Up-regulation of Brain-Derived Neurotrophic Factor Expression by Brimonidine in Rat Retinal Ganglion Cells

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Objectives: Brimonidine tartrate ophth, an α2-adrenergic agonist, is widely used as an antiglaucoma agent for lowering intraocular pressure. Recent studies suggest that brimonidine may be neuroprotective for retinal ganglion cells (RGCs) following optic nerve crush injury. Brain-derived neurotrophic factor (BDNF), a potent neuroprotective factor present in the RGCs, promotes RGC survival in culture and following optic nerve injury. We tested the hypothesis that a possible mechanism of brimonidine neuroprotection is through up-regulation of endogenous BDNF expression in the RGCs.

Methods: A single dosage of brimonidine tartrate ophth solution (0.85-34µM) was injected intravitreally into Sprague-Dawley rat eyes. The fellow eyes of each animal were injected with balanced salt solution (BSS) and used as control eyes. To determine BDNF messenger RNA expression, animal eyes were enucleated and processed for in situ hybridization, or retinas were isolated and processed for Northern blot analysis using rat BDNF radiolabeled riboprobes.

Results: In the control eyes injected with saline, BDNF was present in a minority of the RGCs. Two days after brimonidine injection, the number of BDNF-positive RGCs was increased from 55% to 166%, depending on brimonidine concentrations, when compared with those in the controls. In addition, the BDNF signal intensities in individual RGCs were elevated 50% in brimonidine-injected eyes compared with control eyes. Northern blot revealed a 28% increase of BDNF expression in the brimonidine group compared with the controls (P < .003). No significant difference was observed in BDNF receptor, trkB, expression between brimonidine, or BSS control groups.

Conclusions: A single dose of a low concentration of intravitreal brimonidine is sufficient to significantly increase endogenous BDNF expression in RGCs. These results suggest that brimonidine neuroprotection may be mediated through up-regulation of BDNF in the RGCs. The BDNF should be further investigated regarding its role in the neuroprotective effects reported with brimonidine.

Clinical Relevance: Brimonidine may be (potentially) used clinically as a neuroprotective agent in optic neuropathy, including glaucoma, and ischemic and traumatic optic neuropathy.

Arch Ophthalmol. 2002;120:797-803

GLAUCOMA IS a significant public health problem worldwide.1 The current treatment strategies, including medications and surgery, focus exclusively on lowering the intraocular pressure (IOP), though IOP is just one of the risk factors for glaucoma. Clinical observations and research studies suggest other factors that may play important roles in the development of glaucomatous optic neuropathy. Recent research in neuroscience provides some clues on mechanisms of neuronal degeneration, which is improving the understanding of the pathophysiology of glaucoma. Neuronal degeneration is caused by a variety of primary insults to the neurons, such as mechanical trauma, ischemia, or genetic susceptibility. These primary insults seem to initiate a cascade of events that cause injured neurons to release noxious and degenerative substances, which leads to secondary neuronal degeneration in surrounding cells. The final common themes responsible for neuronal damage and eventual cell death by apoptosis are apparently caused by loss of neurotrophic support or release of excitotoxins such as glutamate. Although the causes of primary neuronal degeneration may be unknown, neuronal degeneration could be halted or at least delayed if a secondary degenerative process could be prevented or modified.2,3 For example, neuroscientists have been using N-methyl-D-aspartate (NMDA) inhibitors to block glutamate effects on neurons, which can cause calcium influx into neurons leading to cell death.4,5

Neuronal degeneration can be grouped into 2 categories depending on...
MATERIALS AND METHODS

ANIMALS

Six- to 7-week-old Sprague-Dawley albino rats (approximately 250 g) were obtained from Charles River Laboratories (Wilmington, Mass). Animals were fed ad libitum with Purina laboratory chow (Ralston Purina, Atlanta, Ga) and water, with room lighting consisting of a 12-hour light/12-hour dark cycle.

BRIMONIDINE INTRAVITREAL INJECTION

Animals were anesthetized with intraperitoneal injections of pentobarbital sodium (Nembutal, Abbott Laboratories, North Chicago, Ill) (75 mg/kg). A 0.2% brimonidine (3.4mM) ophthalmic solution (Allergan Inc, Irvine, Calif) was serially diluted with balanced salt solution (BSS; Alcon Labs Inc, Forth Worth, Tex) from 100-fold to 4000-fold (100-, 500-, 1000-, 2000-, 4000-fold, respectively) to obtain final concentrations from 34µM to 0.85µM (34µM, 6.8µM, 1.7µM, and 0.85µM, respectively). A single dose of 5 µL of diluted brimonidine solutions was injected into vitreous under a dissecting microscope, through a temporal postlimbus spot using Hamilton microinjector (Hamilton Co, Reno, Nev). A 30-gauge needle was first used to make a punch incision 0.5 mm posterior to the temporal limbus, and a Hamilton needle was then inserted through the incision approximately 1.5 mm deep and angled toward the optic nerve until the tip of needle was seen in the center of the vitreous. The lens was occasionally involved, a hard resistance could be felt, and the eye was discarded and not used for the study. Since BSS was used to dilute brimonidine to obtain serial concentrations, 5 µL of BSS was used as a vehicle control and injected into the fellow eyes. Animals were humanely killed 48 hours following injection. At least 3 animals were used for each concentration of brimonidine. Two rats were given a brimonidine injection in only one eye, and the fellow eyes were not given any injection and were processed for in situ hybridization. Five rats with a 1.7µM brimonidine injection in one eye and a BSS injection in the fellow eye, were humanely killed 1 week after injection, and eyes were processed for Northern blot analysis.

TISSUE PREPARATION

Animals were humanely killed with overdose of pentobarbital. Eyes were enucleated, an incision was made in the cornea, and eyes were fixed immediately in 4% formaldehyde in 0.1M phosphate buffer (pH, 7.4). After 15 minutes in the fixative, lenses were removed, and eyes were cut along the corneal optic nerve axis into halves. Tissues were further fixed and cryoprotected overnight in 4% formaldehyde, 0.5% glutaraldehyde, and 20% sucrose in 0.1M phosphate buffer (pH, 7.4). Tissues were embedded in Tissue-Tek OCT compound (Miles Inc, Naperville, Ill) and cryosectioned at a thickness of 10 µm at −21°C. The brimonidine-injected and BSS-injected eye tissue sections were mounted on the same slide and processed identically so that sections could be directly compared, with as little processing variability as possible.

IN SITU HYBRIDIZATION

Standard protocols of riboprobe in situ hybridization were followed as described in detail previously.27,28 Rat BDNF complementary DNA (cDNA) clone was obtained as a generous gift from Genentech Inc (South San Francisco, Calif). It consists of 460 bases of coding region and was inserted into plasmid pGEM-4Z.29 For the generation of antisense and sense BDNF riboprobes, the plasmid was linearized with restriction enzymes EcoRI and HindIII, respectively.30 S-labeled antisense and sense riboprobes were transcribed using the Riboprobe Gemini System according to manufacturer instructions (Promega, Madison, Wis). The tissue sections were pretreated with 10 µg/mL of proteinase K at 37°C for 20 minutes, 0.25% acetic anhydride, and 0.1M triethanolamine for 10 minutes. The tissue sections were then incubated at 50°C on a slide warmer for 18±2 hours with the location of the primary insult—somogenic or axogenic. In somogenic processes such as stroke, the neuronal cell body is damaged in the early course of the disease. In axogenic disorders such as spinal cord injury, amyotrophic lateral sclerosis, and probably glaucoma, the axon is damaged initially, leaving the cell body viable for a much longer time.9 Thus, neuroprotective agents such as neurotrophic factors can be used to promote neuronal survival and to delay cell death.

Brimonidine tartrate (hereafter referred to as brimonidine) ophthalmic solution is an α2-adrenoreceptor agonist that lowers the intraocular pressure (IOP) by reducing aqueous production and by increasing aqueous uveoscleral outflows.10-13 Recent animal studies provide evidence that brimonidine has neuroprotective properties in optic nerve degeneration, which is not related to its IOP-lowering effects. Yoles et al14 studied the effects of brimonidine on optic nerve degeneration in a rat model. They partially crushed the rat optic nerve and immediately injected a single dose of brimonidine or other α2-agonists into the intraperitoneal space. They found a significant increase in the ganglion cell survival rate with α2-agonist intraperitoneal injection, as compared with the control of saline injection. Brimonidine’s effect was most significant—the loss of ganglion cells 2 weeks after crush injury was 3 times lower in the brimonidine-treated group than in the controls. They also found that brimonidine significantly attenuated the decrease in compound action potential amplitude caused by crush injury.14 Their results suggest that α2-agonists, especially brimonidine, may play neuroprotective roles by delaying secondary neuronal degeneration in axogenic optic neuropathy caused by mechanical injury. Wheeler et al15 reported similar neuroprotective effects of brimonidine in a mechanically injured rat optic nerve.15 Furthermore, they found that topical brimonidine significantly delayed rat ganglion cell death caused by acute retinal ischemia and reduced ganglion cell apoptosis as demonstrated by the TUNEL (terminal deoxynucleotidyl transferase-mediated biotin-deoxyuridine 5-triphosphate nick-end labeling) staining techniques.15

What is the underlying mechanism by which brimonidine can promote ganglion cell survival and pro-
the probe solutions containing 5 × 10⁶ cpm/mL of S-labeled probes, 50% formamide, 10% dextran sulfate, 300 mM sodium chloride, 0.5 mg/mL of transfer RNA (tRNA), 10μM DTT, 0.02% Ficoll, 0.02% polyvinyl-pyrolidone, 0.02% BSA, and 1mM EDTA in 10mM Tris-HCl (pH, 8.0). Following hybridization, the slides were rinsed in 4× sodium citrate (SSC) (SSC: 150mM sodium chloride and 15mM NaAc), digested with 20 μg/mL RNase A at 37°C for 30 minutes, washed through descending concentrations of SSC to 0.1× SSC at 60°C to 70°C. The slides were then dehydrated in ethanol, dried, and coated with NTB-2 liquid emulsion (Kodak Inc, Rochester, NY). Following exposure in the dark for 4 weeks, the emulsion was developed, and sections were stained with hematoxylin-eosin.

Brain-derived neurotrophic factor receptor, trkB, and mRNA expression were also examined in brimonidine- and BSS-injected eyes. trkB cDNA clone, a generous gift from the Bristol-Myers Squibb Pharmaceutical Research Institute (Lawrenceville, NJ), was in pGEM-3Z with an insert of 432 base pairs (bp), encoding a portion of the extracellular domain of mouse trkB receptor. Restriction enzymes HindIII and BamHI were used to linearize the plasmid for the generation of antisense and sense probes, respectively. S-labeled antisense and sense trkB riboprobes were transcribed using the Riboprobe Gemini System. In situ hybridization was then performed as described previously.

IMAGE QUANTIFICATION

To determine and compare the numbers of BDNF-positive ganglion cells in the retinas, cells were quantified using computer-enhanced video densitometry (Southern Micro Instruments, Atlanta, Ga). Brain-derived neurotrophic factor mRNA-positive cells were defined as those cells over which silver grains exceed 5 times the background value. Total cell number in the ganglion cell layer was also counted and used as a denominator. Thus, the percentage of BDNF-positive ganglion cells was determined. For each concentration of brimonidine, at least 3 animals were used and 3 tissue sections were counted for each animal eye.

Animal eyes injected with 1.7 μM (2000-fold dilution) brimonidine were used to determine and compare BDNF signal levels in individual ganglion cells between groups, with and without brimonidine injection. Twenty to 30 BDNF-positive cells were randomly selected from each tissue section, and 3 sections were used from each animal. Silver grain densities over individual BDNF-positive cells were determined using computerized densitometry as described previously. Three animals were included for the brimonidine or BSS group. A t-test was used for statistical analysis between the 2 groups.

NORTHERN BLOT

Two groups of rats were used for Northern blot analysis at 48 hours after intravitreal injections (17 rats), and at 1 week after injections (5 rats). Brimonidine (1.7μM) was injected intravitreally in one eye of each animal, and BSS in the fellow eye. Animals were then humanely killed, and retinas were dissected out and pooled in each group. Total retinal RNAs were isolated as described previously. The antisense BDNF RNA probe was synthesized as described previously using [phosphorus-32{32P}] cytidine 5'-triphosphate. Northern blot analysis was performed using standard methods: the total RNA of 30 μg was separated on 1% agarose formaldehyde-denaturing gel. For the 1-week group, 10 μg of RNA was used. The RNA was blotted to 0.2 μm of neutral nylon membranes (Schleicher and Schuell, Keene, NH) and hybridized to a 32P-labeled BDNF probe (3 × 10⁶ cpm/mL). The membrane was then washed in graded SSC, dried, and exposed to a PhosphorImager plate (Molecular Dynamics, Eugene, Ore). Relative abundance of mRNA was quantified by reading the plate. Both bands of BDNF mRNA expression were used to perform the densitometry. For accurate quantification, the same blot was stripped off and hybridized to 32P-labeled β-actin probe. The ratio of BDNF to actin densities was then used for comparison between the brimonidine and BSS control groups. Northern analysis was repeated 5 times for the 48-hour group, and 3 times for the 1-week group.

In the normal adult rat retina, BDNF mRNA expression is present in a subpopulation of ganglion cells as described in our previous studies. Brain-derived neurotrophic factor–positive ganglion cells account for 5% to 6% of the cells in the ganglion cell layer (GCL) and are randomly distributed throughout the retina. In the control eyes injected with BSS in this study, in situ hybridization showed a very similar image as in our prior study: 6.1% of ganglion cells are labeled positive with the BDNF riboprobe (Figure 1 A). This demonstrates that the intravitreal injection used in the present study does not alter the basal level of BDNF expression in the retina.

Forty-eight hours following intravitreal injection of brimonidine, the number of ganglion cells positive
for BDNF significantly increased (Figure 1B-F). Quantification of the BDNF signal revealed a significant increase in the number of BDNF-positive RGCs, and in the intensity of silver grain density in each BDNF-positive RGC (Figure 2A, B). With a 34µM brimonidine injection, 13.7% of ganglion cells are labeled for BDNF mRNA in the GCL—a 125% increase of BDNF-positive cells compared with the BSS control group (P=.014) (Figure 1B and Figure 2A). When more diluted brimonidine solutions were injected, the number of BDNF-positive RGCs continues to be increased. As shown in Figure 2, the number of BDNF-positive RGCs in the GCL reaches the peak when 6.8µM brimonidine was injected (Figure 1C), revealing a 166% increase of BDNF-positive cells compared with the BSS control group (P=.003) (Figure 2A). With 3.4µM and 1.7µM brimonidine, the retinas demonstrate 124% and 119% increases of BDNF cells in the GCL, respectively (P=.012 and P=.006, respectively) (Figure 1D-E and Figure 2A). Even when very low concentrations of 0.85µM brimonidine were injected, there is still a 55% increase of BDNF-positive RGCs in the retina when

Figure 1. In situ hybridization of brain-derived neurotrophic factor (BDNF) messenger RNA (mRNA) expression in the retinas of rat eyes injected intravitreally with a balanced salt solution (BSS) (A) or brimonidine tartrate ophthalmic solutions of 34µM, 6.8µM, 3.4µM, 1.7µM, and 0.85µM (B, C, D, E, and F, respectively). Arrows show BDNF-positive ganglion cells in the ganglion cell layers. A, In retinas injected with BSS, BDNF hybridization signals were present in a subpopulation of ganglion cells. B-F, With brimonidine injections, the numbers of BDNF-positive ganglion cells were significantly increased. Brain-derived neurotrophic factor signals were also significantly more elevated in each of the individual cells in the brimonidine injection group than those in the BSS control. GCL indicates ganglion cell layer; INL, inner nuclear layer; and ONL, outer nuclear layer. Scale bar=100 µm.
Three animals were used for the 1.7µM brimonidine or BSS group. A computerized densitometry (see “Subjects, Materials, and Methods” section). Sections. Silver grain densities over each individual cell were determined using a BDNF-labeled ganglion cells were randomly selected from each of 3 tissue injections, respectively, with \( P=0.01, P=0.003, P=0.01, P=0.006, \) and \( P=0.01, \) respectively (mean ± SD number of rats used was at least 3 for each group). B, BDNF hybridization signal intensity in individual ganglion cells of eyes injected with 1.7µM brimonidine or BSS as control. Between 20 and 30 BDNF-labeled ganglion cells were randomly selected from each of 3 tissue sections. Silver grain densities over each individual cell were determined using a computerized densitometry (see “Subjects, Materials, and Methods” section). Three animals were used for the 1.7µM brimonidine or BSS group. A test showed a 50% increase of BDNF signal intensity in individual ganglion cells in the brimonidine injection group compared with the BSS control group. See Figure 1 for abbreviation expansions.

differences not presented) and eyes with BSS injection. This suggests that 3.4µM of brimonidine intravitreal injection does not alter BDNF mRNA expression in the contralateral eyes.

Although in situ hybridization is an excellent technique for detection of the localization of mRNA signals, it is only a semiquantitative method for signal quantification. To quantify the difference between brimonidine and BSS control groups more accurately, Northern blot analysis was performed using a rat BDNF riboprobe. Total RNAs were isolated from retinas of eyes injected with either 1.7µM brimonidine or BSS. Northern blot showed 2 characteristic bands migrating at 1.6 kilobases (kb) and 4.0 kilobases corresponding to the 2 forms of BDNF mRNA in rodents. β-Actin (Actin) mRNA expression was used as an internal control. B Densitometry analysis of ratio of BDNF to β-actin mRNA signals shows a 28% increase of BDNF mRNA expression in brimonidine as compared with BSS control groups \( (P<.003, n=5) \). One week after a single dose of 1.7µM brimonidine injection, there is still a 12% increase of BDNF signals, but this is not statistically significant \( (P=0.21, n=3) \). See Figure 1 for abbreviation expansions.

**Figure 2.** A, Percentage of BDNF-labeled ganglion cells in the rat retinas with BSS or brimonidine tartrate ophth intravitreal injections. There were significantly more BDNF-positive ganglion cells in all brimonidine concentrations used than in the BSS controls. Specifically, there were 125%, 166%, 124%, 119%, and 55% increases in 34µM, 6.8µM, 3.4µM, 1.7µM, and 0.85µM brimonidine injections, respectively, with \( P=0.01, P=0.003, P=0.01, P=0.006, \) and \( P=0.01, \) respectively (mean ± SD number of rats used was at least 3 for each group). B, BDNF hybridization signal intensity in individual ganglion cells of eyes injected with 1.7µM brimonidine or BSS as control. Between 20 and 30 BDNF-labeled ganglion cells were randomly selected from each of 3 tissue sections. Silver grain densities over each individual cell were determined using a computerized densitometry (see “Subjects, Materials, and Methods” section). Three animals were used for the 1.7µM brimonidine or BSS group. A test showed a 50% increase of BDNF signal intensity in individual ganglion cells in the brimonidine injection group compared with the BSS control group. See Figure 1 for abbreviation expansions.

eye, and the fellow eyes were not injected, and used as natural controls. In situ hybridization showed no difference in BDNF expression between these 2 natural control eyes (data not presented) and eyes with BSS injection. This suggests that 3.4µM of brimonidine intravitreal injection does not alter BDNF mRNA expression in the contralateral eyes.

Although in situ hybridization is an excellent technique for detection of the localization of mRNA signals, it is only a semiquantitative method for signal quantification. To quantify the difference between brimonidine and BSS control groups more accurately, Northern blot analysis was performed using a rat BDNF riboprobe. Total RNAs were isolated from retinas of eyes injected with either 1.7µM brimonidine or BSS. Northern blot showed 2 characteristic bands migrating at 1.6 kilobases (kb) and 4.0 kilobases corresponding to the 2 forms of BDNF mRNA in rodents. β-Actin (Actin) mRNA expression was used as an internal control. B Densitometry analysis of ratio of BDNF to β-actin mRNA signals shows a 28% increase of BDNF mRNA expression in brimonidine as compared with BSS control groups \( (P<.003, n=5) \). One week after a single dose of 1.7µM brimonidine injection, there is still a 12% increase of BDNF signals, but this is not statistically significant \( (P=0.21, n=3) \). See Figure 1 for abbreviation expansions.
The results of our studies clearly demonstrate that brimonidine up-regulates BDNF mRNA expression in rat retinal ganglion cells. The BDNF mRNA expression is elevated 28% 48 hours after a single dose of intravitreal brimonidine as shown on Northern blot analysis (Figure 3). This elevation slowly declined to 12% 1 week after the injection ($P = .21$), suggesting a pulse response of BDNF expression to a single dose of brimonidine. The elevated BDNF expression was the result of more BDNF-expressing ganglion cells and increased signal intensity in individual cells after brimonidine injection (Figures 1 and 2).

Intravitreal injection of brimonidine was used in our study. Questions arise regarding whether topical brimonidine may cause the same effects (ie, up-regulation of BDNF expression in the ganglion cells). A very recent study by Kent et al$^{34}$ showed that topical brimonidine ophthalmic solutions (0.2%) administered to human eyes result in intravitreal concentrations of 185nM ± 500nM.$^{34}$ These results match very well with the intravitreal brimonidine concentrations in our study. Since the adult rat vitreous volume is 56 µL,$^{31}$ the 5 µL of injected brimonidine solutions used in the present study were diluted 12-fold in the rat vitreous. The actual intravitreal concentrations of brimonidine were approximately 70nM, 140nM, 280nM, 560nM, and 2.8µM for injected brimonidine solutions of 0.85µM, 1.7µM, 3.4µM, 6.8µM, and 34µM, respectively. In the Northern blot analysis, 1.7µM of brimonidine solution was used for intravitreal injection because it resulted in a final intravitreal concentration of approximately 140nM, which simulates the concentration achieved by topical brimonidine in the human study.$^{34}$ Since topical brimonidine can result in similar intravitreal concentrations as in our intravitreal injections, topical brimonidine may up-regulate BDNF expression in human retina. However, different species may require different intravitreal concentrations of brimonidine to elevate BDNF expression. Human retinal tissue is needed to examine BDNF expression after brimonidine treatment.

Although brimonidine is a highly selective $\alpha_2$-agonist, it can also bind to $\alpha_{1}$-receptors with low affinity and to nonadrenergic imidazoline receptors with higher affinity. Previous studies have shown that brimonidine’s effect has species differences: brimonidine stimulates a central nervous system imidazoline receptor (Kd=0.48nM) in monkeys to decrease IOP, blood pressure, and heart rate; whereas in rabbits, it stimulates $\alpha_{2}$-receptors (Kd=3.6nM) to decrease IOP.$^{35}$ In the present study, only nanomolar levels of intravitreal concentrations of brimonidine are needed to up-regulate BDNF expression. It is unlikely that brimonidine acts on the $\alpha_{2}$-receptors. BDNF may act on the $\alpha_{1}$-receptor or on the nonadrenergic imidazoline receptor. In vitro cell cultures are needed determine which receptor(s) brimonidine acts on to elevate BDNF expression.

Because it has been reported that as many as 40% to 50% of cells in the GCL are displaced amacrine cells in the adult rat retina,$^{36}$ an obvious question arises as to the type of cells involved with BDNF expression in the GCL. If some BDNF-positive cells in the GCL were amacrine cells, some amacrine cells in the inner nuclear layer should have also been labeled with the BDNF probe, but this was not observed; no BDNF hybridization signal was detected in the inner nuclear layer. Morphologically,
BDNF-labeled cells in the GCL are large and round, which is also consistent with ganglion cells.

Since BDNF can promote ganglion cell survival, it is reasonable to speculate that promotion of ganglion cell survival by brimonidine in an optic nerve injury model or ischemic model is mediated, at least in part, through BDNF up-regulation. It remains to be studied how brimonidine up-regulates BDNF expression. Brain-derived neurotrophic factor is a complex gene with 4 separate promoters located upstream of each 5’ exon. Alternative usage of these promoters and differential splicing result in 4 BDNF mRNAs with different 5’ untranslated exons, which allow multiple points of BDNF mRNA regulation. Activation of the α2-receptor can result in the regulation of multiple signaling pathways. Considering our results, it is possible that some of the pathways hold potential interactions between brimonidine and BDNF. For example, it has been demonstrated that α2-receptor stimulation induces the phosphorylation of mitogen-activated protein kinase, while activation of this kinase can lead to an increase of BDNF gene expression. In addition, α2-receptor agonists have been reported to induce basic fibroblast growth factor expression in photoreceptors. Molecular mechanisms responsible for such interactions remain to be studied.

Submitted for publication July 5, 2001; final revision received November 16, 2001; accepted February 12, 2002.

Dr Cantor is a speaker at the Bureau of Alcon Labs Inc, Allergan Inc, and Merck Co. He receives research support from Alcon Labs Inc, Allergan Inc, Merck Co, Novartis AG, and Pharmacia Co. Both Drs Gao and Qiao contributed equally to this study.

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