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LABORATORY STUDY

Dosage dependence of the effect of *Ginkgo biloba* on the rat retinal ganglion cell survival after optic nerve crush

K Ma[1], L Xu[1], H Zhan[1], S Zhang[1], M Pu[1] and JB Jonas[1,2]

Abstract

Purpose **To investigate the dosage dependence of the *Ginkgo biloba* effect on retinal ganglion cell survival in the rat optic nerve crush model.**

Methods **The study included 56 Sprague–Dawley rats, the right optic nerve of which was crushed in a standardized manner. Two hours after the crush and once daily during the follow-up, the animals received intragastral applications of saline (saline group; $n = 13$), or of a *G. biloba* extract of 0.25% concentration ($n = 14$; low-dosage group), 1% concentration ($n = 15$; medium-dosage group), or 4% concentration ($n = 14$; high-dosage group). At 23 days after the optic nerve crush, the retinal ganglion cells were retrogradely labelled by injecting 3% fluorogold into the superior colliculi of the brain. At 4 weeks after baseline, the animals were killed. Retinal flat mount photographs were assessed for number and density of the retinal ganglion cells.**

Results **The mean survival rate defined as the ratio of retinal ganglion cell density in the right eye with optic nerve crash divided by the retinal ganglion cell density in left eye without optic nerve intervention increased significantly ($P < 0.001$) from $58.4 \pm 9.0\%$ in the saline group to $68.5 \pm 5.7\%$ in the low-dosage group, to $73.7 \pm 6.4\%$ in the medium-dosage group, and to $74.2 \pm 6.8\%$ in the high-dosage group.**

Conclusions **Intragastral applications of a *G. biloba* extract applied after an experimental and standardized optic nerve crush in rats were associated with a higher survival rate of retinal ganglion cells in a dosage-dependent manner.**

[1]Department of Ophthalmology, Beijing Tongren Hospital, Beijing Institute of Ophthalmology, Capital University of Medical Science, Beijing, China

[2]Faculty of Clinical Medicine Mannheim, Department of Ophthalmology, University of Heidelberg, Mannheim, Germany

Correspondence: L Xu or JB Jonas, Department of Ophthalmology, Beijing Institute of Ophthalmology, 17 Hougou Street, Chong Wen Men, Beijing 100005, China
Tel: +49 621 383 2652; Fax: +49 621 383 3803.
E-mail: Jost.Jonas@augen.ma.uni-heidelberg.de

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Introduction

Atrophy of the retina and the optic nerve are the main causes for irreversible visual impairment and blindness worldwide.[1] For some optic nerve and retinal disorders, treatment strategies are available to stop or at least to decrease further progression.[2] For retinal degenerations and dystrophies, as well as many types of optic neuropathies, effective therapies to prevent further cell loss are not generally available. It has, therefore, been desirable to develop treatment strategies by which the retina and optic nerve can be protected from degeneration by helping the retinal cells to survive even under difficult conditions. Recent studies have reported that substances such as ciliary neurotrophic factor,[3–5] brain-derived growth factor,[6] memantine,[7,8] and *Ginkgo biloba* may have a stabilizing or protecting effect on retinal cells in experimental conditions.[9–22] Particularly for *Ginkgo biloba*, potential antioxidant functions have been discussed to be helpful for impaired retinal cells such as Müller cells.[14]

As an effective neuroprotection would clinically be rather important, and since the studies published so far have not been conclusive with respect to a proven clinical benefit of the substances for patients, it was the purpose of this study to reassess the potentially neuroprotective effect of *G. biloba* by examining a dosage-dependent effect on the survival of

retinal ganglion cells after an experimentally induced standardized optic nerve trauma.

Materials and methods

All experiments were carried out in accordance with the statement of the Association for Research in Vision and Ophthalmology (ARVO) for the use of animals in ophthalmic and vision research, and they were approved by the Beijing Tongren Hospital Biomedical Research Panel. Fifty-six male adult Sprague–Dawley rats with a weight of 200–240 g were supplied by the Beijing Institute of Drugs and Bioproduction Research and were housed in a 12-h light/12-h night cycle with free access to food and water. The animals were randomly divided into a low-dosage *G. biloba* study group ($n=14$ rats), a medium dosage *G. biloba* study group ($n=15$ rats), a high-dosage *G. biloba* study group ($n=14$ rats), and a saline group ($n=13$ rats).

All animals underwent a right optic nerve crush as described previously; the left optic nerve was not injured and acted as a control.[23–25] To perform the optic nerve crush, the animals were anaesthetized with an intraperitoneal injection of 6% chloral hydrate in 0.9% sodium chloride. With the aid of a binocular operation microscope (Olympus–85, Olympus Co., Tokyo, Japan), a right lateral canthotomy was performed. The conjunctiva was incised at a distance of 2 mm to the corneal limbus. The retractor bulbi muscle was separated and the optic nerve was carefully exposed. A 40-g power microclip (TKF-2, microclipping, AROSurgical, Newport Beach, CA USA) was used to clamp the optic nerve at about 2 mm behind the globe for 60 s. Throughout the surgery, care was taken to ensure that the operation did not lead to a lesion of the ophthalmic artery. To observe the retinal vessels, a drop of saline covered by a piece of flat glass was put into the rat cornea to compensate the refractive power of the corneal surface. The fundus of the eye, including the retinal vessels, could be visualized using the microscope. At maximum 2 min after the optic nerve crush, the retinal blood circulation was observed to be present in all animals. The conjunctiva and the canthotomy were closed by sutures and an antibiotic ointment was applied to the wound.

Using a flexible catheter, which was introduced through the mouth into the oesophagus of the animals, all rats received intragastral applications of saline or of *G. biloba* 2 h after the optic nerve crush and once daily during the whole follow-up. After each application, the catheter was removed. The animals of the injury and saline-only group received intragastral applications of 0.9% saline in a dosage of 5 ml/kg. The rats of the low-dosage *G. biloba* study group, of the medium-dosage *G. biloba* study group, and of the high-dosage *G. biloba* study group received intragastral applications of an extract of *G. biloba* (EGb761; Promod Pharmaceuticals Co. Ltd Shanghai, China) in concentrations of 0.25% EGb761 5 ml/kg (low-dosage group), 1% EGb761 5 ml/kg (medium-dosage group), and 4% EGb761 5 ml/kg (high-dosage group), respectively. The concentration of 0.25% EGb761 5 ml/kg is equivalent to 12.5 mg EGb761/kg body weight. As EGb761 contains about 24% flavone glycosides (which have usually been taken as the standard substance in *Ginkgo biloba*), a concentration of 12.5 mg EGb761/kg is equivalent to 3-mg flavone glycosides/kg. It roughly equals the dosage of one pill of *G. biloba* given three times daily to a human. The concentrations of 1% EGb761 5 ml/kg and 4% EGb761 5 ml/kg are equivalent to dosages of 12-mg flavone glycosides/kg and 48-mg flavone glycosides/kg.

At 23 days after baseline of the study, the retinal ganglion cells were retrogradely labelled as described in detail previously (Figure 1).[23–27] Briefly, after a craniotomy was performed, a 10-μl Hamilton syringe was stereotactically inserted (model 900, David Kopf Instrument Ltd, Tujunga, CA, USA) and positioned at the centre of superior colliculus. Three microlitres of 3% fluorogold (Fluorochrome Inc., Denver, CO, USA) was injected into both superior colliculi of each hemisphere. This injection procedure usually took about 1 h to be completed. After the tracer injections, the wound was sutured, antibiotic ointment applied, and the animal was allowed to recover. The principal investigator (KM) had a practical experience of the study procedures obtained in more than 50 animals before the start of the present investigation.

At 4 weeks after the optic nerve crush and at 5 days after the fluorogold injection into the superior colliculi, the animals were killed by an overdose of intraperitoneally administered 6% chloral hydrate. The

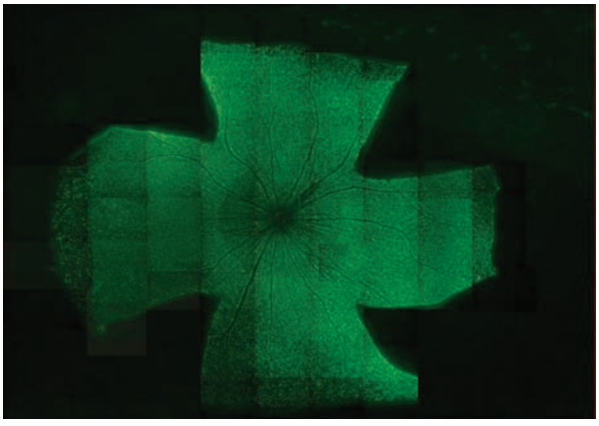

Figure 1 Whole montage of a flat-mounted retina of a normal uncrushed left eye, with the retinal ganglion cells retrogradely labelled by injection of fluorogold into the superior colliculi.

globes were immediately enucleated after the death of the animals. The enucleated eyes were fixed in 4% paraformaldehyde for 2 h. Performing a limbus parallel incision about 1 mm behind the limbus, the cornea was removed and the globe was opened. The retina was isolated from the eyecup, and four radial cuts were performed. The retinas were flat mounted on slides with the vitreal surface of the retina oriented upward. The retina was air-dried and immersed in an antifade mounting medium (Vector Shields, Vector Laboratories, Burlingame, CA, USA) and coverslipped. Photographs were taken with a fluorescent microscope (HA-1 Olympus Ltd, Tokyo, Japan) from each of the four quadrants of the retina at a distance of 2 mm from the optic disc centre with magnification of $\times 125$. The photographs were scanned into a computer (Palaroid Scan 4000). The number of retinal ganglion cells were counted on each photograph automatically using a software system (CPAS software, Daheng software Ltd, Beijing, China).[7] About 500–700 cells per eye were counted. The photographs covered a retinal area of about 0.25 mm^2. The spatial density of the retinal ganglion cells was calculated by the mean number of retinal ganglion cells in the quadrant divided by the area of the quadrant. The survival percentage of retinal ganglion cells was defined as the density of the retinal ganglion cells in the right eye with the optic nerve crash divided by the retinal ganglion cells density in left eye without optic nerve trauma, and then multiplied with 100%. The assessment of the photographs was performed in a masked manner without knowledge to which group the eye examined belonged.

The statistical analysis was performed by using a commercially available statistical software package (SPSS for Windows, version 15.0, SPSS, Chicago, IL, USA). The statistical significance of intergroup differences in the retinal ganglion cell densities was examined by the student's *t*-test for unpaired samples. The statistical significance of intereye differences in the retinal ganglion cell densities was examined by the student's *t*-test for paired samples.

Results

Taking the whole group of animals, the number of labelled retinal ganglion cells was significantly ($P<0.001$) higher in the left eyes without optic nerve crush than in the right eyes with the optic nerve crush (mean ± SD: 1959 ± 183 cells/mm^2; median: 2034 cells/mm^2; range: 1544–2236 cells/mm^2) *vs* 1351 ± 226 cells/mm^2; median: 1318 cells/mm^2; range: 856–1936 cells/mm^2).

The retinal ganglion cell density in the left eyes without optic nerve intervention did not vary significantly ($P=0.10$) between neither the saline group (1883 ± 186 cells/mm^2; median: 1884 cells/mm^2; range: 1608–2184 cells/mm^2) and the whole of the study groups (1983 ± 178 cells/mm^2; median: 2052; range: 1544–2236 cells/mm^2; 95% confidence interval (CI) of the difference between both groups: −222 cells/mm^2 to 23 cells/mm^2) nor each of the groups ($P=0.37$; Figure 2).

The retinal ganglion cell density in the right eyes with optic nerve crush of the rats with intragastral saline application ranged between 856 cells/mm^2 and 1253 cells/mm^2, indicating an interindividual variability of 1–1.46. The retinal ganglion cell density in the right eyes with optic nerve crush was significantly ($P<0.001$) lower in the saline group (1089 ± 137 cells/mm^2; median: 1136 cells/mm^2; range: 856–1253 cells/mm^2) than in the whole of the study groups (1430 ± 184 cells/mm^2; median: 1412 cells/mm^2; range: 1048–1936 cells/mm^2; 95% CI of the difference between both groups: −437 to −244 cells/mm^2).

Correspondingly, the mean survival rate defined as the ratio of retinal ganglion cell density in the right eye with optic nerve crash divided by the retinal ganglion cell density in the left eye without optic nerve intervention was significantly ($P<0.001$) higher in the whole of the study groups with *G. biloba* applications (58.4 ± 9.0%; median: 62%; range:41–74%) than in the saline group with saline applications (72 ± 7%; median: 74%; range: 60–88%; 95% CI of the difference between both groups: −20 to −8%; Figure 3).

The comparison of the different *G. biloba* dosage groups with each other and with the saline group revealed a significant ($P<0.001$) increase in the survival rate of retinal ganglion cells with increasing dosage of *G. biloba* (Figures 3 and 4; $P<0.001$; equation of the regression line survival rate $= 0.61 + 0.052 \times$ group number; correlation coefficient: $r=0.63$; 95% confidence intervals of the slope of the regression line: 0.034, 0.070). The survival rate improved significantly from 58.4 ± 9.0% in the saline group to 68.5 ± 5.7% in the low-dosage *G. biloba* group ($P=0.003$) and from the low-dosage *G. biloba* group to 73.7 ± 6.4% in the medium-dosage *G. biloba* group ($P=0.04$). The medium-dosage *G. biloba* group and the high-dosage *G. biloba* group (74.2 ± 6.8%) did not vary significantly ($P=0.56$) in the retinal ganglion cell survival rate (Figure 3).

Performing a multivariate analysis, with the survival rate of the retinal ganglion cells as dependent variable and the group number and the density of retinal ganglion in the untreated left eye as dependent variables, it is revealed that the survival rate was significantly associated with the group number ($P<0.001$; odds ratio (OR): 0.055; 95% CI: 0.037, 0.073), whereas the retinal ganglion cell density in the untreated left eyes was not significantly associated with the survival rate ($P=0.21$). If the multivariate analysis was performed with the

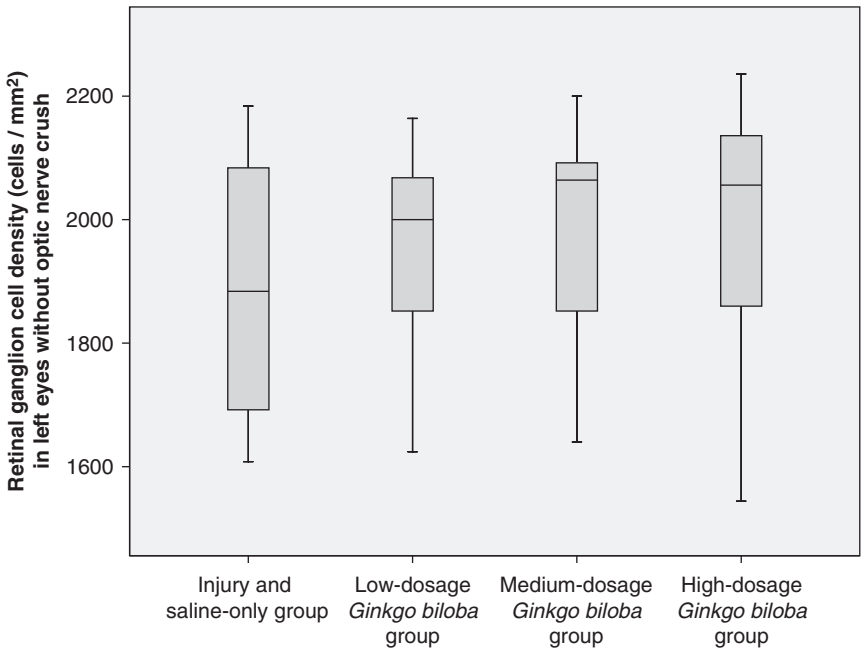

Figure 2 Boxplots showing the distribution of the retinal ganglion cell density of the left eye without optic nerve crush in rats receiving either intragastral applications of saline or *Ginkgo biloba* in increasing concentrations. The retinal ganglion cell density did not vary significantly between neither the injury and saline-only group and the whole of the study groups ($P = 0.10$) nor each of the groups ($P = 0.37$).

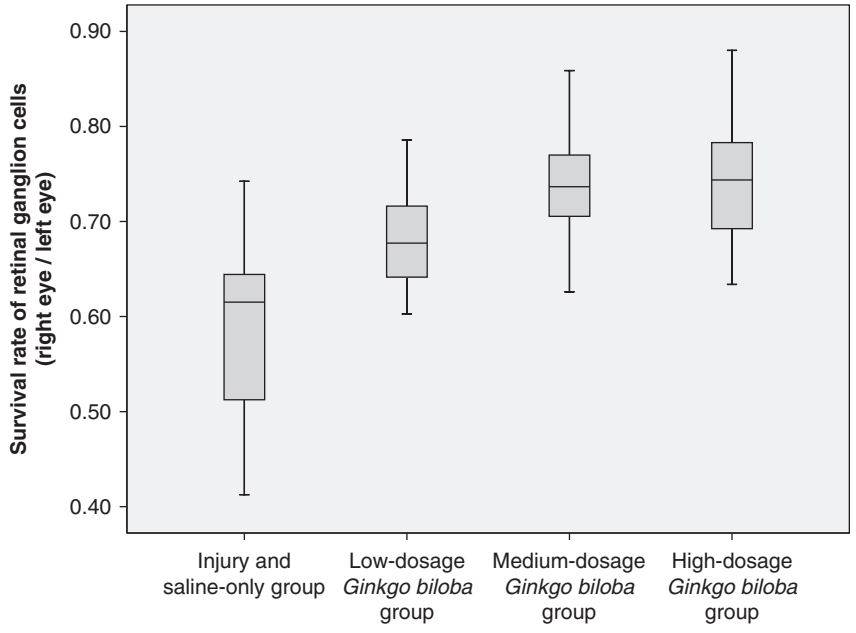

Figure 3 Boxplots showing a statistically significant increase survival rate of retinal ganglion cells (defined as ratio of the density of retinal ganglion cells in the left eyes without an optic nerve intervention compared with the retinal ganglion cell density of right eyes with a standardized optic nerve crush trauma in rats receiving either intragastral applications of saline or *Ginkgo biloba* in increasing concentrations ($P < 0.001$). The survival rate improved significantly from the injury and saline-only group to the low-dosage *G. biloba* group ($P = 0.003$), and from the low-dosage *G. biloba* group to the medium dosage *G. biloba* group ($P = 0.04$). The medium dosage *G. biloba* group and the high-dosage *G. biloba* group did not vary significantly ($P = 0.56$) in the retinal ganglion cell survival rate.

density of retinal ganglion cells in the treated right eye as dependent variable and the group number and the retinal ganglion cell density in the untreated left eyes as independent variables, the density of the retinal ganglion cells in the eyes with an optic nerve crush was highly significantly associated with the group number

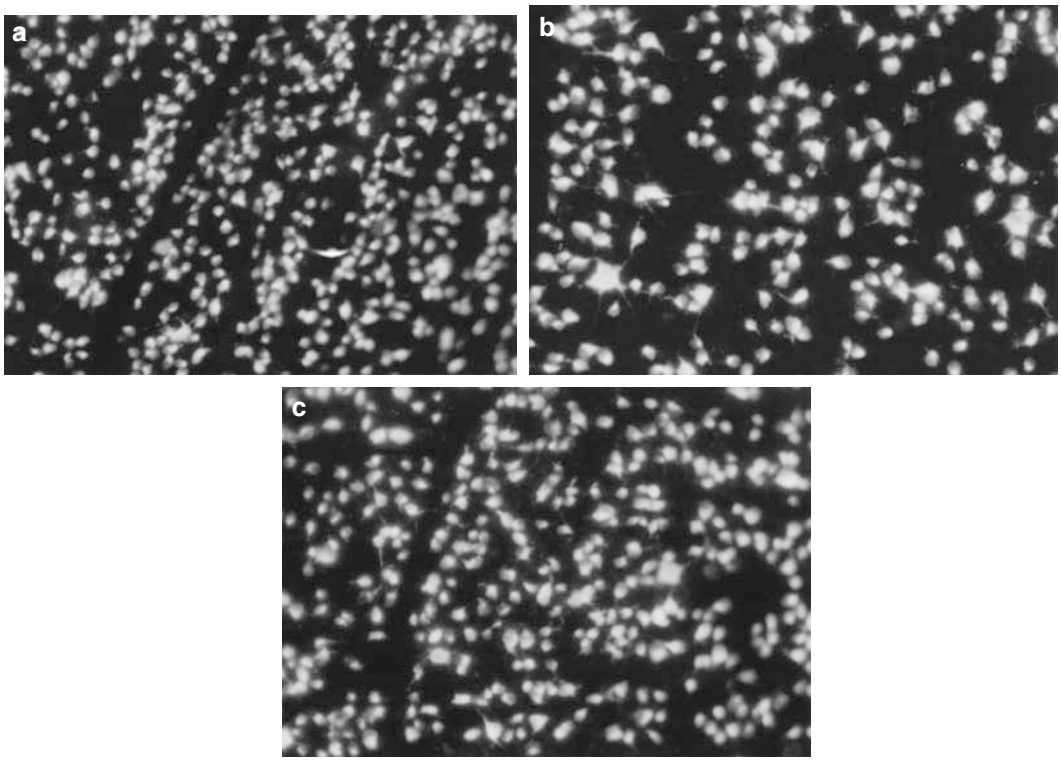

Figure 4 Retinal flat mount photographs taken at 2 mm from the centre of the optic disc in rats of eyes without any procedure (a), or of eyes with an optic nerve crush in animals, which received intragastral applications of saline (saline group) (b) or in animals with intragastral applications of *Ginkgo biloba* (1% EGb761 5 ml/kg; c). The retinal ganglion cells were retrogradely labelled by injection of fluorogold into the superior colliculi.

($P < 0.001$; OR: 110; 95% CI: 74, 146) and with the density of retinal ganglion cells in the untreated left eyes ($P < 0.001$; OR: 0.56; 95% CI: 0.34, 0.78).

Discussion

The results indicate that in the animals included in the investigation, intragastral applications of *G. biloba* were associated with an increased ganglion cell survival at 1 month after an experimental and standardized optic nerve crush, and compared if intragastral saline applications were performed. In addition, the survival rate of the retinal ganglion cells measured as retinal ganglion cell density in the eye with the optic nerve crush divided by the retinal ganglion cell density in the contralateral unaffected eye increased significantly with increasing dosage of *G. biloba* applied. This positive dose–response relationship may further strengthen the conclusion that under experimental conditions, intragastral applications of *G. biloba* may lead to a higher retinal ganglion cell survival.

In recent studies, the rat optic nerve crush model has been widely used for the examination of a potential neuroprotective effect of drugs. In these studies, the crush sites at the optic nerve were chosen from 0 to 3 mm behind the globe.[28–31] Also the device to crush the optic nerve varied between the studies. Buys *et al*[30] used a Dumont no. 5 forceps to crush the optic nerve immediately behind the eyeball. Yoles and Schwartz[31] applied the injury to the optic nerve with a calibrated cross-action forceps. In this study, the optic nerve was exposed by cutting a slit into the optic nerve sheath, and the optic nerve was crushed with a calibrated 40-g microclip for 60 s. This procedure led to a relatively reproducible loss of retinal ganglion cells of about 1–1.46. It may suggest that the technique applied was suitable to detect the differences between the study and the saline groups.

The results of our study are in agreement with previous investigations in which a *G. biloba* extract has the capacity to protect retinal ganglion cells after standardized crush injury In an experimental study, Hirooka *et al*[18] investigated the effect of *G. biloba* extract in rats with unilateral chronic, moderately elevated intraocular pressure. When compared with their contralateral saline eyes with normal intraocular pressure at 5 months after the induction of an intraocular elevation, the retinal ganglion cell loss in eyes with chronic, moderately elevated intraocular pressure was $4.6 \pm 4.5\%$ in the rates receiving *G. biloba* as compared

with 29.8 ± 1.5% in the animals without *Gingko biloba*. Baudouin *et al*[9] reported that *G. biloba* decreases the severity of oedema and neovascularization in an experimental retinopathy generated by the intravitreal production of superoxide radicals. Juárez *et al*[13] confirmed that *G. biloba* can prevent an experimental retinopathy of prematurity in a rabbit model. In a similar manner, *G. biloba* reduced the light damage to photoreceptors and protected against the progression of diabetic retinopathy in experimental animal models.[11,17] In addition, some studies suggested a helpful effect of *G. biloba* for the treatment of glaucoma.[15,17] Two other studies, which are confirmed by our study, may be mentioned.[10,11] Paasche *et al*[14] studied age-related changes of mitochondria in Müller (retinal glial) cells from guinea pigs fed with or without externally applied *G. biloba* extract (EGb 761). The mitochondria of the animals receiving *G. biloba* displayed a significantly enhanced membrane potential and a significantly enhanced index of vitality. The authors concluded that many but not all structural and functional parameters of aging Müller cell mitochondria are impaired by accumulating oxidative damage, and that externally applied radical scavengers, such as *G. biloba* may protect the organelles from the damaging actions of free radicals. Droy-Lefaix *et al*[10] evaluated the antioxidant effect of *G. biloba* on retinas of albino rats submitted to different types of aggressors. On isolated rat retina, *G. biloba* (EGb 761) given orally significantly protected against lipoperoxidation induced by a mixture of ferrous sulphate and sodium ascorbate added to the perfusion solution. With *Ginkgo biloba*, the decrease of the b-wave amplitude of the electroretinogram was less pronounced and the retina survival was increased. *G. biloba* was also effective against ischaemia-reperfusion disorders because of the occlusion of the central retinal artery or by intraocular hypertony. Like other antioxidants, *G. biloba* significantly attenuated, according to a dose–response effect, the free radical injury. The authors noted a *G. biloba* dose-dependent protective effect against acute and chronic chloroquine toxicity to the retina. Another investigation may be mentioned with respect to this study. Cheung *et al*[16] investigated the effects of panax quinquefolius L extract, *G. biloba* extract, and hypericum perforatum extract, in combination or alone, on the survival and regeneration of axotomized retinal ganglion cells in an optic nerve transection model in adult hamsters. The authors observed that treatment with the three substances alone failed to show a neuroprotection to injured retinal ganglion cells. However, treatment with Menta-FX, a mixture of all three substances, significantly augmented the survival of the retinal ganglion cells at 7 days after the axotomy. The treatment with Menta-FX also induced a significant increase in the number of regenerating retinal ganglion cells at 21 days after the optic nerve transection. The authors concluded that herbs can act as a potential neuroprotective agent for damaged retinal ganglion cells, and that the therapeutic value of herbal remedies may be improved by the use of mixtures of appropriate herbs. In contrast to this study, the investigation did not find a significant improvement by the application of *G. biloba* alone, but only in combination with other herbal substances. The reasons for this discrepancy may be the differences in the model of optic nerve damage, and other factors have not yet cleared. Although our study generally confirms the previous studies mentioned above, it extends the observations to an acute optic nerve injury and additionally shows a dosage-dependent effect of *Ginkgo biloba*.

In conclusion, intragastral applications of *G. biloba* given from then and 2 h after an experimental and standardized optic nerve crush daily in rats were associated with a dosage-dependent higher survival rate of retinal ganglion cells. It may suggest a positive effect of *G. biloba* on the survival of the retinal ganglion cells. It remains unclear whether and how far these results from an animal experimental study can be transferred into the clinical situation.

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