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Full Length Research Paper

Fraction V of bovine albumin improves the adherence and survival of adult rat cerebral cortex neurons in primary culture

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Primary embryonic or young neuronal cells are used to study physiology or pathology of neurons. However, they are not optimal as a model for age-related changes in physiology or late-onset disease. Successful culturing of neurons from adult animals, however, has been historically difficult. Because the attack of anoxia and mechanical/chemical force in the course of the single neuron separated from the brain, the adult neocortex neurons may be edema. Since albumin can reduce edema and absorb toxic, we therefore hypothesized that albumin should improve the neuron survival *in vitro*. Here, we report that 0.2-0.5% albumin can accelerate neurons adherence to dish bottom, increase the attached cells number, and improve the viability. Our results indicate that fraction V of albumin is an effective supplier for neocortex neurons culture *in vitro*.

Key words: Adult, neocortex, primary culture, fraction V of bovine albumin.

INTRODUCTION

Alzheimer's disease (AD) is a major public health concern in all countries. Although the precise cause of AD is still unknown (Florent-Béchard et al., 2007), researchers have found that there is marked neuronal death in the neocortex and subcortex (hippocampus and nucleus basalis), especially in the neocortex of AD patients (Berger-Sweeney et al., 2001; Romito-DiGiacomo et al., 2007). The biochemical, functional and morphological characteristics of neocortical and subcortical neurons are different; for example, estradiol (100 nM) inhibits the activation of caspase-3 at an early stage of development (2 DIV) in hippocampal cultures, but not in neocortical cultures (Kajta et al., 2006). Adult neocortical neuronal culturing methods have not been well established; as a result, the most comprehensive studies of AD mechanisms ***in vitro*** are primarily focused on subcortical neurons (Resende et al., 2007; Hatanaka et al., 1988). Therefore, substitutions for cultured adult neurons with proliferating neuron-like cells (e.g., PC12, SN4741) (Biewenga et al., 2005; Okada et al., 2007), cultured young neurons (young neocortical or hippocampal neurons in culture) (Kajta et al., 2006; Resende et al., 2007; Lopes et al., 2007), neural stem cells and neurons from long-term cultures of neonatal neurons (Lesuisse and Martin, 2002), have been used in AD study. Studies involving only subcortical neurons are currently the best, but without neocortical neurons, subcortical neurons do not provide a complete picture of the neuropathology of AD.

Recently, it was found that mature post-mitotic neurons can also reenter the cell cycle (replicate their DNA) in neurodegenerative disorders (Lee et al., 2003 ; Vincent et al., 1997), but these neurons cannot divide and are destined to degenerate or die (Bauer and Patterson, 2005). These growth characteristics suggest that proliferative neuron-like cells (tumor cells or neuroendocrine cells) cannot be used in neurodegenerative studies. Alzheimer's disease is characterized by late life dementia

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Abbreviations: AD, Alzheimer's disease; **NGF**, nerve growth factor; **MTT**, 3-(4,5)-dimethylthiahiazo (-z-y1)-3,5-di-phenytetrazoliumromide.

(Florent-Béchard et al., 2007; Berger-Sweeney et al., 2001; Romito-DiGiacomo et al., 2007; Braak et al., 2003), and as a result, cultured young neocortical or subcortical neurons are not optimal models for the study of AD.

Alternatively, long-term cultures of neonatal neurons or primary cultures of young neurons have been used as models to study development, aging and death (Lesuisse et al., 2002; Akasofu et al., 2006). Usually, cortical neurosphere cells have been harvested from embryonic day 14 mice (Niculescu et al., 2005), but neurosphere cells can also be harvested from embryonic day 16 or 18 mice (Udagawa et al., 2006; Cheng et al., 2004). So, long-term culture methods do not protect new neurons from stem cell contamination. In this culture system, stem cells can divide, differentiate into neurons and serve as a continuing source of neurons. Moreover, after long-term culture, some biochemical characteristics of the cells change (e.g., the developmental stage of neurons cannot be clearly differentiated after long-term culture).

Organotypic cultures are likely to be a good choice for neuroscientific study, especially for study of the function and development of a neural organ (e.g., organotypic cultures were used to examine the mechanism of neural development) (Ghoumari et al., 2002), even for study of AD (Huuskonen et al., 2005); however, the effects of glial cells on neurons were not clearly distinguished from the effects of neurons on neurons in their experiments.

Therefore, there is a significant need to establish a method for the reliable cultivation of healthy adult neocortical neurons. Although methods for culturing primary dorsal root ganglion (DRG) and young neocortical neurons have been documented by several authors (Eide and McMurray , 2005; Nathan et al., 2004; Brewer and Torricelli, 2007), methods for adult neocortical neuronal cultures are still challenging. Developing more reliable and effective methods for neocortical neuronal culturing from adult animals is of great interest.

Above all, it is important to establish a culturing method for adult cortical neurons. Although methods for culturing primary neurons from young animals are easy to perform, methods for culturing primary neurons from adults are more difficult and extremely complex. .

Because the anatomical procedure of harvesting cortical neurons from the brain results in cellular anoxia, improvements of adherence ability (Nathan et al., 2004; Brewer and Torricelli, 2007) and reductions in neuronal edema are very important measures for the survival of cortical neurons ***in vitro.***

Albumin is an important substance needed by the body (Elliott et al., 2007). It combines many types of substances and maintains colloid osmotic pressure (Kass and Lipton, 1989). Albumin not only has neuroprotective effects against stroke (Parkkinen et al., 2007; Koch et al., 2004), but is also a new biological marker of ischemia (Abboud et al., 2007; Dziedzic et al., 2006). Therefore, we hypothesized that albumin could help adult neocortical neurons to live ***in vitro***. Here, we report that albumin can effectively improve the adherence ability and viability of adult cortical neurons ***in vitro***.

EXPERIMENTAL PROCEDURE

Chemicals

Nerve growth factor (NGF) (R and D Systems, Minneapolis, MN, USA), Fraction V of Albumin Bovine (Amresco 0332).

Supplement composition of the neuronal culture medium for

The basic culture medium was DMEM (High Glucose). Some supplements were added to the medium (Table 1).

Animals

We obtained adult Sprague Dawley rats aged 6 weeks or older (body weight, 220–260 g) and young rats aged 4–6 days from our experimental animal center (Central South University, Changsha, Hunan, ROC). All animal procedures were approved by the ethics committee of the XiangYa Hospital, which is affiliated with the Medical College, Central South University, and were in accordance with the European Communities Council Directive (86/609/EEC). All efforts were made to minimize the number of animals used and any potential suffering of those subjects. The rats had unlimited access to food and water before the experiments. After being weighed, the rats were anesthetized with Chloral hydrate (0.4 ml /100 g) and were placed in a supine position on a warming pad (24˚C)

Cerebral cortical neuronal culture methods

(Eide and McMurray, 2005; Nathan et al., 2004; Brewer and Torricelli, 2007; Smith et al., 2000).

1. For each experiment, the rat was anesthetized with 10% Chloral hydrate (0.4 ml /100 g).
2. After the animal was immobilized, the hair was cut with a scissor and the skin above the skull (the scalp) was sterilized with iodine and wiped with 75% alcohol. The skin was cut and peeled carefully from the skull to the edge of the eye and neck. The rat was euthanized with excess Chloral hydrate (another injection of 0.4 ml Chloral hydrate).
3. ▲CRITICAL It was essential for the entire brain to be removed within 5 min after death (Brewer and Torricelli, 2007) and placed in DMEM medium with 5% horse serum and 1% glutamine at 24°C. This was performed within 3 min on average. The cerebral pia mater, hippocampus, blood vessels, cerebellum and white matter of the cortex were dissected carefully from the gray matter under a microscope and were discarded.
4. ▲CRITICAL Only the gray layer of the cerebral cortex from the frontal, apical and temporal lobes (bulbus olfactorius were discarded) were harvested.The residual tissue (gray matter) was chopped with scissors into pieces of about 1-2 mm and digested with 0.1% collagenase, and the pieces were pipetted (caliber 0.1–1 mm) up and down repeatedly for 15-20 min at 30°C. The liquid was filtered through a 0.1-μm pore sieving nylon mesh.
5. ▲CRITICAL The suspension was collected, diluted with liquid medium containing 10% bovine serum and 10% horse serum and centrifuged twice at 100 × g for 5 min. The resulting supernatant was centrifuged at 400 × g for 5 min, and the pellet was resuspended with the medium described in Table 1. Cells (2×10^6 per well) were dispensed into 12-well plates.
6. ▲CRITICAL The wells contained inserts or coverslips precoated overnight with poly-D-lysine (120 mg/ml, Sigma). Before culture, these wells or coverslips precoated with poly-D-lysine must be

Table 1. The supplements for basic culture medium (DMEM) of neurons.

Culture medium	Albumin (0.2~0.5%)	NGF (20ng/L)	10% bovine serum and10% horse serum
DMEM			√
NGF+Serum-containing DMEM		√	√
Albumin-containing DMEM	√		
NGF+Albumin-containing DMEM	√	√	
Neurobasal and B27			

√ There are this supplements for basic culture medium.

washed at least twice by PBS.

7. ▲CRITICAL The initial medium was replaced. To reduce neuroglial cell growth, purification of cortical neurons capitalized on the fact that the attachment time is shorter for neurons (12–24 h) than for neuroglial cells (more than 72 h) (Olson and Holtzman, 1980). After cortical neurons were cultured for 24 h, the culture medium was removed.

▲CRITICAL

The initial medium replacement is the key procedure for a successful culture, and the procedure must be performed using the following steps:

a. Gentle horizontal agitation of the flask will result in the suspension of cellular debris and unattached cells.

b. After removing 20-25% of the medium from the container, the same volume should be replaced with medium (Table 1) and cells must be cultured continuously for an additional 2 h.

c. Repeat step b four times.

d. 10 μM cytarabine should be added to inhibit neuroglial cell growth.

f. After 48 h in the presence of cytarabine, the culture medium should be replaced with medium without cytarabine, ▲CRITICAL or with another neuronal culture medium if researchers consider the disadvantage that albumin can absorb drugs. However, the procedure of medium replacement must be performed strictly according to steps a to c.

Fluorescent immunostaining of MAP2

After a 15 min incubation in 0.3% Triton X-100 in phosphate-buffered saline (PBS) to permeabilize the cells , the cells were incubated for 30 min blocking solution containing 5% bovine serum albumin (BSA). The cells were then switched to a solution containing a rabbit anti-MAP2 antibody (1:1000 in antibody diluents, Abcam, Cambridge, UK), incubated overnight at 24°C, washed in PB. Next, the cells were incubated with a FITC fluorescent-conjugated goat-anti-rabbit antibody (1:50 in antibody diluents, Southern Biotechnology) at room temperature for 30 min, washed in PBS and finally incubated with Hoechest 33258 for 10 min at room temperature and washed in PBS.

Cells were examined using an Olympus fluorescence micro-scope, and images were taken using a digital video camera.

Double Fluorescent immunostaining of MAP2 and GFAP

Similar to step 2.5.1., Double Fluorescent immunostaining was performed to test the purity of neurons with GFAP and MAP2 antibodies. Briefly, after cellular permeabilization with 0.3% Triton X-100, the neurons were incubated overnight at 4°C with rabbit-anti MAP2 (1:1000 in antibody diluents, Abcam, Cambridge, UK). The cells were kept at room temperature for 30 min, washed in PBS, incubated with mouse-anti GFAP (1:600 in antibody diluents, Chemicon) at 37°C for 1 h and washed in PBS. The neurons were incubated with the first secondary antibody, FITC fluorescent-conjugated goat anti-rabbit (1:50 in antibody diluents, Southern Biotechnology), at 37°C for 1 h and washed in PBS. The neurons were then incubated with an additional secondary antibody, PE fluorescent-conjugated goat anti-mouse (1:50 in antibody diluents, Southern Biotechnology), at 37°C for 1 h.

Cells were examined under an Olympus fluorescence microscope and images were taken using a digital video camera.

Analysis of thedose response effect of albumin on the number of attached cell and the purity of the cultured neurons

We plotted a dose-response curve for the number of attached cells after 72 h treatments of cortical cells with albumin at dosages of 0-1.0%. We confirmed the number of attached cells according to the methods mentioned above. Based upon these results, we chose an albumin concentration of 0.45% for MTT test and immunohistochemistry (immunofluorescence).

We identified attached neurons by Nissl staining or MAP2 Fluorescent immunostaining. Briefly, after Nissl staining or MAP2 Fluorescent immunostaining was performed, dishes or slides were viewed under an Olympus phase microscope, and random fields were recorded using a digital camera and analyzed using SimplePCI software (Compix). Determinations were made from at least five separate experiments and the number of attached cells was measured from at least five random visual fields. Finally, based upon the numbers of attached cells per visual field, we estimated the number of attached cells per well/slide.

Measurement of the nuclear integrity of neurons (Figures 1 and 4)

After 4 days or 14 days of culturing, we examined the nuclear integrity of neurons with the fluorescent dye Hoechst 33258. The supernatant was removed and the cells were fixed at room temperature for 30 min. Cells were washed twice with 0.01 mol/L PBS. The staining solution was added, and the cells were incubated for 20 min. The fixing solution was removed, and the cells were mounted with an anti-quench mounting solution. Apoptosis was determined under a fluorescent microscope.

Measurement of the viability of neurons

We examined the viability of young or adult neurons with MTT after

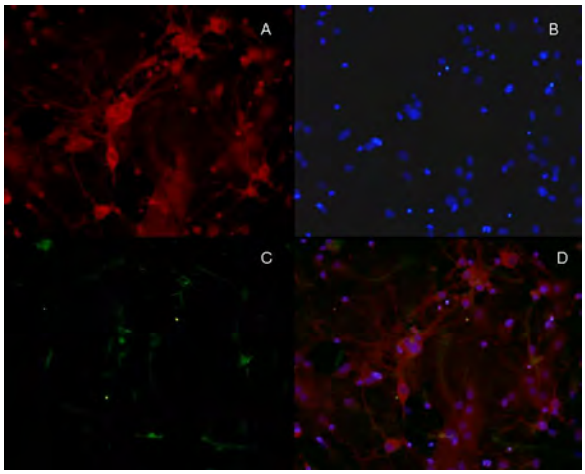


Figure 1. The neurons were cultured for 7 days. The pure rate of harvest of neurons tested by double Fluorescent labeling with GFAP and MAP2 antibodies. Notably, the astrocytes look like polygonal or fusiform cells, not like stellate (or asteroidal) cells; on the contrary, stellate neurons look like astrocytes. Adult neocortical neurons were examined with MAP2-PE fluorescence; the size and morphology of the neurons were altered. Neural networks were beginning to form (**A**). Neuronal nuclear integrity was tested with Hoechst 33258; no apoptotic bodies are observed in this image (**B**). Glias were confirmed with GFAP-FITC. Astrocytes morphology are shuttle, not polygonal cells (**C)**. Overlap image of A, B and C (**D**).

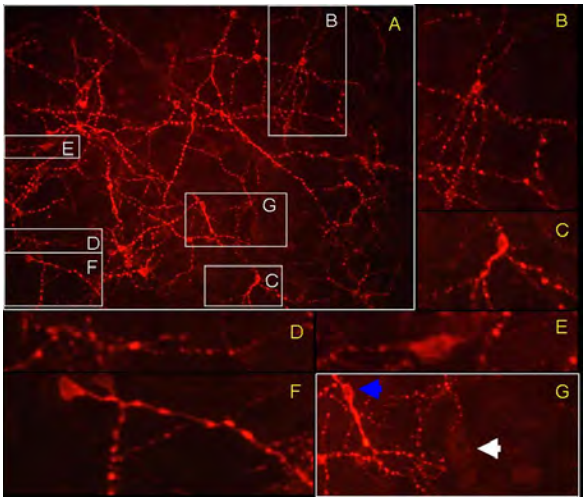


Figure 2. Adult neocortical neurons were cultured for 14 days, and neurons were labeled with a MAP2 antibody (PE red fluorescent immunostaining). All neurons were cultured with NGF+Albumin-containing DMEM, and the albumin concentration was 0.45%. The neural networks are very clear; the neurons look like stellate cells (**B**), pyramidal cells (**C, F**), horizontal cells (**D**) or fusiform cells (**E**); cell size varied significantly. The stellate cell-like neurons had many axons and resembled GFAF-positive astrocytes, but the stellate cells were MAP2-positive (**B**). Notably, astrocytes are usually polygonal or fusiform cells under normal culture conditions. Pyramidal-like neurons had long axons only (**C** and **F**). The blue arrow points to a MAP2-positive cell, which is present in neurons; the white arrow points to a MAP2-negative cell, which indicates non-neuronal cells such as astrocytes. These cells are polygonal cells (**G**).

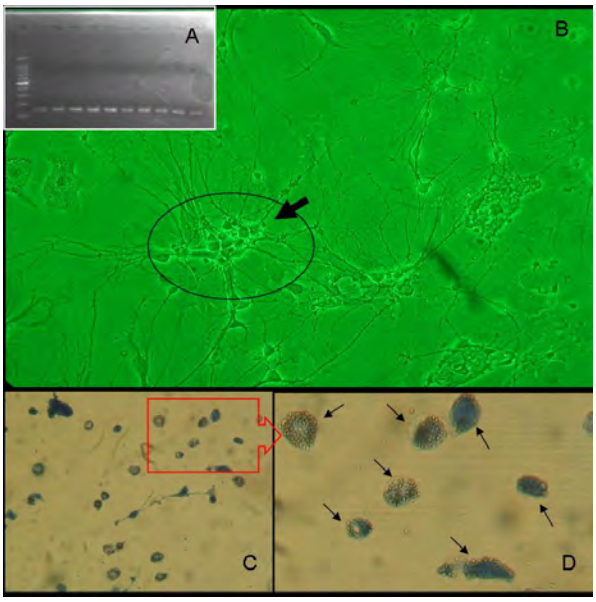


Figure 3. All neurons were cultured with NGF+Albumin-containing DMEM, and the albumin concentration was 0.45%. RT-PCR detection of mRNA for NSE in both young (**lane 2-6**) and adult (lane 6-13) cortical neurons. The young cerebral cortex neurons from postnatal day 6 rats were cultured. Notably, some neurons massed together and resembled neurospheres (**red cycle in B**). A neural network was beginning to form. Adult neocortical neurons were cultured for 2 days (**C** and **D**), and were tested by Nissl staining (**Vogt stain**). We were able to clearly identify the cells outlined with red rectangles (C); all cells were neurons. **D** indicates Nissl bodies.

14 days in culture. Briefly, five holes were taken from every group, and 20 µl MTT (5 g/L) was added. Four hours later, the supernatant was removed and 150 µl DMSO was added into every hole. OD values were determined at a wavelength of 570 nm and at a reference wavelength of 630 nm**.**

Expression of NSE by RT-PCR

Total RNA was isolated from adult cortical neurons. After a reverse transcription reaction, we amplified the NSE gene by PCR. PCR amplification was performed using gene-specific forward and reverse primers (see below for sequences). Primers were all designed according to the Genbank sequence (AU025592) for the product of interest, and the primers were checked in Genbank to exclude the possibility of sequence homology with other genes. The forward primer was CAGGGCTAACCTGTCCCAT and the reverse primer was TGATAGAACAACCTTGTTCCCCT.

The PCR conditions consisted of an initial denaturation step at 94^{o}C for 5 min, then 35 cycles of 94 ^{o}C for 1 min, 64 ^{o}C for 1 min and 72 ^{o}C for 1 min, with a final 6 min extension at 72 ^{o}C.

Statistics

The data are presented as mean values (SD) from three or more experiments (as indicated). The first tests were performed using standard t-tests, and further analysis was conducted using a repeated measure one-way ANOVA, Regression Analysis and Dunnett's post test. P values <0.05 were considered significant, and SPSS version 11.5 was used for statistical analysis.

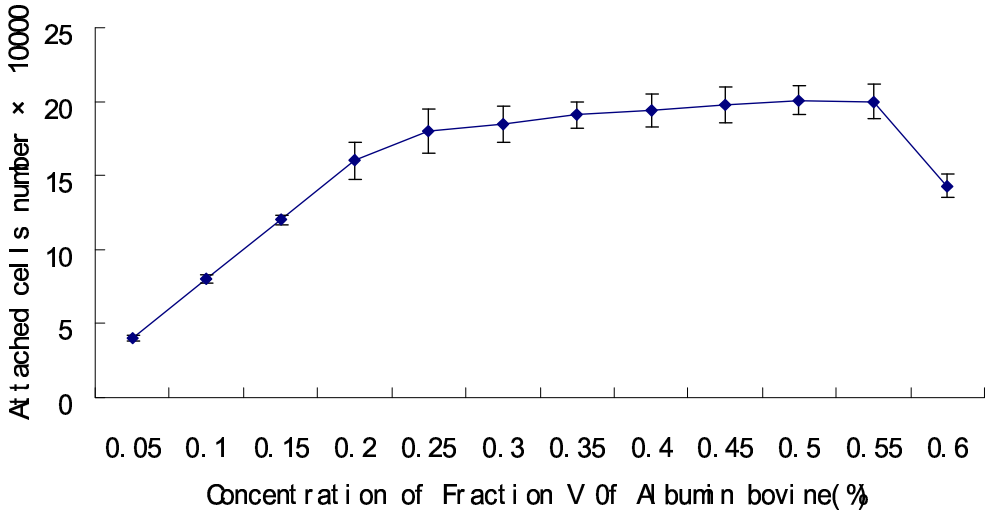


Figure 4. Dose-response curves for the number of attached cells after 72 h of treating cortical cells with albumin at dosages of 0-1.0% were plotted. When the albumin concentration was 0.2%, the attached cells increased greatly, and the attached numbers were enough for biochemical testing; when the albumin concentration was 0.2-0.5%, the attached cell number increased with increasing albumin concentration. However, when the albumin concentration was 0.6%, the attached cells decreased greatly relative to the number seen with 0.5% albumin ($p<0.01$).

RESULTS

We harvested healthy adult cerebral cortical neurons after 7-14 days of culturing (Figures 1 and 2). The number of harvested neurons was sufficient to perform chemical test, for example a PCR test (Figure 3A).

After 7 days of culturing, neural networks began to form (Figure 1). After 14 days of culturing, the neural networks had formed (Figure 2). These neurons appeared similar to granular cells, fusiform cells, pyramidal cells or satellite cells, and the sizes of the neuronal bodies varied. Usually, they had a long axon and short dendrites. Notably, the stellate neurons were easily mistaken for astrocytes. Under normal culture conditions, except in the presence of exogenous stimulation such as LPA (Lysophosphatidic Acid) treatment (Itoh et al., 2006), astrocytes are polygonal or fusiform-like cells, not asteroidal-like (or stellate-like) cells. These morphological characteristics confirm that we harvested neurons from the cerebral cortex (neocortex).

The harvested neurons were very pure

Whether young cerebral cortical neurons or adult cerebral cortical neurons cultured ***in vitro*** were used**,** they survived for over 3 weeks. The purity of cortical neurons harvested on the 3rd day was about 90%, and it was about 95% on the 7th day (Figures 1 and 2).

The nuclear integrity of neurons was examined with the fluorescent dye Hoechst 33258 (Figure 1B)

Irrespective of whether the neurons were cultured for 4 or 14 days, the number and amount of neurons decreased minimally over the course of culture. The nuclear integrity of neurons was examined after 14 days of culturing with the fluorescent dye Hoechst 33258. The results obtained by Hoechst staining were validated in previous studies by the use of TUNEL labeling and produced similar results (Silva et al., 2001). The rate of healthy nuclei was typically over 90% in both young and adult neurons.

The viability of neurons was examined by MTT after 14 days in culture (Table 2)

After 14 days of culturing with DMEM, albumin-containing DMEM, NGF + Serum-containing DMEM, NGF + Albumin-containing DMEM or Neurobasal + B27, we tested neuronal viability with MTT. These results demonstrated that there was no significant difference between NGF + Albumin-containing DMEM and Neurobasal + B27; viability was elevated greatly by NGF in young neurons, but not in adult neurons; it is notable that albumin greatly improved the viability of both young neurons and adult neurons.

The optimal dose of the Albumin for neurons (Figure 4)

Albumin was used at a concentration of 0.05-1%. After the initial medium was replaced, we counted the number of attached cell from five random visual fields. Our results show that the optimal concentration of albumin for neurons is 0.2-0.5%.

Table 2. The OD value of young or adult cortex neurons with different culture medium (n=5).

Culture medium	Young neurons	Adult neurons
Basic culture medium(DMEM)	0.33±0.08	0.29±0.08
NGF+Serum-containing DMEM	0.51±0.07*▲	0.32±0.06
Album-containing DMEM	0.54±0.07*	0.54±0.07*
NGF+Albumin-containing DMEM	0.81±0.08**##	0.79±0.08**##
Neurobasal+ B27	0.84±0.05**##	0.83±0.06**##

*P<0.05, **P<0.01, compare with DMEM in adult neurons or young neurons; ## P<0.01 compare with DMEM, NGF + Serum-containing DMEM, album-containing DMEM, NGF + Albumin-containing DMEM or Neurobasal+B27 in adult neurons or young neurons; ▲P<0.05, the value of NGF + Serum-containing DMEM in young neurons compare with the one in adult neurons.

Comparison of attached cell numbers among cultures grown in DMEM, albumin-containing medium, albumin + NGF-containing DMEM and Neurobasal + B27-containing DMEM (Figure 5A-G).

We also compared the number of attached cells in cultures grown with DMEM, NGF + Serum-containing DMEM, albumin-containing DMEM, albumin + NGF-containing DMEM, or neuronbasal + B27. The results showed that the number of attached cells in the culture grown in DMEM was lowest relative to cultures grown in the other media; NGF + Serum-containing DMEM effectively improved attachment ability, but the effect of NGF + Serum-containing DMEM was much lower than that of albumin-containing DMEM, albumin + NGF-containing DMEM and neuronbasal + B27; however, there was no significant difference among the cultures grown in albumin-containing DMEM, albumin + NGF-containing DMEM or neuronbasal + B27. These results indicate that albumin improves the attachment ability of neocortical neurons.

DISCUSSION

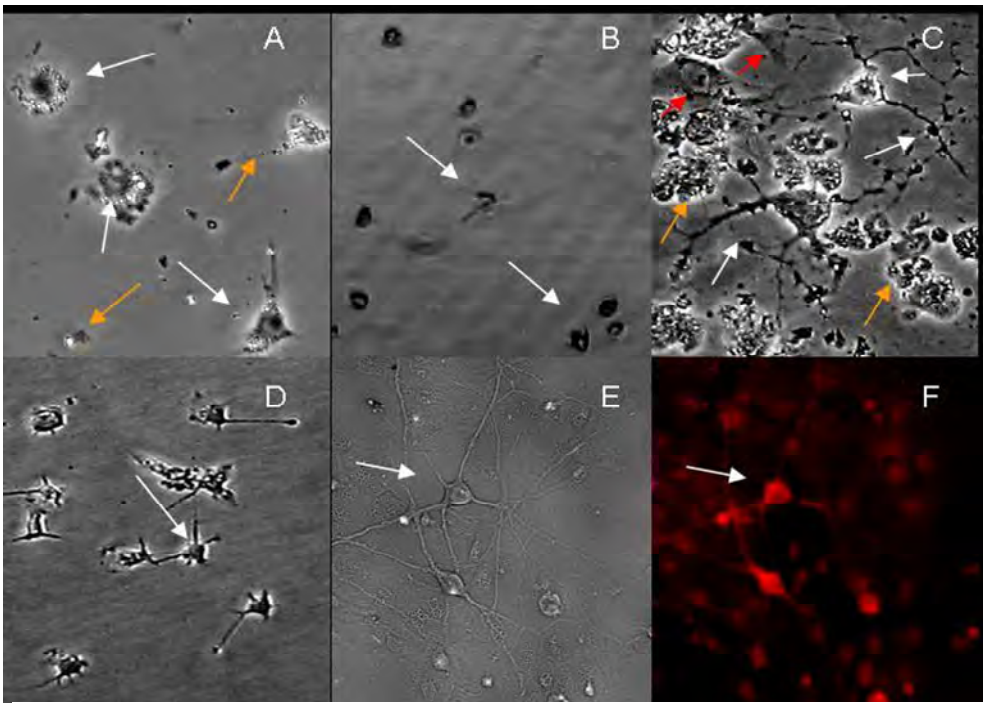
Our results are the first to demonstrate that fraction V of albumin can improve the adherence ability and viability of adult cortical neurons ***in vitro***. In the CNS, cerebral cortex neurons are known to be extremely vulnerable to hypoxia. The exposure of hippocampal CA1 neurons to anoxia for more than 10 min results in cerebral cortical neuron death (Yang and Lin, 2002). In addition, anoxia causes disturbances in glucose, protein and lipid metabolism in neurons (Wen et al., 2003), producing endogenic toxins (Wen et al., 2003; Chou et al., 2003). Therefore, to reduce injury to cerebral cortical neurons by hypoxia or anoxia, several strategies have been employed. First, our surgical procedure removes the brain from the exposed skull within 3 min. Second, our surgery was performed at low temperatures to decrease neuronal metabolism and to increase the possibility of neuronal survival (Sahuquillo and Vilalta, 2007; Dawson et al., 2000; Liu et al., 2007). In particular, our surgery was performed at 24°C (Chou et al., 2003) and not at 4°C (Eide and McMurray, 2005; Nathan et al., 2004), because the optimal temperature for neuronal survival was 24°C (Chou et al., 2003; Sahuquillo and Vilalta, 2007).

Although the measures mentioned above were employed to reduce anoxia, damage from anoxia could not be completely avoided. Anoxia causes disturbances in glucose, protein and lipid metabolism in neurons (Sahuquillo and Vilalta, 2007), resulting in the production of endogenic toxins (Sahuquillo and Vilalta, 2007; Dawson et al., 2000). In adult neocortical neuronal cultures, the number of attached cells was minimal, and most attached cells were swollen. However, this was not observed with young cortical neurons; the viability of attached cells increased greatly. Therefore, it is helpful for neuronal survival to reduce edema ***in vitro***.

Because albumin can reduce edema effectively, it may also improve neuronal viability. Our MTT results demonstrate that serum + NGF-containing DMEM significantly improved young neuronal viability, but did not improve adult neuronal viability. However, albumin-containing DMEM greatly improved neuronal viability in both young and adult neurons. NGF is very important for neuronal survival, axon extension, and especially for neuronal development and differentiation (Yang and Lin, 2002). During development, the axonal regenerative ability and viability of neurons gradually decreases. In adult neurons, albumin can effectively improve viability relative to serum + NGF, but serum + NGF is beneficial for the survival of young neurons.

Our results also demonstrate that fraction V of albumin can enhance the adherence ability of adult cerebral cortex neurons. To improve the adherence ability of neurons, high poly-D-lysine (120 mg/ml) is used for neurons (Nathan et al., 2004). It is known that poly-D-lysine and debris (proteins derived from neurons) are usually positively charged, but cell surfaces are negatively charged. As a result, cellular debris may combine with neurons and interfere with neuronal attachment to poly-D-lysine (Eide and McMurray, 2005). However, albumin is negatively charged and has a small molecular weight; thus, albumin may absorb debris and endogenous toxins, thereby improving attachment ability.

Our results demonstrate that an appropriate concentration of albumin (0.2-0.5%) is helpful for neuronal survival ***in vitro***, and a high concentration of albumin



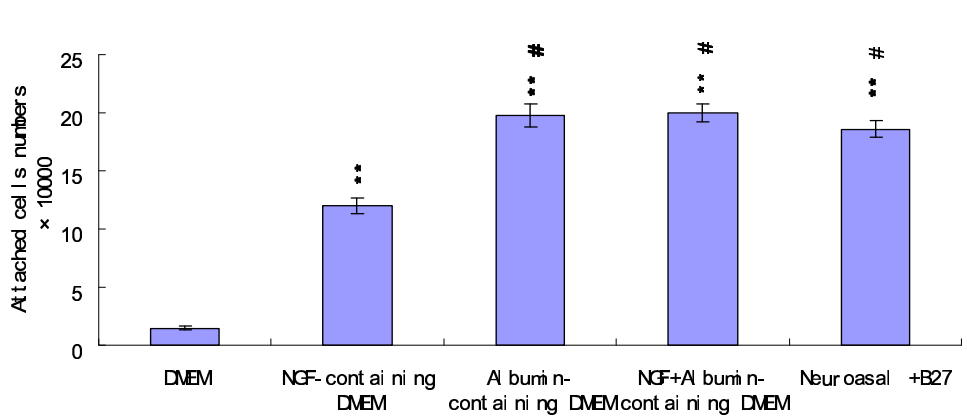


Figure 5. Images of adult cortical neurons after 4 days of culturing. Adult cortical neurons were cultured with DMEM. The neuronal bodies were swollen and a significant amount of cellular debris could be seen (**white arrows**). There is little debris in the image (**yellow arrows**). The axon is short (**A**). Adult cortical neurons cultured with NGF + Serum-containing DMEM. The neuronal body is normal (white arrow) and little debris can be observed in the image. The axon is short (**B**). Adult cortical neurons cultured with Neurobasal + B27. The neurons grow very well, the axon is long (**white arrow**) and a neural network is beginning to form. However, there is a significant amount of debris in the image (**yellow arrows**). Although the purity of the neurons is high, astrocytes can be seen in this image (**red arrows**) (**C**). Adult cortical neurons cultured with Albumin-containing DMEM. The neurons grow well (**white arrows**), and little debris can be seen in the image. Although the axons are long, they are shorter than the ones in C (**D**). Adult cortical neurons cultured with Albumin+NGF-containing DMEM. The neurons grow well (**white arrows**), and the background is very clean (no debris can be seen in the image). The axons are long, and some of the neurons' axons are forming a neural network (**E** and **F**). **E** shows neurons with light microscopy, **F** shows neurons labeled with a MAP2 antibody (PE Fluorescent).The number of attached cells among cultures grown in DMEM, NGF+Serum-containing DMEM, Albumin-containing DMEM, Albumin+NGF-containing DMEM and Neurobasal + B27. ** means for comparison with DMEM, $P<0.01$; # means for comparison with NGF+Serum-containing DMEM, $P<0.05$. Notably, although there was no statistically significant difference between albumin-containing media (Albumin-containing DMEM, Albumin + NGF-containing DMEM) and Neurobasal + B27, the attached cell numbers are more than with Neurobasal+B27 (**G**).

(0.5%) is harmful to neurons. A high concentration albumin may cause serious dehydration, which counters the rapid attachment and growth of neurons.

Notably, medium replacement must be performed strictly according to the description presented in the Experimental Procedures. This technique maintains a relatively stable osmotic pressure, which is very important for cortical neuron survival, especially during the first few days of culturing.

As mentioned above, bovine albumin can improve the ability of neurons to adhere ***in vitro***, and bovine albumin enhances the survival of neurons ***in vitro***. Our adult neocortical neuron culturing method is easy and inexpensive to perform.

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