

## Protective effect of XY99-5038 on hydrogen peroxide induced cell death in cultured retinal neurons

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### Abstract

XY99-5038 is a preparation from a specific formula provided by Dr. Yan Xin, a renowned Traditional Chinese Medicine specialist. This formulation has proven to be effective without side effects but has not been studied under rigorous laboratory conditions. In order to investigate the role of XY99-5038 in protecting neurons from degeneration, we used retinal neuronal culture as a model system to study the protective effects of XY99-5038 against hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) induced cytotoxicity. Basic fibroblast growth factor (bFGF), a potent neurotrophic factor, was employed as comparable agent. Retinas of 0–2 days old Sprague-Dawley rats were isolated, dissociated, and the neurons maintained for 2 weeks in a synthetic serum-free media. Cytotoxicity was determined by MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide) and LDH (Lactate dehydrogenase) assays, whereas apoptotic cell death was evaluated by the TUNEL (TdT-mediated digoxigenin-dUTP nick-end labeling) assay. Treatment with H<sub>2</sub>O<sub>2</sub> significantly induced death of retinal neurons. Pretreatment with XY99-5038 prior to insult greatly inhibited or attenuated H<sub>2</sub>O<sub>2</sub> induced cytotoxicity and apoptosis. These effects were shown to be dose-dependent. Our data also show that the protective effect of XY99-5038 is more potent than that of bFGF. Our data suggest that XY99-5038 could be a potential agent in the treatment of disorders associated with oxidative damage. © 2001 Elsevier Science Inc. All rights reserved.

*Keywords:* XY99-5038; Oxidative stress; Hydrogen peroxide; Apoptosis; Neuroprotection; Retinal neuronal culture; Yan Xin qigong; Traditional Chinese Medicine; External qi

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### Introduction

Over the past three decades, the dual nature of oxygen has come to be fully appreciated. On the one hand, oxygen is indispensable to aerobic existence. On the other hand, it is a ma-

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major endogenous toxicant. Oxidative stress refers to the cytotoxic consequences of oxygen radicals: superoxide, hydroxy radical, and hydrogen peroxide ( $H_2O_2$ ), which are generated as byproducts of normal and aberrant metabolic processes that utilize molecular oxygen. Oxygen radicals can attack proteins, deoxyribonucleic acids, and lipid membranes, thereby disrupting cellular functions and integrity.

It has been demonstrated that oxidative stress plays an important role as a mediator of apoptosis [1,2], and hydrogen peroxide ( $H_2O_2$ ), a by product of oxidative stress, has been implicated in triggering apoptosis in various cell types [3,4]. There is substantial evidence that light can injure retinal neurons, such as photoreceptor cells, by increasing the formation of reactive oxygen species and that antioxidants can rescue light-damaged photoreceptors [5–7]. It is now clear that apoptotic cell death occurs in virtually every cell type, if not all cells. It is also clear that most pathological insults can provoke apoptotic cell death if delivered at a dose below that expected to cause acute, necrotic cell death. Apoptosis has been described in a wide variety of hereditary retinal degenerations [8], in light damaged retinas [7] and following retinal detachment [9]. Other types of retinal degeneration, such as glaucoma and retinal ischemia have also been associated with apoptosis [10,11].

XY99-5038, one of XY Yang Sheng Su (previously named “Yan Xin Qigong nutrition powder”) serial products, is a preparation from a specific formula provided by Dr. Yan Xin according to Traditional Chinese Medicine and qigong theories on health preservation and longevity. This formulation has proven to be effective without side effects for certain degenerative disorders and also shown to delay aging process in many case-based studies [12–15]. In order to investigate the role of XY99-5038 in protecting neurons from degeneration, we used retinal neuronal culture as a model system to study the protective effects of XY99-5038 against  $H_2O_2$  cytotoxicity. Our data showed that XY99-5038 inhibited  $H_2O_2$  induced cytotoxicity, and that  $H_2O_2$ -induced retinal neuron apoptosis could be blocked by XY99-5038.

## Materials and methods

### Materials

Fetal bovine serum, gentamicin sulfate, L-Glutamine, creatine, poly-D-lysine, sodium dodecyl sulfate (SDS), N,N-dimethyl formamide (DMF), MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide), hydrogen peroxide, human recombinant fibroblast growth factor (bFGF) were purchased from Sigma (St. Louis, MO). DMEM/F12 and Tii-Medium were purchased from Gibco-BRL (Grand Island, NY) and JRH Biosciences (Lenexa, KS) respectively. XY99-5038 is prepared from several purely natural and non-toxic plants. XY99-5038 for these experiments was made and supplied by American Biology Engineering Science Research Institute (Los Angeles, CA), and the external qi of qigong were added by Dr. Yan Xin. Since the efficacious components of this preparation are still unknown, total protein level was used as index to estimate the concentration of XY99-5038. The concentrated abstract was filtered with 0.2  $\mu m$  filter and diluted with serum-free culture medium to 1 mg total protein/ml as stock solution for further use. Tissue culture plastic ware and 12 mm diameter coverslips were from Fisher Scientific (Pittsburgh, PA). Cytotoxicity detection (LDH assay)

and apoptosis detection kits were purchased from Boehringer Mannheim Corp (Indianapolis, IN) and Oncor (Gaithersburg, MD) respectively.

#### *Primary cultures of postnatal retinal neurons*

Animals used in these studies were cared for and handled according to the guideline of the Association for Research in Vision and Ophthalmology (ARVO) for the Use of Animals in Vision and Ophthalmic Research and the University of Oklahoma Faculty of Medicine Guidelines for Animals in Research. The preparation of primary cultures of retinal neurons was as described previously [16]. Briefly, retinas of 10–15 rat pups, 0–2 days old, were removed using sterile conditions in a tissue culture hood and a dissecting microscope. The retinas were suspended in 25 ml of DMEM-F12 plus 10% fetal calf serum in a plastic bag and mechanically dissociated. The suspension was first filtered through a 230  $\mu\text{m}$  sieve, rinsed with medium and the filtrate was subsequently passed through a 140  $\mu\text{m}$  sieve and followed by a rinse with undiluted fetal calf serum. The suspension was centrifuged at 800 rpm in a clinical centrifuge for 5 minutes, the supernatant decanted and the cell pellets resuspended in 25 ml of media using a sterile 5 ml pipette. The concentration of cells was determined with a cell counter or hemocytometer and the suspension diluted with medium to  $1 \times 10^5$  cells per ml. The cells (1 ml) were plated in 24 well tissue culture plates on 12 mm coverslips that had been pre-treated overnight with poly-D-lysine (10  $\mu\text{g}/\text{ml}$ ). The cells were maintained in the synthetic serum-free media developed by Bottenstein & Sato [17] as modified by Lillien and Cepko [18]. The cultures at 14–20 days after plating were used for experiments.

To evaluate the potential of XY99-5038 to protect neurons from oxidative damage, we used retinal neuronal culture as an *In vitro* model system to study the protective effects of XY99-5038 against  $\text{H}_2\text{O}_2$  toxicity. We pretreated retinal neuron cultures with XY99-5038 (100 ng/ml) 1 hr prior to exposure to different doses of  $\text{H}_2\text{O}_2$  (30, 60, 100, 300 and 600  $\mu\text{M}$ ) for 24 hrs. Cell viability or cytotoxicity was determined by the MTT and LDH assays respectively [19,20].

#### *MTT assay*

MTT was dissolved at a concentration of 5 mg/ml in PBS. The lysing buffer was prepared as follows: sodium dodecyl sulfate (20% w/v) was dissolved at 37°C in a 50% solution of DMF in deionized water. The pH was adjusted to 4.7. 25  $\mu\text{l}$  of the stock solution of MTT was added to each well and incubated for 2 hours at 37°C, and then 100  $\mu\text{l}$  of the lysing buffer was added. After an overnight incubation at 37°C, absorbance of the samples was read at 562 nm using a microtiter plate ELISA reader.

#### *LDH assay*

To measure LDH (lactate dehydrogenase) activity released from the neuronal cells, we use a commercially available cytotoxicity detection kit. Two controls were performed in each experimental setup. The first was a background control. This provides information about the LDH activity contained in the fresh culture medium. The absorbance value obtained in this control was subtracted from all other values. The second control measured spontaneous LDH release. This provides information about the LDH activity released from the untreated normal cells. Total culture medium were collected and centrifuged to remove contaminating

cells and cellular debris. The volume of media was then measured. To assay LDH inside the cells, 1 ml of 1% Triton-100 was added to the wells, and cells were incubated for 30 min with gentle shaking. The percentage of LDH release was then calculated as: LDH outside the cells/total LDH (inside + outside the cells). For the actual assay, 100  $\mu$ l of each sample was transferred to a 96 well microtiter plate. 100  $\mu$ l of LDH assay reagent (prepared in the dark according to the protocol provided by Boehringer Mannheim Corp) was added to each well and incubated for up to 30 min at room temperature and the absorbance of samples was measured at 490 nm.

#### *TUNEL assay*

The TUNEL (TdT-mediated digoxigenin-dUTP nick-end labeling) for *in situ* apoptosis detection was carried out using a commercially available kit. Positive controls were carried out by treating coverslips with DNase I (1  $\mu$ g/ml) prior to the assay. Negative controls were carried out by omitting TdT from the protocol. Cells were fixed in 2% paraformaldehyde for 10 min at room temperature and washed twice with phosphate-buffered saline (PBS) for 5 min each at room temperature. Staining for the TUNEL technique was performed according to the manufacturer's protocol. TUNEL positive cells were visualized using diaminobenzidine as substrate for the kit's horseradish peroxidase. The results were viewed with a Nikon Eclipse 800 microscope. The pictures were transferred by a digital camera and stored in the computer. The percentage of apoptotic cells were calculated by counting TUNEL positive cells from the digitized image stored in the computer, and by dividing TUNEL positive cells by total cells visualized by nomarski optics in the same field. Three digitized images of similar total cell numbers were selected from each coverslip for counting and averaging, and were considered as one independent experiment. Eight independent experiments were then averaged.

#### *Statistical analysis*

Data were analyzed by means of analysis of variance (ANOVA), and further assessed by Dunnett tests. Statistical differences reaching  $p < 0.05$  were accepted as significant.

## **Results**

There were no significant differences in cell viability (Figure 1) between untreated cultures and treated ones when the dose of  $H_2O_2$  was below 30  $\mu$ M. For 60  $\mu$ M  $H_2O_2$  treated groups, a significant decrease in cell viability was seen after 24 hr exposure. Cell viability decreased to 30–40% at 100  $\mu$ M, and to 10–20% at 300  $\mu$ M  $H_2O_2$  or higher. XY99-5038 at 100 ng/ml significantly inhibits cytotoxicity induced by  $H_2O_2$ . Basic fibroblast growth factor (bFGF), a potent neurotrophic factor/survival factor, at 100 ng/ml also significantly reduced toxicity induced by 60 and 100  $\mu$ M  $H_2O_2$ , whereas this concentration of bFGF did not inhibit cytotoxicity when the cultures were exposed to 300  $\mu$ M  $H_2O_2$  or higher.

To further investigate the protective effect of XY99-5038, the LDH assay, another non-morphological cell toxicity assay was performed. LDH is a stable cytoplasmic enzyme present in all cells, and it is rapidly released into the cell culture supernatant upon damage of the plasma membrane. An increase in the number of dead or plasma membrane-damaged cells results in an increase in the LDH enzyme activity in the culture supernatant. As shown

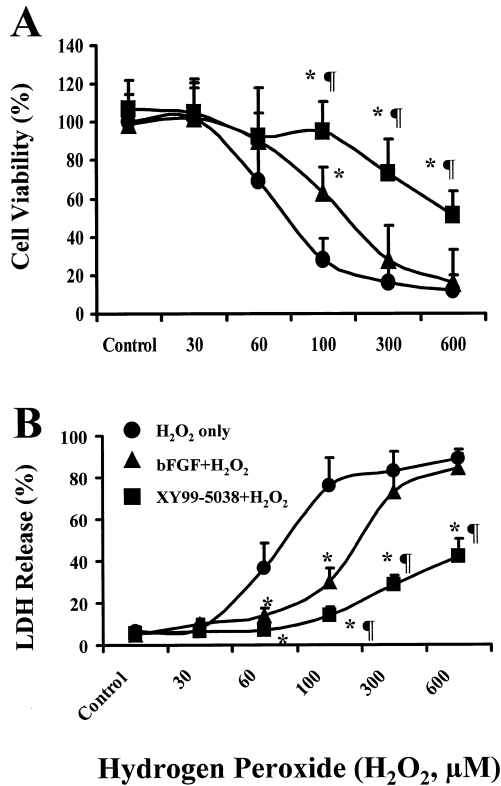


Fig. 1. Inhibition of H<sub>2</sub>O<sub>2</sub>-induced cell death by XY99-5038. Cultures were pretreated with either XY99-5038 (100 ng/ml) or bFGF (100 ng/ml) for 1 hr before being exposed to different concentrations of H<sub>2</sub>O<sub>2</sub> for 24 hrs or were treated only with H<sub>2</sub>O<sub>2</sub> for 24 hrs without any pretreatment. A: MTT assay; B: LDH assay. \* p<0.05 versus the same group of H<sub>2</sub>O<sub>2</sub> exposure without pretreatment; † p<0.05 versus the same concentration of H<sub>2</sub>O<sub>2</sub> exposure without pretreatment (mean ± SD, n = 8).

in Figure 1B, a significant increase in LDH release reflecting cytotoxicity was observed after 24 hr exposure to 60 μM H<sub>2</sub>O<sub>2</sub>. This increase reached a plateau with 300 μM H<sub>2</sub>O<sub>2</sub> exposure. XY99-5038 significantly attenuated this increase in LDH release. The pattern of protective effect of XY99-5038 on H<sub>2</sub>O<sub>2</sub>-induced cytotoxicity determined by LDH assay was similar to that determined by MTT assay.

To examine the dose-dependent effects of XY99-5038, retinal neuronal cultures were pretreated with different concentration of XY99-5038 ranging from 0.001 to 1000 ng/ml (0.001, 0.01, 0.1, 1, 10, 100 and 1000 ng/ml). As shown in Figure 2, different doses of XY99-5038 were added to the cultures 1 hr before exposure to 100 μM H<sub>2</sub>O<sub>2</sub>, and cytotoxicity was evaluated by MTT assay (Fig.2A) and LDH assay (Fig.2B). Significant increase in cell viability was evident with pretreatment with 0.01 ng/ml of XY99-5038. The increase reached to a plateau at 10 ng/ml of XY99-5038 or higher. XY99-5038 at 0.001 ng/ml did not show any protective effect against H<sub>2</sub>O<sub>2</sub>-induced cell death (Fig. 2A). Figure 2B shows that 100 μM H<sub>2</sub>O<sub>2</sub> induced significant LDH release. This increase in release of LDH started to moderate with

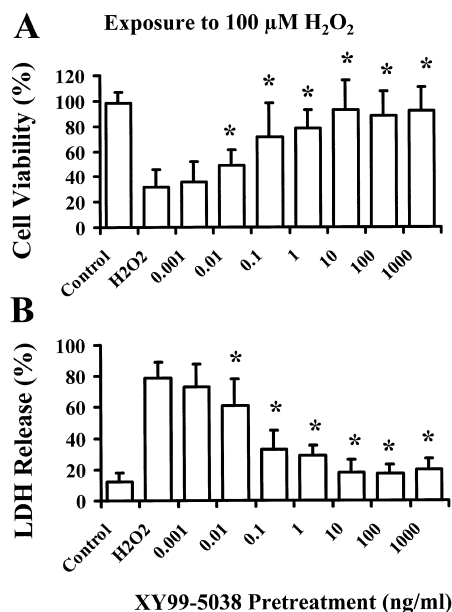


Fig. 2. Dose-dependent protective effect of XY99-5038. Protective effect of XY99-5038 on cultured retinal neurons against  $\text{H}_2\text{O}_2$ -induced cell death, as determined by (A) the MTT assay, (B) the LDH assay. Cultures were pre-treated with different concentrations of XY99-5038 for 1 hr before exposure to  $100 \mu\text{M}$   $\text{H}_2\text{O}_2$  for 24 hrs. \*  $p < 0.05$  versus the same concentration of  $\text{H}_2\text{O}_2$  exposure without pretreatment (mean  $\pm$  SD,  $n = 8$ ).

pretreatment of  $0.01 \text{ ng/ml}$  XY99-5038. Again, the pattern of dose-dependent protective effect of XY99-5038 on  $\text{H}_2\text{O}_2$ -induced cytotoxicity determined by LDH assay was similar to that determined by MTT assay.

The MTT and LDH assays are convenient screening assays for the measurement of cell death but they do not discriminate between necrosis and apoptosis. It has been shown that apoptosis is the mechanism of cell death for a diverse group of neuronal lesions and oxidative stress has been shown to play a role in the apoptotic process. To determine whether XY99-5038 can inhibit  $\text{H}_2\text{O}_2$ -induced apoptosis, the TUNEL assay was applied. A few TUNEL positive staining cells were noted in control cultures (Fig. 3), whereas cultures treated with  $100 \mu\text{M}$   $\text{H}_2\text{O}_2$  for 24 hrs contained large numbers of cells undergoing apoptotic cell death (Fig. 3B). Parallel cultures pretreated with different doses of XY99-5038 ( $0.001$ ,  $0.01$ ,  $0.1$ ,  $1$ ,  $10$ ,  $100$  and  $1000 \text{ ng/ml}$ ) 1 hr before  $100 \mu\text{M}$   $\text{H}_2\text{O}_2$  exposure showed a significant decrease in the numbers of apoptotic cells starting at the concentration of  $0.01 \text{ ng/ml}$  of XY99-5038 (Fig. 3E). The maximum protection was seen at  $10 \text{ ng/ml}$  of XY99-5038 or higher (Fig. 3D), whereas  $0.001 \text{ ng/ml}$  XY99-5038 did not affect  $100 \mu\text{M}$   $\text{H}_2\text{O}_2$ -induced apoptosis (Fig. 3C).

To investigate whether the addition of XY99-5038 to the cultures after  $\text{H}_2\text{O}_2$  exposure could enhance or attenuate the protective effect, cultures were treated with  $100 \text{ ng/ml}$  XY99-5038 and  $100 \mu\text{M}$   $\text{H}_2\text{O}_2$  exposure for 24 hrs at same time (0 hr pretreatment). Similar cultures were also treated with same concentration of XY99-5038 at 2, 4 and 6 hrs after  $100 \mu\text{M}$   $\text{H}_2\text{O}_2$  exposure. As expected and shown in Fig. 4A, the cultures exposed to  $100 \mu\text{M}$   $\text{H}_2\text{O}_2$  for

24 hrs revealed large numbers of cells undergoing apoptotic cell death, whereas the simultaneous addition of 100 ng/ml XY99-5038 and 100  $\mu$ M H<sub>2</sub>O<sub>2</sub> caused a dramatic decrease in the numbers of apoptotic cells (Fig. 4B). In XY99-5038 post-treated groups, 2 hr post-treated cultures showed a significant reduction in the numbers of apoptotic cells (Fig. 4C) whereas 4 hr and 6 hr XY99-5038 post-treated groups did not show statistically significant protective effect (Figs. 4D and 4E). The percentage of TUNEL positive cells (Fig. 4E) in control cultures from eight independent experiments was only 2–5%, whereas H<sub>2</sub>O<sub>2</sub>-treated cultures exhibited 65% positive cells. Addition of XY99-5038 and H<sub>2</sub>O<sub>2</sub> at the same time to the cultures significantly reduced the positive cells to approximately 10%, whereas 2 hr, 4 hr or 6 hr XY99-5038 post-treated cultures showed approximately 20%, 50% and 60% apoptotic cells respectively.

## Discussion

In the present study, we used H<sub>2</sub>O<sub>2</sub> as a toxic stimulus in a retinal neuron culture model to examine the protective role of XY99-5038. We chose H<sub>2</sub>O<sub>2</sub> to induce retinal neuron death in our cell cultures because: 1) oxidative stress is believed to be an important mediator of neuronal cell death and has been postulated to contribute to the pathogenesis of retinal degeneration; 2) H<sub>2</sub>O<sub>2</sub> is a precursor of highly oxidizing, tissue-damaging radicals such as hydroxyl radicals and is known to be toxic to many systems; 3) exogenous H<sub>2</sub>O<sub>2</sub> can enter the cells and induce cytotoxicity due to its high membrane permeability [21]; 4) H<sub>2</sub>O<sub>2</sub> has been reported to trigger apoptosis in various cell types [4]. We found that exposure of cultured retinal neurons to H<sub>2</sub>O<sub>2</sub> induces cell death. We have also noted that at concentrations of 100  $\mu$ M H<sub>2</sub>O<sub>2</sub>, most TUNEL positive cells exhibited an apoptotic morphology. At present, neither the precise sequence of events nor the critical triggering mechanisms involved in H<sub>2</sub>O<sub>2</sub>-induced neuronal injury has been established. Inhibition of mitochondrial activity has been shown to result in apoptosis [22,23]. The MTT assay is a colorimetric assay for the non-radioactive quantification of cell viability and is based on the cleavage of the yellow tetrazolium salt MTT to purple formazan crystals by metabolically active cells. The formation of formazan is thought to take place via intact mitochondria. A decrease in number of living cells results in a decrease in the total metabolic activity in the sample. Our MTT assays demonstrated that the reduction of metabolic activity was significant, suggesting an inhibition of mitochondria function by H<sub>2</sub>O<sub>2</sub> in this culture system.

We demonstrate here that XY99-5038 protects against H<sub>2</sub>O<sub>2</sub>-induced retinal cell death. The protective effect was dose-dependent with half-maximum effect at 0.1 ng/ml. Like many other formulations of Chinese Traditional Medicine, the components/ingredients in this formula remain to be discovered. The dilemma in understanding the efficacy of this formulation in treatment of diseases has been the uncertainty of what components or other factors incorporate the external qi [24] in the preparation and how those compositions or factors delay or prevent or even cure certain diseases. Limited information is known at present time. It has been determined that XY99-5038 contains high concentration of Vitamin C and some other beneficial amino acids (data not shown). However, the concentration of these components in XY99-5038 that were added to our cultures in this experiments were relative low and they may not play the major role in its protective effect against H<sub>2</sub>O<sub>2</sub>-induced retinal cell death ob-

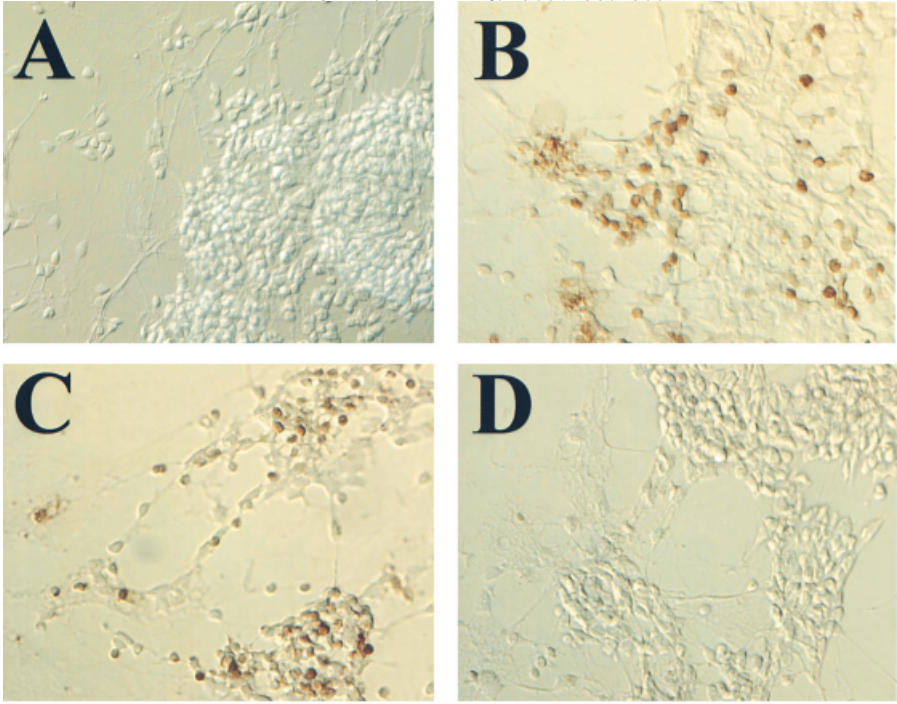


Fig. 3.

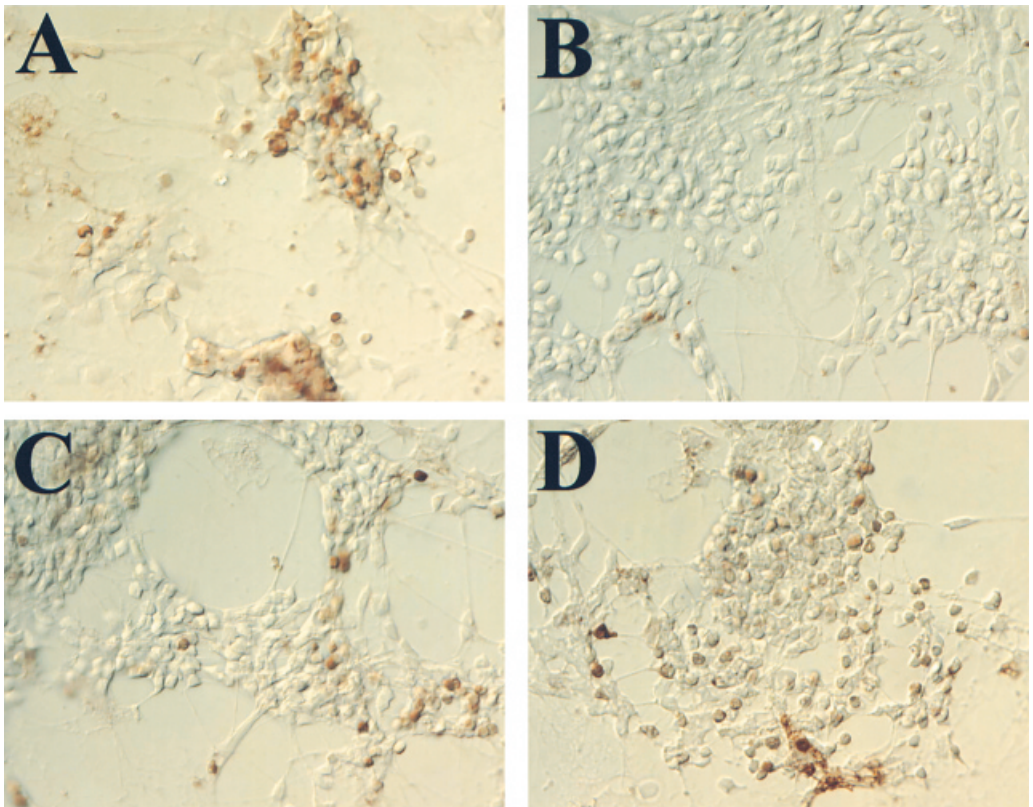


Fig. 4.



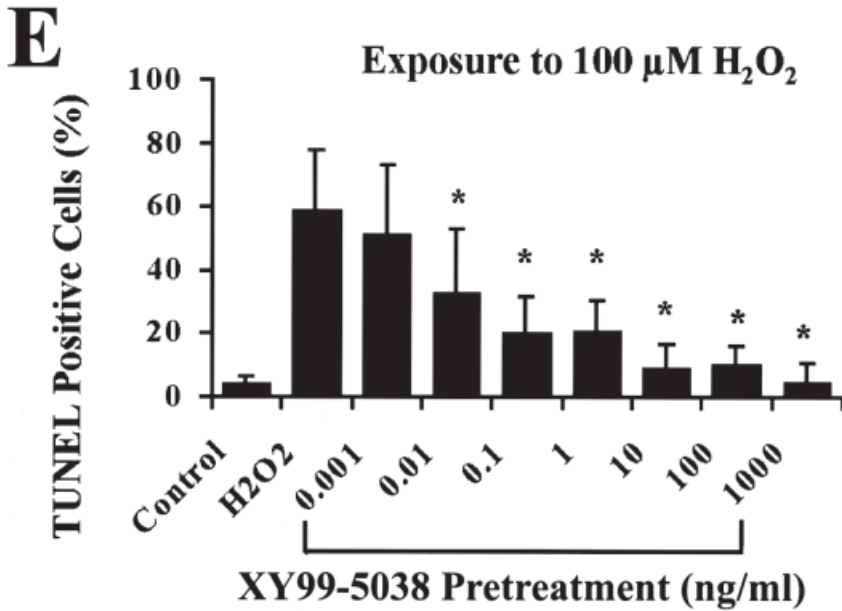


Fig. 3 (continued). XY99-5038 inhibits  $\text{H}_2\text{O}_2$ -induced retinal neuron apoptosis. A: Untreated normal control. B: Exposure to 100  $\mu\text{M}$   $\text{H}_2\text{O}_2$  only. C: Pretreated with 0.001 ng/ml XY99-5038 1 hr before exposure to 100  $\mu\text{M}$   $\text{H}_2\text{O}_2$  for 24 hrs. D: Pretreated with 10 ng/ml XY99-5038. E: Dose-dependent inhibition of XY99-5038 on 100  $\mu\text{M}$   $\text{H}_2\text{O}_2$ -induced apoptosis as determined by TUNEL assay. \* $p < 0.05$  versus the same concentration of  $\text{H}_2\text{O}_2$  exposure without XY99-5038 pretreatment (mean  $\pm$  SD,  $n = 8$ ).

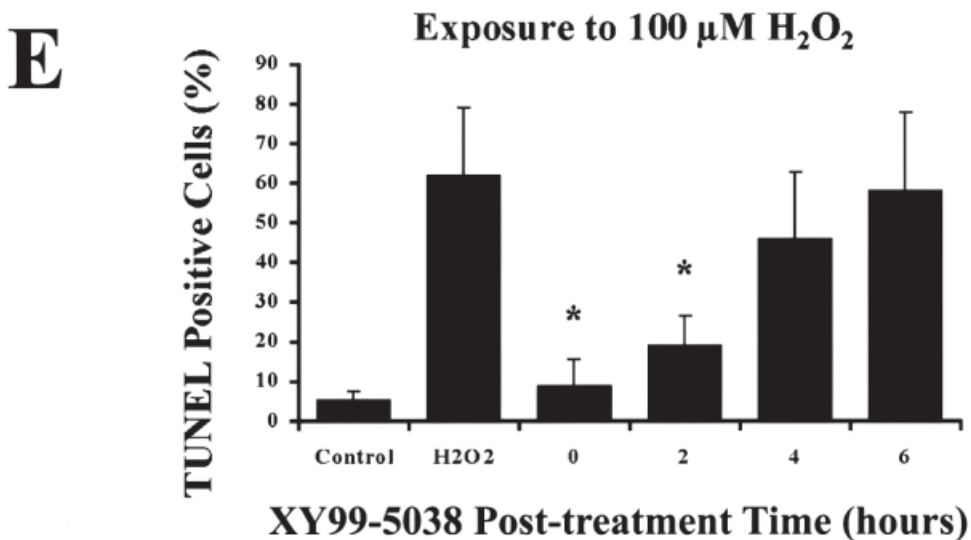


Fig. 4 (continued). Protective effects of XY99-5038 on  $\text{H}_2\text{O}_2$ -induced apoptosis in cultured retinal neurons as a function of treatment time.  $\text{H}_2\text{O}_2$  (24 hrs) and XY99-5038, when present, were used at 100  $\mu\text{M}$  and 100 ng/ml. A:  $\text{H}_2\text{O}_2$ . B: Additions of XY99-5038 and  $\text{H}_2\text{O}_2$  at same time. C: Treatment with XY99-5038 2 hr after  $\text{H}_2\text{O}_2$  exposure. D: Treatment with XY99-5038 4 hr after  $\text{H}_2\text{O}_2$  exposure. E: Time-dependent inhibition of XY99-5038 on 100  $\mu\text{M}$   $\text{H}_2\text{O}_2$ -induced apoptosis. \* $p < 0.05$  versus  $\text{H}_2\text{O}_2$  exposure without XY99-5038 treatment (mean  $\pm$  SD,  $n = 8$ ).

served in present study. It has been observed that Vitamin C levels in excess of 50  $\mu\text{M}$  are necessary to optimally maintain flexor tendons from adult animals in organ culture [25]. Vitamin C at 60  $\mu\text{M}$  has been proved to protect against  $\text{H}_2\text{O}_2$ -induced DNA damage [26]. As calculated from our concentrated abstract, Vitamin C concentration in our stock solution (1 mg/ml) of XY99-5038 was 50  $\mu\text{M}$ . The maximum dose of XY99-5038 used in this study was 1000 ng/ml with vitamin C level at 0.05  $\mu\text{M}$ . It is known that there is no antioxidant effect when concentrations of Vitamin C are lower than 1  $\mu\text{M}$  [27]. As described before, XY99-5038 is from a specific formula provided and processed by Dr. Yan Xin and was believed to be therapeutic in a wide variety of clinical conditions [12–15] but it has not been studied under rigorous laboratory conditions. We report the first laboratory evidence that XY99-5038 has objectively measurable effects. The mechanism(s) underlying the protective effects of XY99-5038 remains to be elucidated. Therefore, we simply let the results speak for themselves.

In conclusion,  $\text{H}_2\text{O}_2$ , a free radical generator, significantly induces cell death in cultured retinal neurons and pretreatment of the cells with XY99-5038 inhibits this process in a dose-dependent manner. Application of the TUNEL assay demonstrates that the  $\text{H}_2\text{O}_2$ -induced cell death occurs by an apoptotic mechanism and that XY99-5038 prevents apoptosis. XY99-5038 could be a useful in the development of treatment strategies for degenerative disorders.

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