

# Neuroprotective Effect of Inosine on Axotomized Retinal Ganglion Cells in Adult Rats

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**PURPOSE.** To explore the potential survival-promoting effect of inosine on axotomized retinal ganglion cells (RGCs) of adult rats in vivo.

**METHODS.** The left optic nerves (ON) in the subject rats were transected at 1.5 mm from the optic disc. Repeated intraperitoneal injections or single intraocular injection of inosine were administered. The RGCs were retrogradely labeled with a gold fluorescent dye and the density of surviving RGCs in number per square millimeter of retina was calculated in whole-mounted retinas. The functional integrity of the blood-retinal barrier (BRB) after ON transection was evaluated with an intravenous injection of Evans blue.

**RESULTS.** In control animals, the mean density of surviving RGCs (number per square millimeter) of the whole retina was  $207 \pm 68$  at 2 days (taken as the normal value),  $927 \pm 156$  at 7 days, and  $384 \pm 33$  at 14 days after surgery. Repeated intraperitoneal injections (75 mg/kg for each injection) of inosine significantly enhanced RGC survival at 14 days after ON transection ( $500 \pm 38$ ), whereas no significant difference in the densities was detected at 7 days ( $974 \pm 101$ ), even when the dosage of inosine was doubled ( $1039 \pm 61$ ). At this time point, however, a single intraocular injection of inosine significantly increased the density of surviving RGCs ( $1184 \pm 156$ ). Moreover, more RGCs around the optic disc were rescued when inosine, administered either intraperitoneally or intraocularly, showed a beneficial effect on RGC survival. No breakdown of the BRB after ON transection was detected with the method used in the study.

**CONCLUSIONS.** These findings demonstrate that inosine could protect axotomized RGCs in vivo after ON transection. (*Invest Ophthalmol Vis Sci.* 2004;45:662-667) DOI:10.1167/iovs.03-0281

Inosine, a purine derivative, has revealed itself in protecting the myocardium from ischemia by its production of adenosine triphosphate (ATP).<sup>1,2</sup> It can also protect glial cells of the nervous system during glucose deprivation.<sup>3</sup> In the presence of glial cells, it has been demonstrated that inosine can prevent cocultured mouse spinal cord neurons against injury by respi-

ratory inhibitors.<sup>4</sup> Recently, we further demonstrated its direct neuroprotective effect on PC12 cells against high concentrations of zinc sulfate.<sup>5</sup> Although exogenous inosine has been shown to stimulate collateral sprouting of intact neurons in the contralateral cerebral cortex of rat with corticospinal tract hemisection<sup>6</sup> or cerebral hemisphere stroke,<sup>7</sup> it remains to be determined whether inosine can improve resistance to insult of neurons in adult mammals. It has been demonstrated in the adult rat that most retinal ganglion cells (RGCs) die after optic nerve (ON) transection.<sup>8-11</sup> In the present study, we investigated the potential effect of inosine on the survival of axotomized RGCs in adult rats.

Although inosine, when administered systemically, has been found to pass through the normal blood-brain barrier to a certain extent,<sup>12-14</sup> changes in the blood-brain barrier certainly affect its concentration in the tissue. The possible breakdown of the blood-retinal-barrier (BRB) after ON transection, as happens in some experimental or pathologic conditions,<sup>15-17</sup> was thus examined.

The present study showed that inosine effectively protected RGCs from the ON insults, which may have clinical implications.

## MATERIALS AND METHODS

Fifty-four adult female Sprague-Dawley rats (The Laboratory Animal Center, The Fourth Military Medical University), weighing 200 to 220 g, were used. All animals underwent left intraorbital ON transection while under deep anesthesia induced by intraperitoneal injections of 1% pentobarbital sodium (50 mg/kg body weight). The animals were divided into two groups (group A and group B). Group A, consisting of 48 rats, was used to explore the potential effect of inosine on the survival of axotomized RGCs. Group B, including the remaining six rats, was designed to ascertain functional integrity of the BRB after ON transection. This investigation adhered to the principles regarding the care and use of animals stated by the American Physiological Society and the Society for Neuroscience and the ARVO Statement for the Use of Animals and Ophthalmic and Vision Research.

## Surgical Procedures and Inosine Administration

With the use of a surgical microscope, the left ON was exposed through a superior temporal approach, and the dura sheath of the nerve was cut open longitudinally, taking care to avoid the ophthalmic artery. The ON was then gently detached from the sheath and completely transected with a pair of iris scissors at 1.5 mm from the optic disc. A small piece of foam (Gelfoam; Pharmacia & Upjohn, Uppsala, Sweden) soaked in a 5% gold fluorescent dye (Fluorogold; Fluorochrome, Inc., Denver, CO) was inserted at the site of the stump of the ON. After that, the wound was sutured.

Eighteen animals received repeated intraperitoneal injections of inosine (Sigma-Aldrich, St. Louis, MO) every 8 hours, starting 1 day before ON transection until the day they were killed. In 12 rats, each injection contained 75 mg/kg body weight of inosine, and the animals were allowed to survive for 7 or 14 days ( $n = 6$  for each time point). Having found that the protective effect was not statistically significant at 7 days, the dosage of inosine was doubled in another six rats (150 mg/kg body weight) and examined 7 days after ON transection. In

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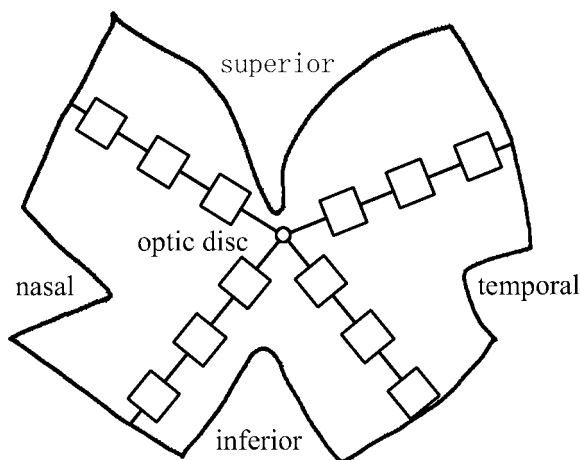
Supported by the Grants 30100187 and 30070799 from the National Foundation of Natural Science of China.

Submitted for publication March 19, 2003; revised September 10, 2003; accepted October 9, 2003.

Disclosure: **B. Hou**, None; **S.-W. You**, None; **M.-M. Wu**, None; **F. Kuang**, None; **H.-L. Liu**, None; **X.-Y. Jiao**, None; **G. Ju**, None

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**FIGURE 1.** Schematic diagram showing the sampled fields in the retina. The squares represent the sampled fields of  $500 \times 500 \mu\text{m}$  at 0.5, 1.5, and 2.5 mm from the optic disc.

other 12 animals, 1 day before ON transection, inosine of  $250 \mu\text{g}$  in  $5 \mu\text{L}$  of sterile water ( $n = 6$ ) or normal saline of the same volume ( $n = 6$ ) was injected into the vitreous space at the cornea-sclera junction as demonstrated by Kermer et al.<sup>18</sup> and the animals were killed 7 days later. The remaining 18 animals served as the control without any treatment and survived for 2, 7, or 14 days ( $n = 6$  for each time point).

### Counting of RGCs and Statistics

The left eye of each rat in group A was removed immediately after they were killed with a lethal dose of pentobarbital sodium. The retina was dissected in 4% paraformaldehyde in 0.1 M phosphate buffer, postfixed in the same fixative for 1 hour, rinsed three times in 0.1 M phosphate buffer, cut into four quadrants, flatmounted on a glass slide in antifade medium (Vectashield; Vector, Burlingame, CA), and coverslipped. To evaluate RGC survival after complete transection of the ON, the retinas were examined under a fluorescence microscope (model BX-60; Olympus, Tokyo, Japan) in the UV mode. The gold-labeled RGCs were identified by somata full of intense and diffuse fluorescent granules. They were counted in a double-blind manner along the midlines of the four quadrants starting from the optic disc to the peripheral border of the retina at  $500\text{-}\mu\text{m}$  intervals, in an eye-piece square grid framing a field of  $500 \times 500 \mu\text{m}^2$  (Fig. 1). The density of labeled RGCs in the whole retina was calculated by averaging the number of all sampled fields in each retina.

The fluorescent gold dye used (Fluorogold; Fluorochrome, Inc.) is a neuronal retrograde tracer. No transneuronal leakage has been reported. However, phagocytosis of the debris of degenerated labeled neurons may be a source of miscounting. In our pilot experiments, no glial fibrillary acidic protein (GFAP)-immunoreactive Müller cells were labeled. A small number of OX-42-immunoreactive microglia contained the gold label, however, characterized by their rod-shaped somata with intensive gold labeling in lumps, rather than the granular form that appeared in the RGCs. Thus, the labeled microglial cells could be easily identified and excluded from RGC analysis. Because the death of RGCs does not occur within 3 days after ON transection,<sup>8,19,20</sup> the density of RGCs at 2 days after axotomy in the control animals was taken as the norm.

The data were analyzed with the two-tailed Student's *t*-test on computer (Origin ver.7.0, Microcal Corp. Northampton, MA).

### Evaluation of Function of BRB

Six animals in group B were allowed to survive for 7 or 14 days ( $n = 3$ , for each time point) after ON transection. Two hours before perfusion, the animals were anesthetized with an intraperitoneal injection of pentobarbital sodium ( $50 \text{ mg/kg}$  body weight) and followed by injection

of  $30 \text{ mg/mL}$  of Evans blue (Sigma-Aldrich) in normal saline through the exposed femoral vein over 10 seconds at a dosage of  $45 \text{ mg/kg}$  body weight. Immediately, the rats turned visibly blue, confirming successful distribution of the dye. The rats were perfused through the ascending aorta with normal saline at  $37^\circ\text{C}$ . The perfusion lasted for 2 minutes at a physiological pressure of approximately  $120 \text{ mm Hg}$ . Then, both eyes of the rat were immediately enucleated and deep frozen. The eyeballs were cryosectioned horizontally at  $25 \mu\text{m}$  and examined under a fluorescence microscope (BX-60; Olympus). The right eyes served as the internal control.

## RESULTS

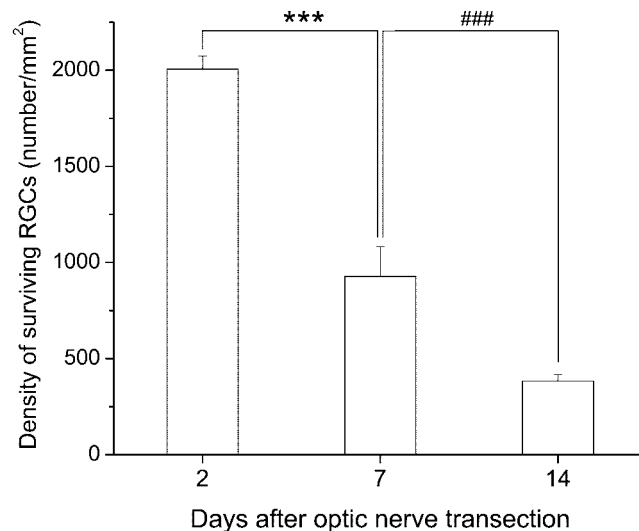
### Effect of Inosine on the Survival of Axotomized RGCs

The density of surviving RGCs in the whole retina (number per square millimeter) at 2 days after ON transection in control animals was  $2007 \pm 68$  (mean  $\pm$  SD), which dropped sharply to  $927 \pm 156$  at 7 days ( $P < 0.001$ ) and to  $384 \pm 33$  at 14 days ( $P < 0.001$ ; Fig. 2). With the density at 2 days taken as normal, approximately 54% of all RGCs had died at 7 days, and 81% were lost at 14 days.

In the animals treated with intraperitoneal injections of inosine at  $75 \text{ mg/kg}$ , there was no significant difference in the density of surviving RGCs at 7 days ( $974 \pm 101$ ) in comparison to the control ( $P > 0.05$ ). At 14 days, the density of the RGCs was found to be significantly higher in the treated group than that in the control ( $500 \pm 38$ ;  $P < 0.001$ ). When a higher concentration ( $150 \text{ mg/kg}$ ) of inosine was used, the RGC density of the whole retina increased to  $1039 \pm 61$  at 7 days. The increase was not prominent enough to be statistically significant ( $P > 0.05$ ; Fig. 3). A single intraocular injection of inosine ( $250 \mu\text{g}$  per animal), however, increased RGC survival significantly at 7 days ( $1184 \pm 156$ ;  $P < 0.05$ ; Fig. 4).

### Distribution of RGCs and Their Response to Axotomy

In the control animals at day 2 after ON transection (taken as the normal number of RGCs), the density of RGCs was found to be higher the shorter the distance from the optic disc:  $2286 \pm 201$ ,  $2043 \pm 98$ , and  $1693 \pm 216$  at 0.5, 1.5, and 2.5 mm from the optic disc, respectively. The differences be-



**FIGURE 2.** Density of surviving RGCs in the whole retina of control animals after ON transection. \*\*\* $P < 0.001$ , between 2 and 7 days; ### $P < 0.001$ , between 7 and 14 days.

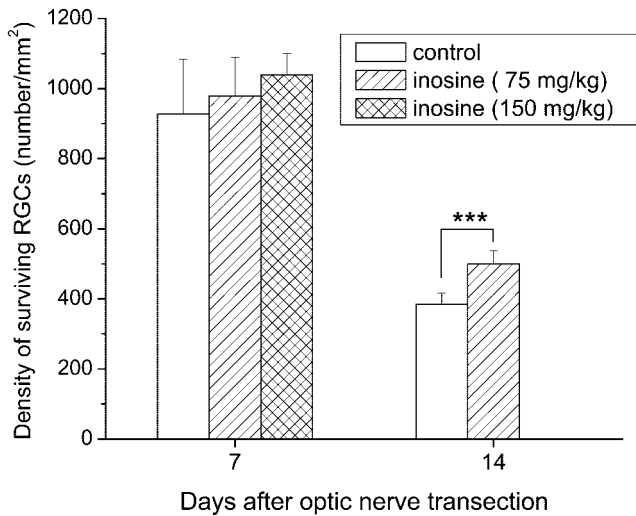


FIGURE 3. Density of surviving RGCs in the whole retina after ON transection when treated with an intraperitoneal injection of inosine. \*\*\**P* < 0.001 compared with the control.

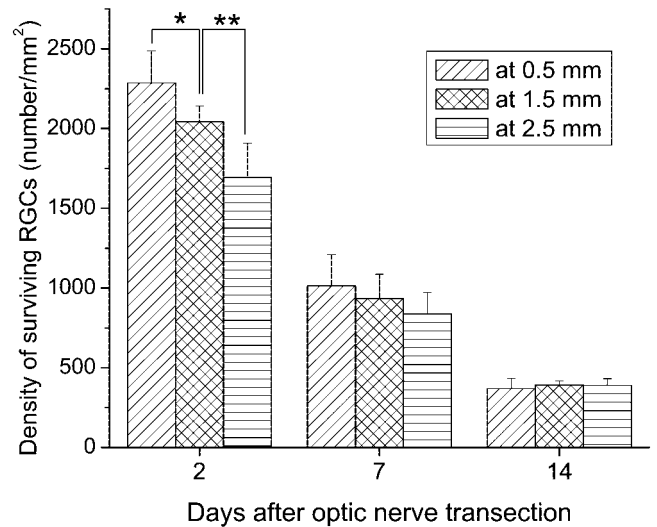


FIGURE 5. Density of surviving RGCs at different distances from the optic disc in the control animals after ON transection. \**P* < 0.05; \*\**P* < 0.01.

tween each two distance-distinguished samples were statistically significant (*P* < 0.05 between 0.5 and 1.5 mm; *P* < 0.01 between 1.5 and 2.5 mm). The RGC density at 1.5 mm from the optic disc was close to the average value in the whole retina (Fig. 5).

At 7 days after ON transection, the densities of RGCs at 0.5, 1.5, and 2.5 mm from the optic disc decreased to 1012 ± 195, 934 ± 151, and 837 ± 136, respectively. At 14 days, the RGC densities at the corresponding loci were 370 ± 64, 391 ± 26, and 390 ± 41, respectively. There was no statistically significant difference in density between any two distance-distinguished samples at each time point (*P* > 0.05; Fig. 5). The percentages of surviving RGCs at different distances from the optic disc at 7 and 14 days are shown in Table 1. It was found that the closer to the optic disc, the smaller the percentage of surviving RGCs. Statistically, how-

ever, the percentages between different loci were significant only at 14 days.

### Differential Effects of Inosine on the Survival of Axotomized RGCs at Different Loci

The effects of inosine on the survival of axotomized RGCs at different loci of the retina were not equal. For intraperitoneal administration of inosine, no apparent protective effect was demonstrated at 7 days in the present study. Thus, only those data showing definite protective effect of inosine were further analyzed. Compared with the control, when the subjects were examined at 14 days, intraperitoneal injections of inosine at 75 mg/kg, significantly enhanced the surviving RGC density at 0.5 mm (519 ± 46; *P* < 0.001) and 1.5 mm (527 ± 65; *P* < 0.001), but not at 2.5 mm, from the optic disc (455 ± 64; *P* > 0.05; Fig. 6).

In the animals treated with intraocular injection of inosine, the protective effect was evaluated at 7 days. A significant increase in density of surviving RGCs occurred at 0.5 mm (1388 ± 181; *P* < 0.01), but not at 1.5 (1161 ± 203; *P* > 0.05) or 2.5 mm, from the optic disc (1001 ± 125; *P* > 0.05; Fig. 7).

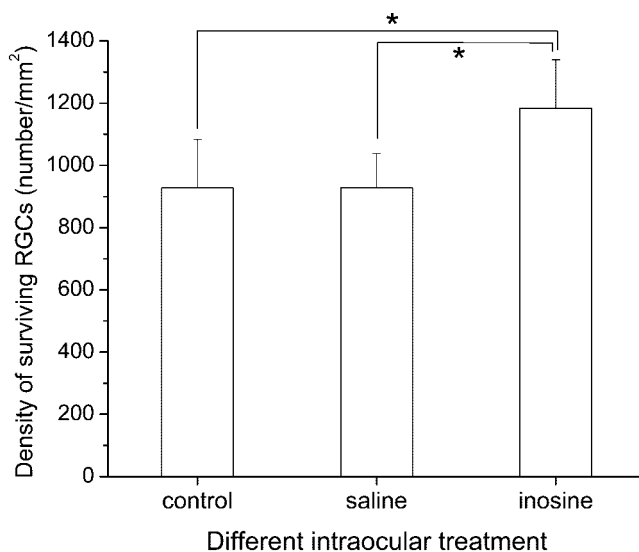


FIGURE 4. Comparison of density of surviving RGCs in the whole retina 7 days after ON transection when treated with an intraocular injection of saline or inosine. \**P* < 0.05.

### Evaluation of the BRB

No Evans blue was detected in the retinas of the left eyeballs, either at 7 or 14 days after ON transection, although intense red fluorescent Evans blue was present in the sclera and the choroid (Fig. 8).

TABLE 1. Percentages of Surviving RGCs at Different Distances from the Optic Disc in the Control Animals

Distance from Optic Disc (mm)	2 days	7 days	14 days
0.5	100	44	16
1.5	100	46	19*
2.5	100	49	23†

\* *P* < 0.05, between 0.5 and 1.5 mm.

† *P* < 0.01, between 1.5 and 2.5 mm.

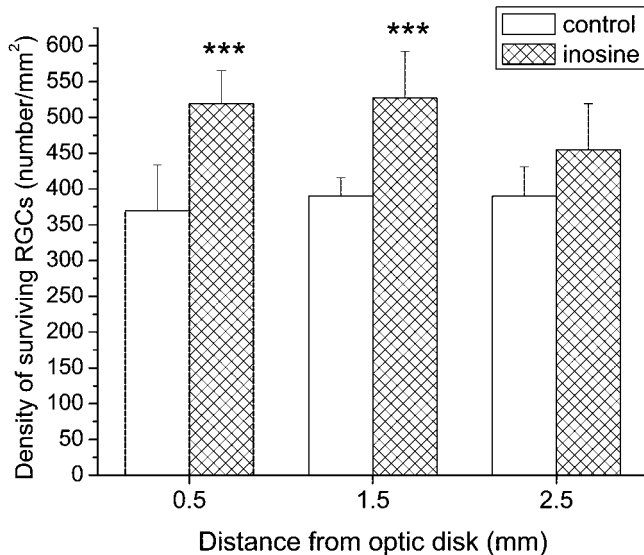


FIGURE 6. Differential effects of intraperitoneal inosine on the survival of RGCs at 14 days after ON transection, according to distance from the optic disc. \*\*\* $P < 0.001$  compared with the control.

## DISCUSSION

It has been well documented that RGCs die quickly after severe ON injuries in adult rats.<sup>8,10,11</sup> There is a 50% and 80% approximate loss of RGCs in the retina at 7 and 14 days, respectively.<sup>8</sup> Our results confirmed those in other studies.

Inosine, through its production of ATP, has been shown to have a protective effect in various kinds of cells.<sup>1,2</sup> It has further been reported that inosine can, directly or indirectly, prevent cultured neurons against injuries by respiratory inhibitors.<sup>4,5</sup> The results of the present study showed that inosine protects RGCs after ON transection. Repeated intraperitoneal injections of 75 mg/kg of inosine significantly enhanced the survival of RGCs at day 14 after ON transection, but not at day 7. Possible explanations for the lack of significant protective effect at day 7 could be: (1) The RGCs that died before 7 days were too severely injured to be

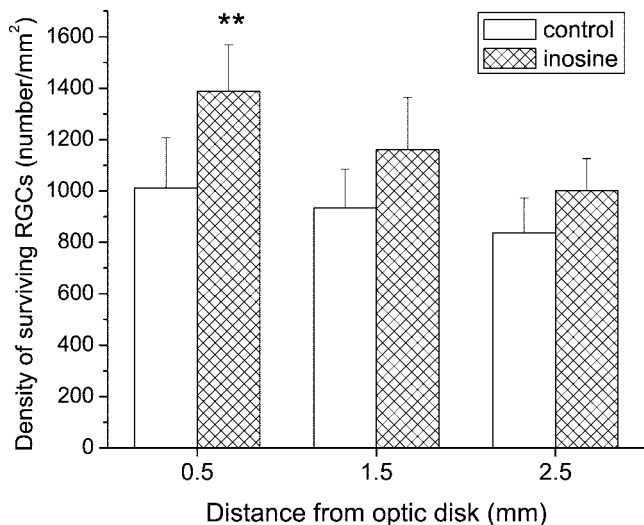


FIGURE 7. Differential effects of intraocular inosine on the survival of RGCs 7 days after ON transection, according to distance from the optic disc. \*\*\* $P < 0.01$  compared with the control.

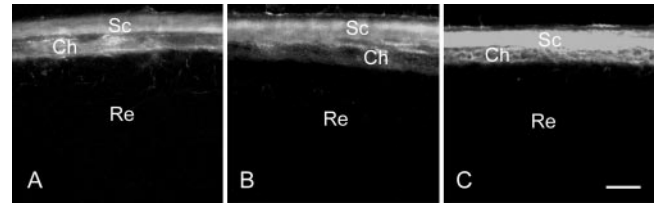


FIGURE 8. Micrographs of horizontal sections of the eyeballs. The highlighted parts indicate leakage of Evans blue. (A) Control and (B) 7 and (C) 14 days after ON transection. Sc, sclera; Ch, choroid; Re, retina. Bar, 50  $\mu$ m.

rescued by inosine. Many of them died of necrosis. (2) The amount of inosine that reached the retina was not enough. The study of an increased dosage of inosine lends support to the second possibility. The density of surviving RGCs was increased when the dosage was doubled, yet still it did not reach a statistically significant level. This may be partly due to the limitation of the ability of inosine to pass through the BRB. It has been shown that inosine crosses the blood-brain barrier, but not freely.<sup>12-14</sup> Because the BRB, a homologue of the blood-brain barrier, is often damaged during various experimental or pathologic conditions,<sup>15-17</sup> we examined the possible change in BRB in our experimental condition. It turned out to be that a change in the BRB was not detectable after ON transection with the Evans blue method we used. By intraocular injection of inosine to circumvent the BRB, we showed inosine to be effective in protecting against RGC loss before 7 days after injury. This hypothesis has also been supported by in vitro studies, demonstrating that the protective effect of inosine works in a dose-dependent manner.<sup>4,5</sup>

In the present study, the density of RGCs was found to be higher the closer to the optic disc, consistent with previous reports.<sup>11,21,22</sup> After ON transection, the absolute number as well as the percentage of RGC loss was greater nearer to the optic disc. Apparently, this is because the site of severance of the ON was closer to the somata of RGCs near the optic disc, thus the RGCs were more seriously damaged.<sup>8,11</sup> One would expect that the RGCs less seriously damaged would be more easily rescued. Paradoxically, in the present study inosine exhibited better protective effect for RGCs closer to the optic disc. This is not due to a difference in the concentration of inosine reaching at different loci of the retina, because the intraocular injection of inosine showed similar results. Furthermore, we did not detect any change of BRB at all parts of the retina, when using the Evans blue method. A possible explanation of this differential effect may lie in the difference of cell populations at different loci of the retina, thus causing a different response to inosine. It has been reported that rat RGCs can be classified into three types according to the size of their somata: large, medium, and small.<sup>21</sup> Their counterparts in the cat have been shown to exhibit different susceptibility to the axotomy and responsiveness to trophic factors.<sup>25</sup> Moreover, the densities of medium and small cells, occupying 30% and 65%, respectively, of all RGCs of rats, increase sharply toward the center of the retina.<sup>21</sup>

The mechanisms involved in the neuroprotective effect of inosine remain to be clarified. There is compelling evidence that death of RGCs after axotomy can be ascribed primarily to apoptosis,<sup>8,24-28</sup> but at least a subset of axotomized RGCs die by necrosis.<sup>29-32</sup> ON transection was shown to induce an increase of nitric oxide synthase activity in RGCs and the production of reactive oxygen species.<sup>30,33-35</sup> These peroxides can efficiently induce exces-

sive activation of poly(ADP-ribose) polymerase (PARP), which results in depletion of nicotinamide adenine dinucleotide (NAD<sup>+</sup>) and subsequent cellular ATP depletion, ultimately leading cells to apoptosis<sup>30,33-35</sup> or necrosis.<sup>36,37</sup> It has been demonstrated that RGC-specific PARP is activated and upregulated after ON transection.<sup>32</sup> Furthermore, intravitreal injections of 3-aminobenzamide, a highly selective PARP inhibitor, significantly increased the density of surviving RGCs 14 days after ON transection.<sup>32</sup> Inosine may compete with NAD<sup>+</sup> to bind PARP because of its structural similarity to NAD<sup>+</sup>, finally stopping the depletion of ATP.<sup>38</sup> In our previous study, inosine was demonstrated to protect PC12 cell from zinc insult, which is believed to be mediated through PARP. Thus, in the present study, inosine might directly rescue a subset of axotomized RGCs from death mediated by activation of PARP. In addition, previous investigations<sup>3,39</sup> have shown that inosine could protect glial cells through purine nucleoside phosphorylase (PNP), a purine metabolic enzyme that can cleave inosine into hypoxanthine and ribose-1-phosphate. The final product of hypoxanthine is uric acid, a scavenger of peroxides.<sup>40</sup> PNP is reported to be rich in glial cells,<sup>41</sup> the protective effect of inosine on mouse spinal cord neurons can be demonstrated only in the presence of glial cells.<sup>4</sup> Presumably, the RGC protective role of inosine in the present study may be assisted by its beneficial effect on glial cells.

Inosine has been shown in the present study to be protective of axotomized RGCs in vivo. It may also be applicable to the treatment of other neurodegenerative diseases characterized by progressive RGC death, such as glaucoma.

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