatic decreases in GFAP (**A**) and Iba-1 (**B**) immunostaining following chronic treatment with minocycline. Bars; left panel=500 μ m, right panel=20 μ m. GFAP and Iba-1 immunostaining was quantified, normalized to plaque load, and expressed as % area; minocycline-treated mice showed significantly reduced astrocytosis (**C**) and microgliosis (**D**) compared to saline-treated mice. Values are mean $\pm$ SD.; n = 8 mice/group. * $p < 0.01$.

Figure 4.

Minocycline decreased expression of vascular inflammatory genes in Tg2576 mice. **A.** QPCR was performed on cortical extracts using primers indicated. mRNA for MMP9, NOX4, CD45, s-100, and Iba1 were significantly reduced in minocycline-treated mice compared to control (n = 8 mice/group). Gelatin zymography (**B**) revealed that minocycline treatment reduced both MMP-2 and MMP-9 activities in the brains of Tg2576 mice compared to controls (n=3 mice/group) (**C**). Values are mean $\pm$ SD.; * p < 0.05.

Figure 5.

Minocycline increased vascular ZO-1 and Col IV immunostaining in Tg2576 mice. Confocal microscopy of ZO-1 (A), Col IV (B) and lectin-positive microvessels (A-B, bar=50μm) in the cortex of Tg2576 mice treated with saline or minocycline. Minocycline-treated mice showed greater microvascular ZO-1 (C) and Col IV (D) immunostaining compared to saline-treated mice. Values are mean ± SD.; n = 8 mice/group. * $p < 0.01$.

Figure 6. Chronic minocycline treatment reduced hemorrhage frequency in 5×FAD/ApoE4 mice. **A.** 5×FAD/ApoE4 mice treated with minocycline (n=8) for 8 weeks showed fewer microhemorrhages compared to mice treated with saline (n=8). CAA load (**B**, Congo Red-stained vessels), and plaque burden (**C**, HJ3.4 immunostained and X-34 stained plaques) were similar between groups. **D.** Soluble and insoluble Aβ_{x-40} and Aβ_{x-42} (quantified with ELISA) showed no differences between groups. Values are mean ± SD. **p* < 0.05, ***p* < 0.01.