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RESEARCH ARTICLE

# Velvet antler polypeptide is able to induce differentiation of neural stem cells towards neurons *in vitro*

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## Abstract

**OBJECTIVE:** To investigate the neural differentiation capacity of water extraction of velvet antler.

**METHODS:** Velvet antler (Cervus Nippon Temminck) polypeptide (VAP) was used to differentiate neural stem cells (NSCs) towards neurons in the study. Firstly, we obtain the polypeptides of VAP by water extraction. Secondly, we observed the morphology, assayed the factors in the media by enzyme-linked immunosorbent assay, and detected the special neural molecules by immuno fl uorescence staining. NSCs were cultured on the cell climbing film. After neuronal differentiation, differentiated NSCs were mounted for immunocytochemistry with immunofluorescence technique.

**RESULTS:** The differentiating cells look like neuron, some special factors, such as Glial cell line-derived neurotrophic factor, nerve growth factor, in the media can be detected while differentiated neuron -like cells can express the special neural molecules.

**CONCLUSION:** Differentiation of NSCs towards neurons can be induced by velvet antler polypeptide.



**Keywords:** Cornu cervi pantotrichum; Glial cell line-derived neurotrophic factors, Nerve growth factor; Neural stem cells

## INTRODUCTION

Human cerebral ischemia and neurodegenrative diseases such as Parkinson's disease (PD) and Alzheimer's disease (AD) are caused by a loss of neurons and glia in the brain or spinal cord.[1,2] Though neurons and glial cells have successfully been generated from several kinds of stem cells such as embryonic stem cells (ESCs),[3] induced pluripotent stem cell,[4] mesenchymal stem cells[5] and neural stem cells (NSCs),[6] it seems that protocols of neural differentiation or co-culture system reported were difficult, complicated and not suitable for routine use in clinical practice. Therefore, stem cells-based cell therapies, transplantation included, should be developed.

NSCs have the special capacities of self-renew and multilineage differentiation, which make them possible to compensate the lost neurocytes and cure neurodegenerative diseases such as Parkinson's disease (PD) and Alzheimer's disease (AD). So it is necessary to develop the convenient protocol which can differentiate NSCs towards neurons.

Deer velvet antler, containing numerous regulatory substance, chemicals and components, such as polysaccharide, phosphatide, hormone, mineral composition, polypeptide and protein, is the young antler which is still not ossified and full of velvet of the male deer Cervus Nippon Temminck.[7]

The water extract of velvet antler has been widely used in the treatment of some diseases because several axon growth promoters have been contained in the antler or its extract, which could promote the organ regeneration. Some papers have reported that the extract of velvet antler could differentiate NSCs, derived from neonatal rats or fetal rats' brain, towards neurons. However, NSCs derived from brain of neonatal rats or fetal rats are different from ones from adult rats. In this study, we aimed to investigate how the polypeptide from velvet antler influences differentiation of NSCs towards neurons from the brain of adult rats.

## METHODS

### *Materials*

Neural stem cells (NSCs, NE4C cell line) were purchased from Cells Resource Center of Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences (Shanghai, China). Dulbecco's modified Eagle medium (DMEM) /F12 were purchased from Thermo Scientific, Waltham, MA, USA. The primary antibody (goat anti neuron-specific enolase, NSE) was purchased from Aves Labs, Inc., China, while the other primary antibody (rabbit anti-proliferating cell nuclear antigen, PCNA) was purchased from Cell Signaling Technology, Inc., China. The secondary antibodies, rat anti-goat IgG conjugated to phycoerythrin (PE), rat anti-rabbit IgG conjugated to fluorescein Isothiocyanate (FITC) were purchased from Beyond, China. Non-essential amino acids (NEAA, 100 ×) and Fetal bovine serum (FBS) were purchased from Life Technologies Corporation, Carlsbad, California, USA. Enzyme linked immunosorbent assay (ELISA) quantitation kit (NGF and GDNF) were purchased from Abnova, Carlsbad, CA, USA. Dishes and 24-well plates were purchased from Corning corporation, NY 14831, USA. Basic fi broblast growth factor (bFGF) and nerve growth factor (NGF) were purchased from PeproTech Inc., Rocky Hill, NJ, USA. Dimethyl Sulphoxide (DMSO) was purchased f from Fisher Chemical. 3-(4,5-dimethylhioazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was purchased from Sigma-Aldrich (St. Louis., MO, USA).

### *Extraction of velvet antler polypeptide*

Extraction of velvet antler polypeptide (VAP) was performed as follows.[8,9] Fresh velvet antler (1 kg), purchased from Jilin Yunlu Group, were chop into pieces, rinsed several times with precooled distilled water until the small pieces are completely bloodless, minced into paste, added 5000 mL pre-cooled homogenate (acetic acid solution, pH 3.5) and repeatedly homogenized with a colloid mill. Homogenate was centrifuged at for 20 min 8500 rpm and 4 ℃, and supernate was obtained, added 95 % ethanol to the supernate (final conc., 60 %), placed at 4 ℃ and continued stirring. 4 h later, supernate was centrifuged at 8500 rpm for 20 min again, and after, and new supernate was obtained. Supernatant was isolated, concentrated by rotary evaporation under vacuum until a volume of 500 mL remained, and then lyophilized the concentrate to obtain crude extract of velvet antler polypeptide, and stored at − 20 ℃.

### *Cells culture*

Neural stem cells were cultured in DMEM) / F12 supplemented with 15% FBS, 2 mM L-Glutamine, 1 mL NEAA (100 ×), 100 units/mL penicillin and 100 mg/mL streptomycin, named normal medium (NM), at 37 ℃, 5% $CO_2$ and 95% humidity. Cell culture media were renewed every three days, up to the confluence of the monolayer. Cells were washed upon formation of confluent cultures, and then detached from the culture flasks or dishes with trypsin-EDTA.

### *Cell viability assay*

NSCs were seeded into 96-well micro-culture plates at a concentration of $1 \times 10^4$ cells per well. After 12 h incubation, cells were exposed to extraction of VAP at concentrations from 100 to 10 μg/mL, positive control treated with 20 ng/mL bFGF & 20 ng/mL NGF and negative control (medium control). After 48 h, 10 mL of MTT solution (5 mg/mL in phosphate buffered solution) was added to the culture medium and incubated at 37 ℃ for a further 4 h. After removing unconverted MTT, 150 mL of DMSO was added to each well and the plates shaken to dissolve the reduced MTT crystals (formazan); the optical density was measured on a microplate reader at a wavelength of 490 nm. The average of cell viability was evaluated by MTT tetrazolium dye assay. Each experiment was performed three times.

### *Neuronal differentiation of NSCs*

NSCs were seeded into polylysine-coated 24-well plates and divided into three groups: VAP group, positive control group and negative control group. The VAP group was treated with 50 μg/mL VAP, the positive control group was treated with 20 ng/mL bFGF and 20 ng mL NGF together, negative control group was treated with normal medium. The protocol of neuronal differentiation of NSCs was as follows (Figure 1).