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The effect of Ginkgo biloba on the expression of intermediate–early antigen (c-fos) in the experimentally induced anosmic mouse

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Abstract

Objective: Treatment of olfactory dysfunction is very difficult and has limited modality. Treatment with steroids has been used in patients with olfactory dysfunction but the side effects of steroid need to be weighed against its potential benefits. In the present study, the effect of systemic administration of dexamethasone and EGb 761 on damage to olfactory mucosa produced by zinc sulfate was examined. Expression of the immediate-early antigen (IEG), c-fos, in the olfactory bulb and piriform cortex was used to determine the effects of treatment.
Methods: Young adult CD1 mice (6 to 8 weeks old, male) were used. After anosmic mice were made by bilateral intranasal irrigation with 0.2 ml of 5% (0.17 M) zinc sulfate, anosmia was confirmed by a food finding test. Four groups of anosmic mice were studied: a steroid group (steroid injection group, $n = 12$), an EGb group (EGb injection group, $n = 12$), a steroid-EGb group (steroid and EGb injection group, $n = 12$), and a control group (anosmic mice and no Tx. $n = 12$). The olfactory bulb and piriform cortex of four mice in each group were obtained at 1, 2, and 3 weeks after instillation of zinc sulfate by cardiac perfusion, and immunohistochemical staining for c-fos was also performed to evaluate brain activity. In approximately 10 well-defined glomeruli of the olfactory bulb and in one side of the piriform cortex, c-fos (+) cells were counted. Statistical analyses were performed by Kruskal–Wallis one-way analysis of variance (ANOVA) by rank.
Results: In all experimental groups, c-fos (+) cells increased in a time-dependent manner. The combination treatment of steroid and EGb was the most effective and the no-treatment group the least effective 1 week later after zinc sulfate irrigation. However, 3 weeks later after zinc sulfate irrigation, there was no statistically significant differences in the number of c-fos positive cells among all 4 groups (3 treatment groups and the control group).
Conclusion: The combination treatment of EGb and steroid enhanced the regeneration of the olfactory pathway after olfactory mucosal injury by zinc sulfate. Our study suggests that EGb could be an effective treatment option for olfactory dysfunction.

Keywords: Olfactory dysfunction; Ginkgo biloba; Steroid

1. Introduction

Olfactory dysfunction can cause significant complications such as loss of appetite, weight loss, and the onset of depression at all ages. Olfactory dysfunction is caused by obstructive nasal and sinus disease, post-upper respiratory infection, head trauma, aging, congenital conditions, toxins, and idiopathic issues. Obstructive nasal and sinus disease can be treated by surgical and medical therapy but there are no proven therapies for other etiologic categories. Generally steroid treatments have been used for olfactory dysfunction patients but their effects are not consistent. Vitamin A, mineral treatments, aminophylline, and so on have been proposed for the treatment of olfactory dysfunction but their results have not always been satisfactory [1].

The leaves and seeds of Ginkgo biloba and its extract, EGb 761, have been used since ancient times in Asian cultures to treat many diseases. Extracts of Ginkgo biloba

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leaves contain approximately 300 chemicals. It is not clear which of these components are responsible for their biological actions but it is thought that the flavonoids and the ginkgolides are the principal active components. The effects of EGb 761 have been studied clinically and experimentally on an extensive range of disorders and diseases, including cerebral insufficiency, Alzheimer's disease, multi-infarct dementia, tinnitus, vestibular disorder, and so on. Although the neuroprotective mechanism of EGb remains unexplored, several lines of evidence suggest that EGb allows mitochondria to maintain their respiratory activities under ischemic conditions, and may play a protective role in homeostasis of inflammation and oxidative stress. The scavenging activity of free radical of EGb has been proven in different *in vitro* and *in vitro* studies [2,3].

In the present study, the effect of systemic administration of dexamethasone and EGb 761 on olfactory mucosa damaged by zinc sulfate was examined. Expression of the immediate-early antigen (IEG), c-fos, in the olfactory bulb and piriform cortex was used to determine the effects of treatment.

2. Materials and methods

2.1. Animals

Young adult BCF1 mice (6 and 8 weeks old, male) weighing between 20 and 25 g served as subjects in this study. Thirty-six mice were randomly allocated into the study group (nine groups of four animals each), and 12 mice were allocated to the control group (three groups of four animals each).

This experiment was performed in accordance with the ''Guide for the Care and Use of Laboratory Animals,'' as approved by the National Research Council and accredited by the Association for Assessment and Accreditation of Laboratory Animal Care, International (AAALAC International).

2.2. Anosmia model by zinc sulfate

Anosmic mice were created by bilateral intranasal irrigation with 0.2 ml of 5% (0.17 M) zinc sulfate in dH_2O, by using 1 cm^3 syringe fitted with a blunt-end, polished, 25-gauge needle. Anosmia was confirmed by a food finding test as described previously [4].

2.3. Ginkgo biloba extract and steroid treatment

After mice had been confirmed to have anosmia by the food finding test, we divided mice into four groups by the type of treatment. After then, steroid was intraperitoneally administered to group 1 mice at a dose of 1 mg/kg a day; steroid and EGb 761 to group 2 mice at a dose of 1 mg/kg and 100 mg/kg a day respectively; EGb 761 to group 3 mice at a dose of 100 mg/kg a day; but the control group had no treatment.

Each group was then subclassified into three subgroups (groups A, B, and C) by the time of sacrifice. In group A: the animals were killed in the first week following treatment; in group B: the animals were killed in the second week; in group C: the animals were killed in the third week.

2.4. Tissue preparation and staining

Mice were transferred to the laboratory at least 24 h before treatment or perfusion in order to avoid c-fos activation. We used the cardiac perfusion technique, in which the mice were anesthetized, the chest cavity was opened, and the mice were perfused via the left ventricle with 1% paraformaldehyde (Sigma Chemical Company, St. Louis, MO, USA) in citrate buffer (pH 4.2), which was prewarmed to 37 °C to prevent vasoconstriction. The perfusion lasted for 30 min under a pressure of 120 mmHg to clear the blood from the vessels. Immediately after perfusion, the mice were decapitated and the olfactory bulb and brain were enucleated. Harvested tissues were fixed in 10% paraformaldehyde for 24 h, embedded in paraffin blocks, and sliced. The slice thickness was 4–5 μm.

2.5. Immunohistochemical staining

Tissue blocks were cut in serial coronal sections on a cryostat and then rinsed in phosphate-buffered saline (PBS) for 30 min. The sections were washed in PBS for 15 min, followed by 3% hydrogen peroxide for 2 min to quench endogenous peroxidase activity. Sections were placed in PBS containing 10% goat serum for 20 min. They were then incubated in a primary antibody to c-fos (1:5000 dilution, CalBiochem, San Diego) overnight at 4 °C. After sections were rinsed in PBS, they were incubated in N-histofine goat anti-rabbit IgG (Nichirei, Tokyo Japan) for 1 h. After a serial rinse in PBS and Tris buffer, the c-fos reaction product was made visible by incubating sections with hydrogen peroxide and diaminobenzidine. The sections were then washed in distilled water, mounted in PBS, and air-dried overnight. Sections were subsequently cleared in ascending alcohol and xylene baths. Permount medium was used as a coverslip. Sections were examined under a light microscope. The c-fos reaction product appeared as dark brown staining in the cell nucleus (Figs. 1 and 2).

2.6. Analysis and statistics

In approximately 10 well-defined glomeruli of the olfactory bulb and in one side of the piriform cortex, c-fos (+) cells were counted. All data are presented as the mean ± S.D. (standard deviation). Statistical comparisons between each of the groups were made by Kruskal–Wallis one-way analysis of variance (ANOVA) by rank. A p value of less than 0.05 was considered statistically significant. The