Medicated rat serum containing Gengnianchun decoction reduces apoptosis of pheochromocytoma cells insulted by amyloid beta protein

Jun Li¹, Bin Li¹, Wen-jiang ZHOU², Fan-gui ZHAO¹, Da-jin LI¹, Wen-jun WANG¹
1. Department of Integrated Traditional Chinese and Western Medicine, Gynecology and Obstetrics Hospital, Fudan University, Shanghai 200011, China
2. Department of Experimental Animal, Fudan University, Shanghai 200023, China

Objective: To investigate the effects of medicated rat serum containing Gengnianchun (GNC) decoction and its protection to pheochromocytoma cells (PC12 cells) from amyloid beta (Aβ[25-35]̄)-insulted apoptosis and to find the possible mechanism.

Methods: Medicated rat serum was prepared by administering ovariectomized Sprague-Dawley (SD) rats with GNC decoction. The effects of medicated rat serum on viability of PC12 cells were evaluated by cell counting kit-8 (CCK-8) assay. The PC12 cells were cultured with different doses of Aβ[25-35] to induce a model of Alzheimer’s disease in vitro. Then, the protective effects of medicated rat serum on Aβ[25-35]-insulted PC12 cells were evaluated by using CCK-8 assay to detect the cell viability, using Annexin V-FITC/propidium iodide (PI) flow cytometry to detect cell apoptosis rate and using Western blotting assay to analyze the expressions of Bcl-2, Bax and active caspase-3 proteins.

Results: PC12 cells cultured with 20% medicated rat serum containing GNC decoction for 24 h or 48 h had higher viability than those cultured with normal culture medium (P<0.05). After 24- or 48-hour treatment of different concentrations of Aβ[25-35], cell viabilities were all decreased as compared with normal medium (P<0.05). Cells underwent apoptosis, which showed the neurotoxicity of Aβ[25-35]. The cell apoptosis induced by Aβ[25-35] was significantly decreased in PC12 cells which were pretreated with 20% medicated rat serum or nerve growth factor (NGF) according to CCK-8 assay and Annexin V-FITC/PI flow cytometry (P<0.05). The ratio of Bax expression to Bcl-2 expression and the expression of active caspase-3 were decreased in the cells treated with medicated serum or NGF as compared with the cells cultured with Aβ[25-35] only.

Conclusion: The GNC-mediated rat serum at concentration of 20% can promote viability of Aβ[25-35]-insulted PC12 cells and decrease the cell apoptosis by regulating the expressions of Bcl-2, Bax and active caspase 3.

Keywords: Alzheimer’s disease; apoptosis; medicated serum; amyloid beta; Bcl-2

中药更年春含药血清对β淀粉样蛋白诱导的PC12细胞凋亡的影响

李君¹, 李斌¹, 周文江², 赵永桂¹, 李大金¹, 王文君¹
1. 复旦大学妇产科医院中西医结合科，上海 200011
2. 复旦大学医学院实验动物科学部，上海 200032


方法: 通过对去势雄性大鼠灌服中药更年春含药血清,细胞计数试剂盒(cell counting kit-8, CCK-8)检
测不同浓度含药血清对PC12细胞存活率的影响，不同浓度Aβ25-35作用于PC12细胞，建立阿尔茨海默病细胞模型，CCK-8法检测含药血清对损伤细胞的保护作用，流式细胞术检测PC12细胞在Aβ25-35和含药血清共同培养下的细胞凋亡率。蛋白印迹法检测各组细胞Bcl-2、Bax和半胱氨酸天冬氨酸特异性蛋白酶3（caspase-3）的蛋白表达情况。

结果：与空白血清相比，20%含药血清培养PC12细胞24 h或48 h后可促进其增殖（P＜0.05）。Aβ25-35对PC12细胞有细胞毒性，并呈剂量依赖性地降低细胞存活率（P＜0.05），导致细胞凋亡。CCK-8法和流式细胞术检测均显示，20%含药血清对Aβ25-35诱导的PC12细胞损伤有保护作用（P＜0.05），其作用效果类似于阳性对照药神经生长因子。蛋白印迹法检测显示，与模型组比较，含药血清组Bax和Bcl-2蛋白表达的比值和caspase-3蛋白表达均降低。

结论：更年期含药血清可抑制Aβ25-35损伤的PC12细胞的存活率，并减少PC12细胞凋亡。其机制可能与调控Bcl-2家族蛋白的表达而实现抗凋亡作用有关。

关键词：阿尔茨海默病；凋亡；含药血清；β淀粉蛋白；Bcl-2

Perimenopausal syndrome (PMS)，a common disease in aged women, is due to the decline of ovary function and decrease of estrogen level. Its typical manifestations are hectic fever, perspiration, insomnia, menstrual disorder, atrophy of genital tract, the change of mental status and even dementia. Alzheimer's disease (AD) is an age-related neurodegenerative disease characterized by the progressive degeneration and loss of neurons in the brain, which has been correlated with the appearance of neurofibrillary tangles and senile plaques\[^{[1]}\]. AD is also one type of dementia. Amyloid beta protein (Aβ) is the major component of senile plaques and considered to have a causal role in the development and progress of AD and this hydrophobic polypeptide is proteolytically produced from amyloid precursor protein\[^{[2]}\]. The sympathetic nerve pheochromocytoma cells (PC12 cells) simulate the behavior of neurons including differentiation, synapse formation and growth cone expansion\[^{[3]}\]. Therefore, this peripheral cell line represents a useful approach for studying the cellular pathophysiology of AD. The former clinical trials had demonstrated that the decoction Gengnianchun (GNC) was effective in treating PMS\[^{[4]}\], especially in improving the learning ability and memory\[^{[5]}\]. The previous study reported that the medicated rat serum containing GNC improved the learning ability and memory of ovariecetomized Sprague-Dawley (SD) rats\[^{[6]}\]. In this study, we observed the protective effects of medicated rat serum on Aβ25-35-induced PC12 cell apoptosis and hoped to supply evidence for its possible mechanism.

1 Material and methods

1.1 Reagents and equipments Horse serum and Ham's F12 culture media were purchased from Hyclone Laboratories (UT, USA). Fetal calf serum (FCS) was purchased from Invitrogen-Gibco (Carlsbad, CA). Aβ25-35 was obtained from Sigma-Aldrich Inc. (St. Louis, MO, USA).

2.5 S nerve growth factor (NGF) was obtained from Promega (Madison, WI, USA). Fluorescein isothiocyanate (FITC)-Annexin V apoptosis detection kit was purchased from BD Biosciences (Germany). Cell counting kit-8 (CCK-8) was obtained from Dojindo Laboratories (Japan). Rabbit anti-Bax monoclonal antibody, rabbit anti-Bcl-2 polyclonal antibody and rabbit anti-active caspase-3 polyclonal antibody were obtained from Abcam (Cambridge, UK). All other chemicals were purchased from common commercial suppliers.

1.2 Preparation of medicated rat serum One unit of decoction Gengnianchun which was 138 g in weight in total, was composed with 12 g of Herba Epimedii Brevicornus (Yinyanghao), 15 g of Radix Rehmanniae (Shendihuang), 12 g of Rasux Paonae Alba (Baishao), 12 g of Semen Cuscutae (Tusizi), 9 g of Poria (Fuling) and Fructus Lycii (Gouqizi), Carapax et Plastrum Testudinis (Gujia), Rhizoma Anemarrhenae (Zhimu), Radix Morindaes Officinalis (Bajitian), Herba Cynomorii Songarici (Roucongrong), Cortex Phellodendri Amurensis (Huangbai), Rhizoma Coptidis (Huanglian). The dry extract of this decoction was prepared by Tianjiang Medicine Company of Shanghai Jahwa United Company. Forty female SD rats [License No. SCXK (Hu) 2005-0001], 3 months old and weighing (250 ± 30) g, were ovariecetomized and had rested for 7 days. These rats were divided into two groups. One group was intragastrically administered with GNC (25 g extract was dissolved in 100 mL normal saline) at a dose of 5 g/kg (according to the weight of dry extract of decoction GNC and rat's surface area), and the
other group was administered with normal saline (NS) with the same volume for five days. One hour after the last administration, the serum was obtained by centrifuging (3000 x g, 10 min). The serum was sterilized by vacuum filtration and stored at -20°C.

1.3 Cell culture PC12 cells originating from rat pheochromocytoma were obtained from the Shanghai Cell Bank of Institute of Cell Biology, Chinese Academy of Sciences. The cells were cultured routinely in Ham’s F12 nutrient medium supplemented with 15% heat-inactivated horse serum and 5% FCS at 37°C in a humidified atmosphere of 5% CO2. The medium was changed every 48 h and cells were plated at an appropriate density (0.5 x 10^4 cells/cm^2). Before treating with the medicated serum, NGF or Aβ25-35, the cells were serum-starved and cultured in culture medium only for 24 h to get the cell cycle synchronization.

1.4 Cell viability CCK-8 assay was used to determine cellular mitochondrial dehydrogenase activity which reflects initial cell death. PC12 cells were plated in 96-well plates at a density of 0.5 x 10^4 cells/cm^2 and 100 μL for every well. Before treated with the medicated serum and NGF, cells were cultured with Aβ25-35 for 2 h which was dissolved in deionized distilled water and incubated at 37°C for 72 h to form aggregated amyloid protein. After the incubation, cells were treated with 10 μL CCK-8 solution every well for 2 h and the absorbance at 450 nm was measured. Results were expressed as optical density (OD) compared to control cells (normal group, cultured with routine culture medium), indicating the loss of cell growth and viability. To assess the effects of rat serum (medicated or not) on PC12 cells, sera at concentrations of 5%, 10%, 20% were used to culture the cells for 24 h or 48 h. For detecting the neurotoxicity of Aβ25-35 to PC12 cells, Aβ25-35 at concentrations of 5, 10, 20 and 50 μM/L were used to culture the cells for 24 h or 48 h. To find protective effects of medicated serum on Aβ25-35-insulted PC12 cells, 20% medicated rat serum containing GNC decoction (MRS group) and 20% non-medicated rat serum (NRS group) were added to Ham’s F-12 medium and 50 ng/mL NGF (NGF group) was added to routine culture medium. The routine culture medium without Aβ25-35 (normal group) and with 20 μM/L Aβ25-35 in routine culture medium (untreated group) were used to culture cells as control. PC12 cells were pretreated with medicated rat serum or NGF for 2 h before 20 μM/L Aβ25-35 exposure.

1.5 Apoptosis rate and flow cytometry analysis

After exposure to medicated rat serum, NGF, or Aβ25-35 for the indicated dosages and time, PC12 cells were harvested by centrifuging (1000 x g, 5 min) and washed with cold PBS twice, and then resuspended in 100 μL binding buffer with about 10^6 cells. Then 5 μL of FITC-Annexin V and 5 μL propidium iodide (PI) was added. Cells were gently oscillated and incubated for 15 min at 25°C in the dark. After adding 400 μL of binding buffer to each tube, cells were analyzed by flow cytometry within 1 h. Cells that stained positive for FITC-Annexin V and negative for PI were considered to be in apoptosis. Cells that stained positive for both FITC-Annexin V and PI were considered either in the end stage of apoptosis, or necrosis or already dead. Cells that stained negative for both FITC-Annexin V and PI were alive and not undergoing measurable apoptosis. The flow cytometry apparatus was FACS Calibur from Becton Dickinson Company and the data were analyzed with software CellQuest.

1.6 Western blotting After exposure to medicated rat serum, NGF or Aβ25-35 for the indicated dosages and time, approximate 5 x 10^6 cells were washed with cold PBS three times, and collected by scraping and centrifugation. Collected cells were lysed with 150 μL ice-cold lysis buffer, and then sonicated. The cell-free supernatants were recovered by centrifugation at 12,000 x g for 30 min at 4°C. Loading buffer was added to each supernatant, which was subsequently boiled for 10 min and then 15 μL lysate was electrophoresed on 12% sodium dodecyl sulfate (SDS) polyacrylamide gel. Proteins were then transferred onto polyvinylidene difluoride (PVDF) membrane. After blocking of the membrane with 5% non-fat dry milk in the Tris-buffered saline containing 0.1% Tween-20 for 1 h at 25°C, the blots were incubated for 12 h at 4°C with the appropriate dilution of the studied antibody (anti-Bcl-2 was used at 1:1000 dilution, anti-Bax at 1:2000, anti-active caspase-3 at 1:1000). After washing, the blots were incubated with peroxidase-conjugated secondary antibody (1:5000) in the second reaction. Binding of antibodies was detected by enhanced chemiluminescence using the enhanced chemiluminescence (ECL) detection system. The relative density of the protein bands was quantified by densitometry using mouse tubulin antibody as compare.

1.7 Statistics Results were presented as X±s. Statistical significance was analyzed by using one-way analysis of variance followed by Fisher’s LSD-t post hoc test. P<0.05 was considered statistical difference.
2 Results

2.1 Proliferation of PC12 cells cultured with medicated serum and non-medicated serum The medicated rat serum containing GNC decoction was effective to proliferation of PC12 cells at an appropriate concentration. Cells were treated with medicated rat serum (MRS group) or non-medicated rat serum (NRS group) at concentrations of 5%, 10%, 20% for 24 h and 48 h. Cell viability was detected by CCK-8 assay. When cultured for 24 h, 20% medicated rat serum could raise the proliferation of PC12 cells as compared with routine culture medium and 20% normal rat serum (P < 0.05). But medicated serum at concentrations of 5% and 10% could not raise the viability as compared with the routine culture medium (Figure 1A). After 48-hour culture, 20% medicated rat serum could raise proliferation of PC12 cells as compared with 20% normal rat serum (P < 0.05) and was nearly the same with routine culture medium. Whereas medicated serum at concentration of 5% and 10% could not raise the viability as compared with routine culture medium (Figure 1B). The normal ovariec-tomized rat serum did not have the same effects as the medicated rat serum.

![Figure 1](image)

**Figure 1** Proliferation rate of PC12 cells cultured with medicated serum containing GNC or non-medicated rat serum Cell viability was determined by CCK-8 assay. PC12 cells were treated for 24 h (A) or 48 h (B). Values were the x ± s of three different experiments performed in triplicate. * P < 0.05, vs normal group.

2.2 Cytotoxicity of Aβ25-35 to PC12 cells Aβ25-35 was cytotoxic to PC12 cells in a dose-dependent manner. Cell viability insulted by Aβ25-35 was assessed by CCK-8 assay. The viability rate of PC12 cells showed that Aβ25-35 has neurotoxicity in a concentration-dependent manner. Both for culturing 24 h or 48 h, the cell viability in Aβ25-35 groups was decreased significantly as compared with the normal group (P < 0.05), decreased by 13.10% for 5 μmol/L group, 22.63% for 10 μmol/L group, 34.13% for 20 μmol/L group and 47.36% for 50 μmol/L group when cultured for 24 h (Figure 2A); decreased by 33.13% for 5 μmol/L group, 43.44% for 10 μmol/L group, 51.33% for 20 μmol/L group and 65.66% for 50 μmol/L group when cultured for 48 h (P < 0.05) (Figure 2B).

2.3 Protective effects of medicated rat serum on Aβ25-35-induced cytotoxicity in PC12 cells The medicated rat serum protected against the Aβ25-35-induced cytotoxicity and apoptotic PC12 cell death. PC12 cells treated with Aβ25-35 at 20 μmol/L were pretreated with 20% medicated rat serum (MRS group), 20% normal ovariec-tomized rat serum (NRS group) or 50 ng/mL NGF (NGF group) for 24 h. Aβ25-35 was added 2 h after the addition of the rat serum and other chemicals (Figure 3). All the cells were treated with Aβ25-35 to induce cell apoptosis except the normal group. The cells were protected against the Aβ25-35-induced apoptosis when pretreated with 20% medicated rat serum and NGF as compared with treated with 20 μmol/L Aβ25-35 only (P < 0.05), however, the 20% normal rat serum did not protect the cells. When cultured for 24 h, MRS group and NGF group had higher cell viability than the untreated group (P < 0.05), and these two groups had nearly the same cell viability, without significant difference.
2.4 Protective effects of medicated rat serum on Aβ35-42-induced apoptosis in PC12 cells
The medicated rat serum was effective to reduce the apoptosis in PC12 cells insulted by Aβ35-42. FITC-
Annexin V is used to quantitatively determine the percentage of cells within a population that are actively undergoing apoptosis. It relies on the property of cells to lose membrane asymmetry in the early phases of apoptosis. PI is a standard flow cytometric viability probe and is used to distinguish viable from nonviable cells. The apoptosis rate was high when treated with Aβ35-42. The use of medicated rat serum and NGF attenuated the apoptosis as compared with Aβ35-42 (P < 0.05) (Figure 4), and these two groups had nearly the same cell viability, without significant difference.

2.5 Preventive effects of medicated serum on Aβ35-42-induced apoptotic signaling
The medicated rat serum inhibited the Aβ35-42-induced apoptosis with down-expression of pro-apoptotic proteins and up-expression of anti-apoptotic proteins. The expressions of Bcl-2, Bax and active caspase-3 proteins were determined by Western blotting. After treatment, we examined the protein expressions of Bcl-2, Bax and the active caspase-3 to determine whether the inhibition of these cell death-associated proteins was responsible for the inhibitory effect of the medicated serum on Aβ35-42-induced PC12 cell death. The medicated serum pretreatment prevented the Aβ35-42-induced up-regulation of Bax and down-regulation of Bcl-2 and thus reduced the Bax/Bcl-2 ratio (Figure 5A). Consistent with the inhibitory effect on Bax expression, the pretreatment of medicated serum also inhibited the expression of the active caspase-3 (Figure 5B). The ratios of Bax expression to Bcl-2 expression in the MRS group and the NGF group were decreased as compared with the untreated group (P < 0.05). The result of the expression of active caspase-3 was consistent with the ratio of Bax/Bcl-2 (Figure 5).
Figure 4  Protective effects of medicated rat serum on Aβ1-42-induced apoptosis in PC12 cells
Cell apoptosis rate was determined by Annexin V-FTTC/PI flow cytometry. In the figure, the transverse axis showed the value of Annexin V and the erect axis showed the value of PI. PC12 cells were treated for 24 h. Values were the \( \pm s \) of three different experiments. \( \Delta P<0.05, \text{vs untreated group.} \)

Figure 5  Preventive effects of medicated serum on Aβ1-42-induced apoptotic signaling
PC12 cells were treated with 20 \( \mu \)mol/L Aβ1-42 as the treatment in the flow cytometry and the expressions of Bcl-2, Bax and active caspase-3 were examined by Western blotting using tubulin as compare. The densitometric values were shown as \( \pm s \) of three separated experiments. \( \Delta P<0.05, \text{vs untreated group.} \)
3 Discussion

According to the data showed in the epidemic investigation, dementia or marked loss of intellectual function is a common and important medical problem. Furthermore, the prevalence of mild cognitive impairment, thought to be a preclinical stage of AD, is estimated to be between 20% and 30% in elderly people. The social and economic implications are the greatest among women because their life expectancy and risk of AD are greater than for men. A key neuropathologic feature of AD is the abnormal deposition in extracellular brain plaques of Aβ protein. Although the mechanism of Aβ-induced neurotoxicity remains obscure, numerous mechanisms for the neuronal cell death in AD have been proposed. One of these is the “Aβ toxicity” hypothesis, which suggests that deposition of Aβ is a primary event in the pathological cascade for AD which may be mediated by several mechanisms. Aβ<sub>25-35</sub> have been shown to induce apoptosis in several different neuronal cells. Thus we used aggregated Aβ<sub>25-35</sub> to induce the apoptosis in the PC12 cells. It showed Aβ<sub>25-35</sub> affected the cells in a dose-dependent manner.

Bcl-2 family members such as Bax and Bcl-2 play a key role in the mitochondrial apoptotic pathway and are believed to be implicated in the progress of apoptotic cell death induced by reactive oxidative species-generating agents such as Aβ<sub>25-35</sub>. Anti-apoptotic Bcl-2 and pro-apoptotic Bax, which resided upstream of mitochondria, focused much of their efforts at the point of mitochondria. Bcl-2 was appeared to inhibit the mitochondria depolarization, while Bax induced mitochondria depolarization. Thus, the down-regulation of Bax expression and up-regulation of Bcl-2 expression by the medicated rat serum containing GNC decoction could be related to its anti-apoptotic effect. With the mitochondrial membrane’s potential depletion by reactive oxidative species, the permeability transition pore (PTP) opened and intermembrane proteins were released out of the mitochondria, which in turn activated a downstream executive caspase-3 and cell death. Caspase-3 activation led to DNA breakage, nuclear chromatin condensation and cell apoptosis. The suppressive effect of medicated serum on the expression of activated caspase-3 and its activity further suggested its anti-apoptosis effect.

It is widely recognized that the main kinds of drugs for treating AD include anti-apoptosis compounds, antioxidant compounds, substances with a mitochondrial impact and anti-amyloid substances. Potentially these strategies will slow down or stop the progressive evolution of the dementia, and could be used as an early pharmacological intervention. However, based on the pathogenesis of AD, improving cell survival has been the primary objective of most therapeutic approaches. Recently, there have been an increasing number of drug discovery efforts on medicinal herbs to find potential drug candidates to treat neurologic dysfunction such as AD like Angelica gigas, Saururus chinensis and Schizandra chinensis mixture, the ethylether fraction of Gastrodia elata, baicalein and baicalin from S. baicalensis. In particular, it was demonstrated that curcumin, a potential drug candidate to treat AD, could inhibit the formation of Aβ oligomers and fibrils, bind plaques, and reduce Aβ in vivo. Thus, it is reasonable to anticipate that rigorous analyses of herbs with neuroprotectivity against Aβ insult may yield potential drug candidates to treat AD.

Our findings are in agreement with previous studies where treatment with GNC was shown to protect neurons in ovariectionized rats. GNC was effective to elevate the degree of Morris water maze test in ovariectionized rats and the ratio of the humid weight to dry weight of brain. GNC could ameliorate the disorder of cells in hippocampus, the cell shrinkage and vacuolization. These findings, taken together, supported that neuroprotection of medicated serum were effective to improve the learning and memory function. The present research demonstrated that medicated rat serum with GNC decoction protected PC12 cells from Aβ-induced apoptosis. The viability rate tested by CCK-8 assay and the apoptosis rate by flow cytometry indicated that the medicated serum with appropriate concentration could reduce the apoptosis insulted by Aβ<sub>25-35</sub>, which is the most toxic part of Aβ. The down-regulation of the Bax/Bcl-2 expression ratio and the expression of active caspase-3 suggested that neuroprotectivity of GNC may be mediated by the regulation of Bcl-2 family members. The effect of GNC could suggest its effective use in treating AD.

Collectively, the treatment for AD is still a hard problem. Our findings provided supportive evidence for the clinical application of the GNC decoction widely, as well as more suggestions in the traditional therapy which may prevent or retard the development of neurologic dysfunction such as AD.
REFERENCES


