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XY99-5038 PROMOTES LONG-TERM SURVIVAL OF CULTURED RETINAL NEURONS

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We have previously reported that XY99-5038, a preparation from a specific formula of Traditional Chinese Medicine, could effectively inhibit hydrogen peroxide-induced retinal cell death. In the present study, we investigated the possibility of XY99-5038 to prolong neuronal survival in a long-term retinal neuronal culture. Basic fibroblast survival factor (bFGF), a potent neurotrophic factor, was employed as comparable agent. Retinas of 0–2 days old Sprague-Dawley rats were isolated and dissociated. The cells were maintained in tissue culture for up to 9 weeks in a synthetic serum-free media. XY99-5038 (100 ng/ml) or a vehicle was added to culture every 3–4 days, starting at the first week of culturing. The number of cells were counted and compared for each time point and treatment. Cell viability was also determined by MTT assay, whereas apoptotic cell death was evaluated by the TUNEL assay. XY99-5038 treatment significantly reduced cell loss, increased cell viability, and inhibited apoptosis in this long-term retinal neuronal culture. 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 beneficial to the prolongation of neuron survival.

Keywords aging, apoptosis, basic fibroblast growth factor, Yan Xin Life Science Technology

We have recently reported that XY99-5038 inhibited hydrogen peroxide (H_2O_2) induced cytotoxicity in cultured retinal neurons, and that H_2O_2-induced retinal neuron apoptosis could be blocked by XY99-5038 (Yan et al., 2001). The overall evidence indicates differentiated cells in vivo accumulate damage over lifetime that results in gradual loss of function and increased probability of degeneration (Rubin, 1997; Drachman, 1997). It has been demonstrated that aging is associated with increased intensity of oxidative damage (Stadtman, 1998; Sohal & Orr, 1998), and that oxygen radicals plays a role in degenerative senescence of the nervous system (Herman, 1995; Pahlavani & Van Remnen, 1997; Markesbery, 1997). The attractive feature of the oxidative stress hypothesis is that it can account for cumulative damage associated with the delayed onset and progressive nature of aging-related disorders, such as Parkinson's disease and Alzheimer's disease as well as retinal degeneration (Carney, 2000; Beatty et al., 2000).

XY995038 is one product in the XY-S series of Yan Xin Life Science Technology (YXLST) research products. It is abstracted from a nontoxic pure plant formula; it includes dozens of nontoxic plant ingredients that are beneficial to life. XY995038 has been undergoing research and study for ten years (Yan et al., 2000; Fong, 1997; Hang et al., 1997; Wang et al., 1997). YXLST is named after Prof. Yan Xin (Yan is the family name), who is a chief physician in China and a World renowned Traditional Chinese Medicine specialist and scientist (Yan et al., 1999). He has held senior positions such as guest professor, adjunct professor, research professor, visiting professor, advising professor, research scientist etc. in leading high education and research institutions in China and the West (Yan et al., 1999). Within his research projects during 1992 to 1998, with reference to Yuhay Fong's original ancestral recipe, and based on the principles of nutrition and life science, Prof. Yan applied integrated preparation and special treatment to produce "Yan Xin Qigong Nutritional Powder" (Fong, 1997; Hang et al., 1997; Wang et al., 1997). Based on that, according to the principles of traditional Chinese medicine and the progress in modern life science research, Prof. Yan conducted further integration, filtering and selection, and finalized the ingredients of XY995038. XY995038 is specially processed by Yan Xin Life Science technology™, a technological achieve-

ment of Yan Xin Qigong ® that received state certification for "Optimization of Industrial Antibiotics Production Strains" on April 20, 1990 in China (Yan et al., 1999). XY995038 is produced by American Biology Science Research Institute using modern big-engineering technologies and special refining methods for nutritional preparation. In the ten-year-long research, it has achieved not only ideal results in the laboratories but also good results in certain clinical studies (Fong, 1997; Hang et al., 1997; Wang et al., 1997; Yan et al., 2000; 2001).

Numerous studies performed on cultured human fibroblasts (Wistrom & Villeponteau, 1990), lymphocytes (Pawelec et al., 1997), and several other types of mammalian cells (Augustin-Voss et al., 1993) demonstrated that normal dividing cells have a finite lifetime in vitro (Hayflick, 1998). During the last decade, significant progress was made in culturing of highly differentiated cell, such as neurons. It became possible to consider the application of long-term primary neuronal cultures to study age-related changes in vitro (Kuroda et al., 1995; Puttfarcken et al., 1996; Porter et al., 1997; Aksenova et al., 1999; Potter & DeMarse, 2001; Troadec et al., 2001). Long-term neuronal culture has been used as aging and neurological research tool to study the effects of long-term exposure to aluminum, a risk factor for Alzheimer's disease, on the cultured cortical neurons (Kuroda et al., 1995). It has been demonstrated that age-induced beta-amyloid neurotoxicity in long-term cultured rat hippocampal cells can be inhibited by antioxidants as well as other agents that provide protection against oxidative damage (Puttfarcken et al., 1996). Age-dependent increases in the density of Ca^{2+} channels in long-term culture of hippocampal cells have been reported to be analogous to patterns seen in neurons of in vivo aged animals (Porter et al., 1997). Age-dependent changes of neuronal survival, protein oxidation and creatine kinase BB expression in long-term hippocampal cell culture have also been described as aging in dish (Aksenova et al., 1999). Using long-term mesencephalic culture, Troadec and coworkers demonstrated that noradrenaline promotes survival and function of dopaminergic neurons (Troadec et al., 2001). All these suggest that long-term primary neuronal culture could serve as model for studying some aspects of aging changes (Porter et al., 1997; Potter & DeMarse, 2001). In present study, we used long-

term retinal neuronal culture to study the effects of XY99-5038 on prolongation of neuronal survival in vitro.

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), and human recombinant basic fibroblast growth factor (bFGF) were purchased from Sigma (St. Louis, MO, USA). DMEM/F12 and Tii-Medium were purchased from Gibco-BRL (Grand Island, NY, USA) and JRH Biosciences (Lenexa, KS, USA), respectively. XY99-5038 was prepared from several purely natural and nontoxic plants. For these experiments, XY99-5038 was made and supplied by American Biology Engineering Science Research Institute (Los Angeles, CA, USA). Because the efficacious components of this preparation are still unknown, total protein level was used as an index to estimate the concentration of XY99-5038. The concentrated extract was filtered with a 0.2 μm filter and diluted with a serum-free culture medium to 1 mg total protein/ml as stock solution for further use. Tissue culture plasticware and 12-mm diameter coverslips were bought from Fisher Scientific (Pittsburgh, PA, USA). Apoptosis detection kits were purchased from Oncor (Gaithersburg, MD, USA).

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 (Cao et al., 1999, 2000). 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 μm sieve and rinsed with medium. The filtrate was subsequently passed through a 140 μm sieve, followed by a rinse with undiluted fetal calf serum. The suspension was centrifuged at 800 rpm in a clinical centrifuge for 5 min, 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 was diluted with medium to 1×10^5 cells per ml. The cells (1 ml) were plated in 24 well tissue culture plates on 12 mm coverslips that had been pretreated overnight with poly-D-lysine (10 μg/ml). The cells were maintained in the synthetic serum-free media developed by Bottenstein and Sato (Bottenstein et al., 1979), as modified by Lillien and Cepko (Lillien & Cepko, 1992).

Cell Counting

Cell counting was performed by staining the cells with DAPI (4',6-diamino-2-phenylindole) and compared for each time point and treatment. The results were viewed at 20× objective with a Nikon Eclipse 800 microscope under ultraviolet light. The pictures were recorded and transferred by a digital camera and stored in the computer. Five digitized images of similar total cell numbers were selected from each coverslip for counting and averaging, and were considered as one independent experiment. A total of nine independent experiments were averaged for each time point and treatment.

Immunocytochemistry

Cells grown in culture on poly-D-lysine coated coverslips were fixed for 30 min in 2% paraformaldehyde in Tris buffered saline, and then rinsed 3 times with 1.0 ml of 0.1 M Tris-HCl, pH 7.5. Nonspecific binding sites were blocked by 2% normal goat serum for 30 min. Cells were incubated overnight with monoclonal antirhodopsin antibody, as well as polyclonal antirecoverin antibody in 0.1 M Tris-HCl buffered saline containing 1% normal goat serum, and

then rinsed three times with 1.0 ml of the same buffer. Subsequently, rhodopsin was visualized using biotinylated goat antimouse IgG, followed by incubation with streptavidin conjugated to Texas-Red. Recoverin was detected using goat antirabbit IgG conjugated to FITC. Cells treated without primary antibody served as control and were unlabeled. Cells were also treated with normal rabbit or mouse IgG at the same concentrations as the primary antibodies, served as additional control, and were unlabeled. The cells on cover slips were then mounted with antifade mounting medium and viewed and photographed with an Eclipse 800 Nikon microscope equipped with fluorescence and Nomarski optics and a digital camera. The pictures were transferred by a digital camera and stored in the computer.

MTT Assay

MTT was dissolved at a concentration of 5 mg/ml in PBS. The lysing buffer was prepared as follows: sodium dodecyl solfate (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 μl of the stock solution of MTT was added to each well and incubated for 2 h at 37°C, and then 100 μl of the lysing buffer was added. After overnight incubation at 37°C, absorbance of the samples was read at 562 nm using a microtiter plate ELISA reader.

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 μg/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. Nine 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 $p < .05$ were accepted as significant. The regression analysis was performed using Sigma Plot software package.

RESULTS

Delaying Cell Loss in Long-Term Retinal Neuron Cell Culture by XY99-5038

Morphology and immunocytochemistry of retinal neuron in week-one culture are shown in Figure 1. Most of the cultured cells were rounded neuron-like cells with long processes and were identified by specific retinal neuron markers (Cao et al., 2000). Figure 1 shows numerous recoverin-positive and rhodopsin-positive cells among a background of unlabeled cells that are visualized with Nomarski optics. Rod photoreceptors that were positive for both proteins appear yellowish green or yellowish red, while cone photoreceptors and bipolar cells that only have recoverin protein are green.

XY99-5038 (100 ng/ml) or vehicle at the same volume was added to the cultures every 3–4 days starting at the first week of culturing. The concentration of XY99-5038 at 100 ng/ml was determined based upon our prior work with known effective doses in protecting against H_2O_2-induced retinal neuron cell death in the same culture system (Yan et al., 2001). Since our preliminary results show that the cell numbers do not change significantly up to week 2, we used week 1

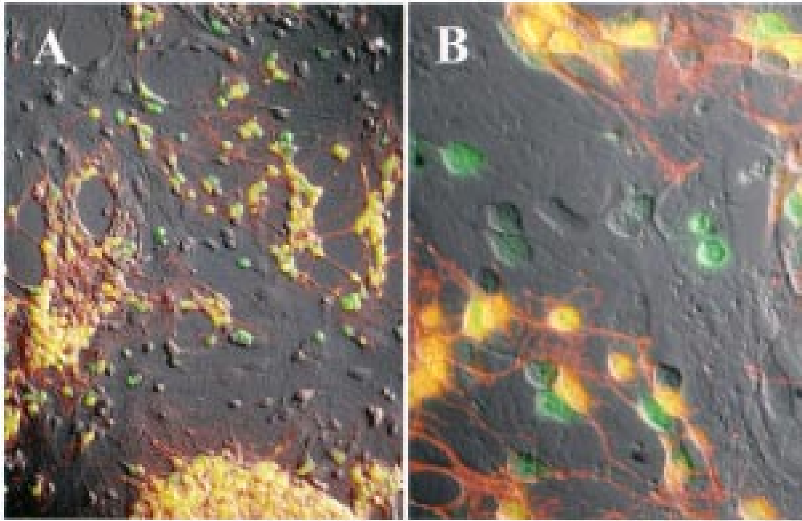

FIGURE 1. Cultured postnatal retinal neurons labeled antirecoverin and rhodopsin antibodies. Field was viewed with filters which allow the Nomarski image, fluorescent (recoverin, green), and Texas-Red (rhodopsin, red) stained cells to be seen simultaneously. (**A**) Low magnification. (**B**) High magnification. (See Color Plate I at end of issue.)

age as a reference time point in our investigation of the effects of XY99-5038 on the delay of age-related changes in long-term rat retinal neuronal cultures. The cells were plated in 24-well tissue culture plates and maintained for up to 9 weeks. Figure 3 shows that a noticeable age-related cell loss started to occur after 3 weeks in culture. A significant loss of cells was observed in the culture that reached the age of week 4 (Figures 2 and 3). By week 5, the number of cells progressively declined. By week 6, although 30% of DAPI-stained cells were observed, most cells lost dendritic processes and cell bodies had significant shrinkage (Figure 2). After week 6, less than 10–20% DAPI-stained cells were seen (Figure 3). XY99-5038 treatment almost completely prevented age-dependent cell loss up to week 6. Although significant loss of cells was seen in XY99-5038-treated groups by weeks 7, 8, and 9, XY99-5038 treatment was still able to significantly reduce the number of the cell loss in comparison with vehicle-treated group at the same culturing age (Figure 3). Basic fibroblast growth factor (bFGF), a potent

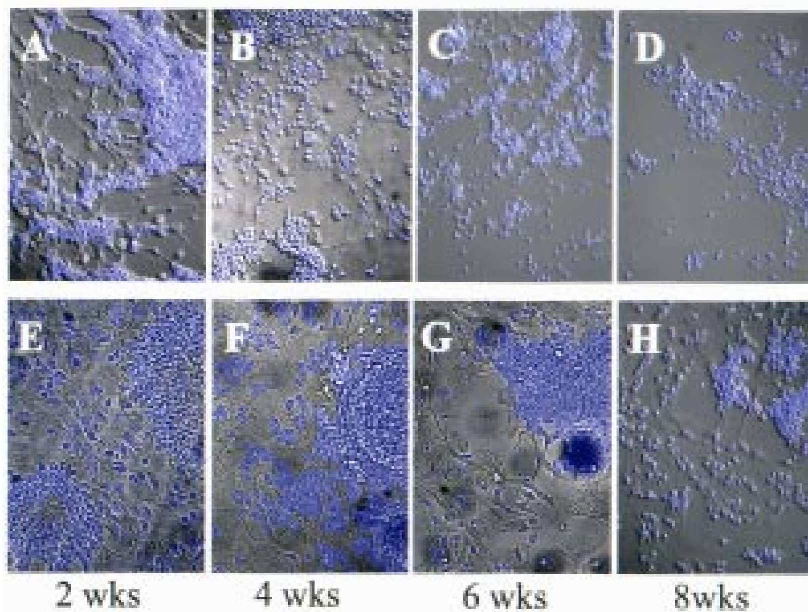

FIGURE 2. Delay of age-dependent cell loss by XY99–5038. Cells were extracted from Sprague-Dawley rats at 0–2 days, kept in serum-free media until appropriate time point, and then fixed on coverslips. XY99-5038 (100 ng/ml) was added to cultures every 3–4 days, starting at the first week of culturing. All cells were stained with DAPI and viewed under ultraviolet light at 20× overlaid with Nomarski image. (**A**) week 2, nontreated; (**B**) week 4, nontreated; (**C**) week 6, nontreated; (**D**) week 8, nontreated; (**E**) week 2, XY99–5038 treated; (**F**) week 4, XY99–5038 treated; (**G**) week 6, XY99–5038 treated; (**H**) week 8, XY99–5038 treated. (See Color Plate II at end of issue.)

neurotrophic factor/growth factor, at 100 ng/ml also significantly reduced cell loss by weeks 3 and 4, whereas this protection was limited by week 5 and longer (Figure 3).

A significant loss of cells was observed in the cultures that reached the age of week 4. By weeks 5 and 6, the number of survival cells progressively declined. Morphological observation of aging cell cultures revealed the presence of neurons with vacuolated soma and beaded or fragmented neurites. By weeks 7 and 8, only 30–40% of survival cells was observed. We used the data of cell counts to determine the probability of survival ($P(t) = N(t)/N(o)$) at different culture ages. $N(t)$ = the changes of the number of viable neurons per

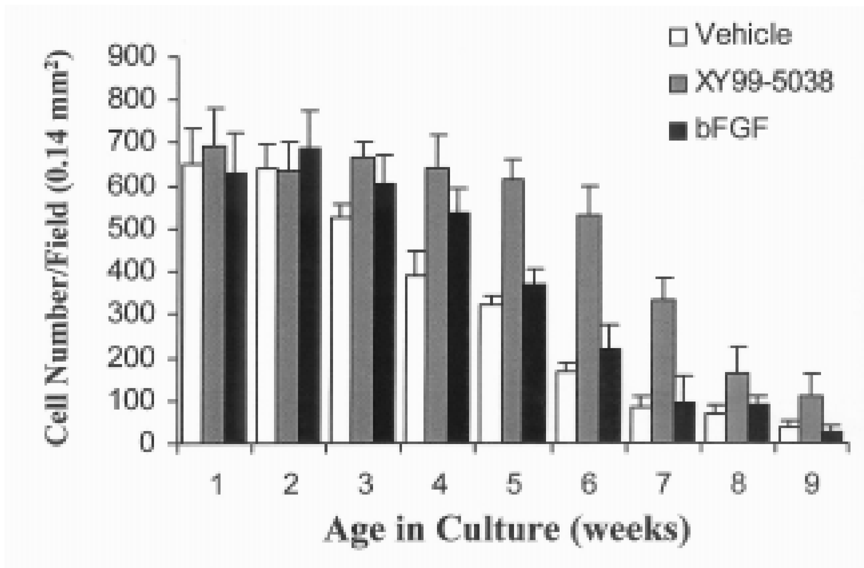

FIGURE 3. Quantitation of cell number. Cells from Sprague-Dawley rats were fixed at weekly time points. The five fields from each coverslip were chosen and viewed at 20× objective of microscope under ultraviolet light, and the numbers of five fields were averaged as one independent experiment (mean ± SD, n = 9).

digitized image. N(o) = the amount of viable neurons per digitized image at 4 days in culture. In order to investigate whether Gomperts law of exponential growth of mortality with age is applicable to the cell death in this long-term neuronal cultures, we calculated values of P(t) were approximated with formula of a Gomperts-type P(t) function:

$$P(t) = e^{-R_0(e^{k(t - t_0)}/K)}$$

Nonlinear regression analysis demonstrated that the experimented data of retinal neuronal cell survival in long-term culture could be successfully fitted with the theoretical “Gompertsian” survival curve (R^2 = 0.985) (Figure 4a). The fitted model is N(t)/N(o) = exp (–0.05635628*exp[0.4993406]) (Figure 4b). For XY99-5038 treatment, the model is inappropriate with R^2 only 0.2. The lack of fit is also clearly seen from plot (Figure 4c). The difference between the vehicle-treated and XY99-5038-treated groups is significant.

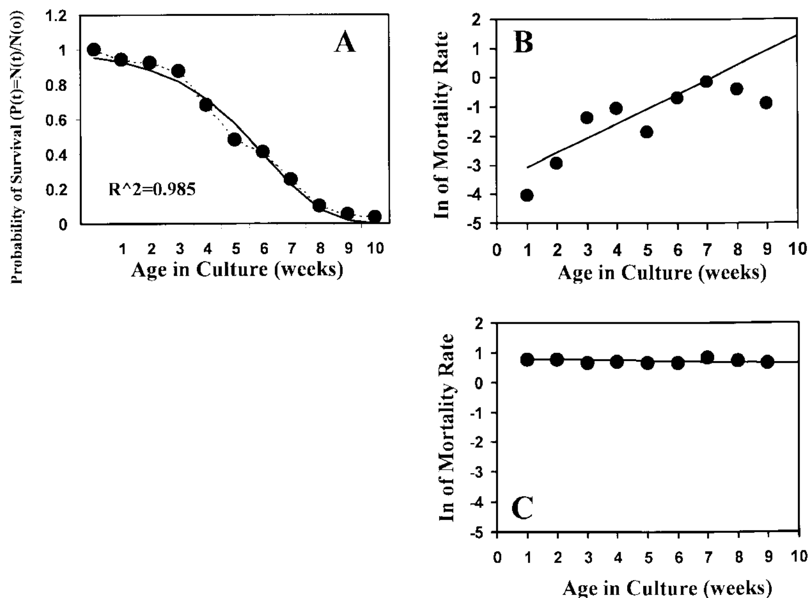

FIGURE 4. Long-term viability of neurons in culture. (**A**) The original data of the probability of neuronal survival and corresponding theoretical "Gompertsian" survival curve are determined by means of nonlinear regression. (**B**) The In of mortality curve corresponds with neuronal survival data. The straight line is the theoretical Gompertsian line. (**C**) The In of mortality curve corresponds with prolongation of retinal neuronal survival in culture by XY99–5038.

Inhibition of Age-Dependent Decline in Cell Viability by XY99-5038

Cell viability was determined by the MTT assay. The MTT assay is a colorimetric assay for the nonradioactive 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 the number of living cells results in a decrease in the total metabolic activity in the sample. As shown in Figure 5, there were no significant differences in cell viability between vehicle-treated cultures and XY99-5038-treated ones when cells were maintained in culturing within 2 weeks. At week 3, a rapid decrease in cell viability was seen in vehicle-treated group. Cell viability decreased to 40% at week 4, and to 16% at week 6 and maintained

this low level afterward. XY99-5038 (100 ng/ml) significantly inhibit-age-dependent decline in cell viability up to week 6. XY99-5038 treatment was still able to increase cell viability in comparison with vehicle-treated group at the same culturing age after week 6 in culture. These data of MTT assay are consistent with that shown in Figure 4. Furthermore, the protective effect of XY99-5038 is more potent than that of bFGF (Figure 5).

XY99-5038 Inhibits Aging-Induced Apoptosis in Cultured Retinal Neurons

It has been demonstrated that aging enhances apoptosis and susceptibility to apoptosis in several types of intact cells (Higmi & Shimokawa, 2000). To determine whether age-dependent loss in our primary retinal neuronal culture is due to apoptosis, the TUNEL assay was applied to the cultures at different ages. The results of our study show that only few TUNEL-positive cells were noted in the culture within the first week, whereas at week 3, a significant increase in TUNEL-positive cells was seen (Figure 6A). This increase in TUNEL-positive staining was more prominent at week 6 (Figure 6B). By week 9, most

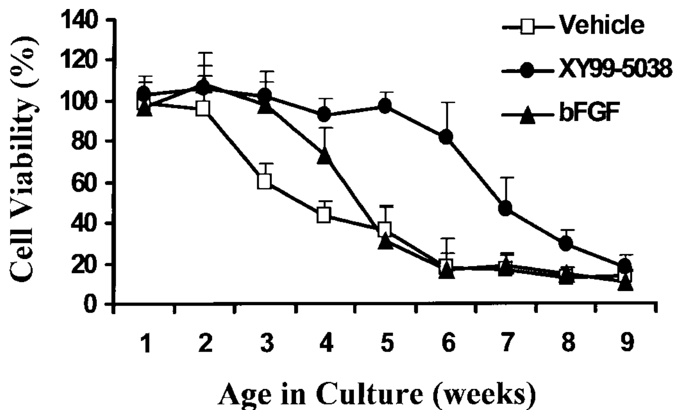

FIGURE 5. Increase of cell viability by XY99–5038. XY99–5038 or bFGF was added to cultures of treated group at final concentration of 100 ng/ml every 3–4 days, starting at the first week of culturing. For control group, the culture medium was replaced as vehicle by the fresh medium without XY99–5038 every 3–4 days. The viability was monitored with MTT activity for the time indicated (mean ± SD, n = 9).

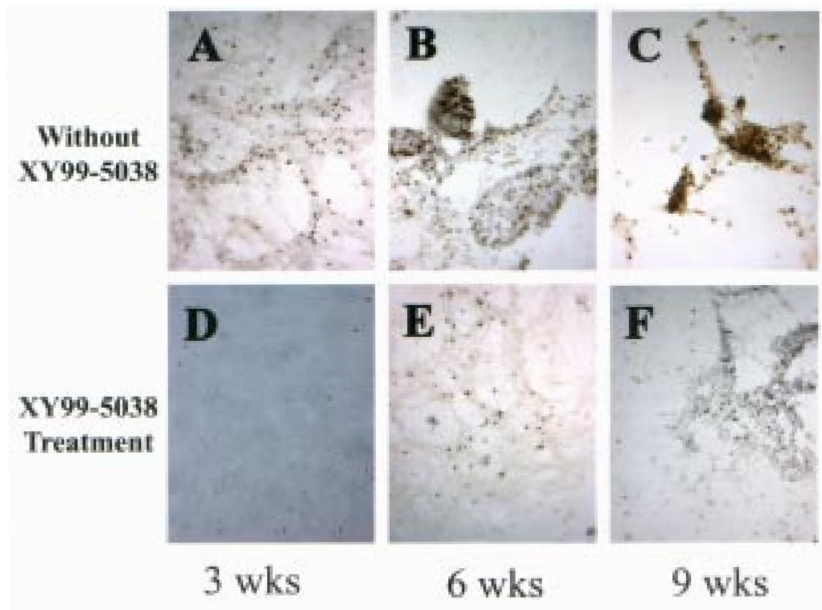

FIGURE 6. Inhibition of age-dependent apoptosis by XY99–5038. Apoptosis in the culture was determined by TUNEL Assay. Figures A, B, and C are without XY99–5038 treatment, whereas Figures D, E, and F are XY99–5038 treated. (**A**) week 3; (**B**) week 6; (**C**) week 9; (**D**) week 3, (**E**) week 6; and (**F**) week 9. (See Color Plate III at end of issue.)

cells were apoptotic cells (Figure 6C). As shown in Figure 7, the percentage of TUNEL-positive cells in week-1 or week-2 cultures were from 4–8%, whereas week-3 culture exhibited 26% positive cells. The positive cells reached 35% at week 4, 57% at week 5, 61% at week 6, 69% at week 7, 86% at week 8, and 88% at week 9.

As mentioned before, XY99-5038 (100 ng/ml) was added to cultures every 3–4 days starting at the first week of culturing. As shown in Figures 6A and 6D, by week 3, XY995038 inhibits age-dependent apoptosis compared to the same age of cells in the culture. There was significant reduction of apoptotic cells at week 6 with XY99-5038 treatment (Figure 6E). By week 9, XY99-5038 treatment was still able to reduce the apoptotic cells (Figure 6F) compared to the same cultured age of vehicle-treated group (Figure 6C). As shown in Figure 7, XY99-5038 treatment completely inhibits age-dependent apoptosis within the first 4 weeks. By weeks 5 or 6,

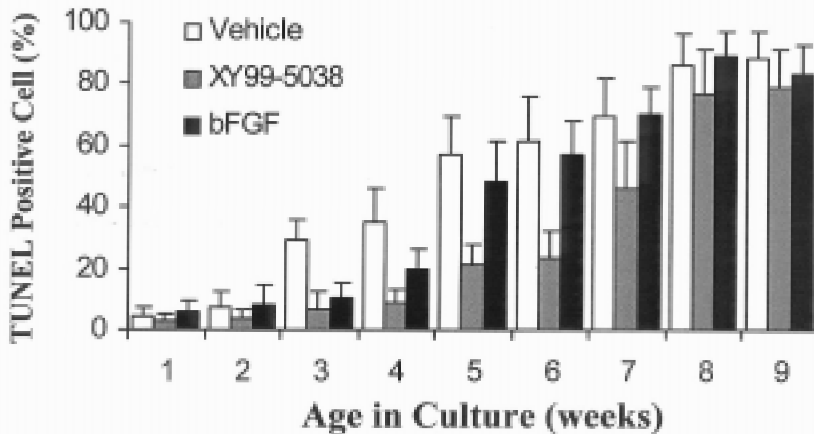

FIGURE 7. Quantitation of apoptotic cells. Apoptotic cells were detected with TUNEL Assay. Three digitized images of similar total cell numbers were selected from each coverslip for counting and averaging, and were considered as one independent experiment. Percentage of TUNEL-positive cells = TUNEL-positive Cells/Total Cells (Mean ± SD, n = 9).

the percentage of apoptotic cells was decreased from 57–61% in vehicle-treated groups to 21–23% in XY99-5038-treated groups. Although a significant increase in apoptotic cells in XY99-5038-treated group was seen by week 7 and after, XY99-5038 treatment was still able to significantly decrease apoptotic cells in comparison with vehicle-treated group at the same culturing age. Consistent with that shown in Figure 5, XY99-5038 is more efficient to inhibit apoptosis than bFGF (Figure 6).

DISCUSSION

Aging is one of the main factors for degeneration of the nervous system (Toescu et al., 2000). In most neurodegenerative diseases, incidences of the disease increase with aging. Aging results in the growth of the risk of death, and the quantity describing this risk is mortality. The Gomperts law of exponential growth of mortality holds for the majority of living organisms that are used as experimental models in gerontological studies (Gomperts, 1825; Strehler & Mildvan, 1960; Witten, 1985; Doubal & Klemera, 1997).

Corresponding to the exponential mortality rate is the Gompertsian survival curve that is described by the formula given in the Results section. Our study demonstrated that retinal neuronal survival in long-term primary culture proceeds according to Gomperts law (Figure 4). The neuronal retina is a postmitotic structure and is vulnerable to many insults including age-related changes, which affect its function. A complex of genetic, programmed, and variable factors producing the age-related changes affect retinal neuronal survival and can result in retinal neuronal degeneration. Long-term neuronal culture has been used as an aging and neurological research tool for understanding the changes of aging, their impact on disease, and for the development of agents able to modify age-related changes and to prevent neurodegeneration (Kuroda et al., 1995; Puttfarcken et al., 1996; Aksenova et al., 1999; Troadec et al., 2001). Age-dependent increases in the density of Ca^{2+} channels in long-term culture of hippocampal cells have been reported to be analogous to patterns seen in neurons of in vivo aged animals (Porter et al., 1997). The results of our study suggest that long-term retinal neuronal cultures may also be used for studying some aspects of aging as a factor in retinal degeneration.

Apoptotic events reflect an interplay between intrinsic signaling events that rely on cytokines, neurotransmitters, and growth factors and responses to extrinsic events that increase levels of radical oxygen species. Although many hypotheses have been proposed to explain the aging process, the exact mechanisms are not well defined. Recent accumulating evidence indicates that the apoptosis is involved in aging processes. In various cells, apoptosis enhances the elimination of damaged and dysfunctional cells presumably caused by oxidative stress. In these cases, the incidence of apoptosis plays an important role in the aging process. Oxidative stress is an important factor in the induction of apoptosis in the aging process (Higami & Shimokawa, 2000). Some reports demonstrated an age-enhanced susceptibility to apoptosis in T cell in C57BL/6 mice (Chrest et al., 1995) and human subjects (Potestio et al., 1998). In hepatocytes, the rate of intrinsic apoptosis increases with aging in B6C3F1 mice and Fischer 344 rats in vivo (Higami et al., 1997; Muskhelishvili et al., 1995). The loss of neurons, which are nondividing cells, is closely associated with functional impairments such as dementia and motor

neuron disability in neurodegenerative diseases. We have recently reported that XY99-5038 effectively inhibited H_2O_2-induced cytotoxicity, and that H_2O_2-induced retinal neuron apoptosis could be blocked by XY99-5038 (Yan et al., 2001). It has also been demonstrated that age-induced beta-amyloid neurotoxicity in long-term cultured rat hippocampal cells can be inhibited by antioxidants, as well as other agents that provide protection against oxidative damage (Puttfarcken et al., 1996). The effect of XY99-5038 on the delay of age-induced changes in vitro observed in the present study is probably through reducing oxidative stress-induced apoptosis. However, the inhibitory effects of XY99-5038 on age-dependent apoptosis observed in our present study is probably not caused by its vitamin C content. The amount of XY99-5038 as added to our cultures in this experiment was 100 ng/ml. It has determined (Yen et al., 2001) that at this concentration the vitamin C level was 0.005 μM. It is known that there is no antioxidant effect when concentrations of vitamin C are lower than 1 μM (Cheng et al., 1988).

As mentioned in the previous report, the components of this formula remain to be discovered, as for many other formulations of Chinese Traditional Medicine. The dilemma in understanding the efficacy of this formulation in promoting neuron survival has been the uncertainty of what are the components or other factors in XY99-5038 formula and how those compositions or factors delay aging process. Limited information is known at the present time. Therefore, we simply let these objectively measurable results speak for themselves. In conclusion, we report the laboratory evidence that XY99-5038 has objectively measurable effects on the delaying of aging process. The mechanism(s) underlying this effect of XY99-5038 remains to be elucidated.

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