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Effects of Lycii Fructus on Primary Cultured Chicken Brain Cells

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Abstract □ Effects of Lycii Fructus on primary cultured chicken embryonic brain cells were studied by microscopic observation, determination of the activity of pyruvate dehydrogenase complex (PDHC), and syntheses of protein, RNA and DNA. The brain cells were prepared from the brains of 10-day-old chicken embryos and cultured with a deficient medium. The activity of PDHC in the brain cells cultured with a deficient medium was increased to 1.8 times by the addition of 30 μg/m*l* of the total methanol extract of Lycii Fructus. To seek the active fraction, total methanol extract was further fractionated by the polarity. The survival rate of neuronal cells was significantly increased by the addition of 100 μg/m*l* of the buthanol or aqueous fraction. At this concentration, the significant increase of the syntheses of protein and RNA, but not of DNA, indicates that the fractions may act on the neuronal cells which are known to be non-dividing cells.

Keywords □ Lycii Fructus, chicken brain cells, primary culture, survival rate, pyruvate dehydrogenase complex, syntheses of protein, RNA, DNA.

The Lycii Fructus, fruit of *Lycium chinensis* (Solanaceae) has been widely used for the treatment of fatty liver[1], hypertension, diabetes[2] and as a tonic in Oriental Medicine. Up to date, only several components such as betain[3], zeaxanthin[4] and withanolides[5] were isolated from the Fructus. Also, the lowering effect of the Fructus on serum glucose and cholesterol level was reported[2]. However, the correlation between the biological activities and the isolated components of the Fructus seemed not be thoroughly studied. It might be due to the difficulties for developing the proper bioassay systems. Therefore, the primary cell culture technique providing intact cells[6] which has advantages over the classical bioassay methods has been employed to solve the common problems which could encounter on the isolation of bioactive components from natural products.

The present investigation examines the biological effect and mode of action of the each fraction of Lycii Fructus upon the survival rate of neuronal cells and neurite outgrowth from the neuronal cells in primary cultured chicken embryonic brain cells.

EXPERIMENTAL METHOD

Plants

Fruits of *Lycium chinensis* were purchased from a local store of Agriculture Co-operative Union (Jindo Island, Chunnam, Korea) and they were identified by Dr. Dae S. Han, professor emeritus, College of Pharmacy, Seoul National University.

Animals

Chicken embryos were purchased from Hung-Il Hatchery (Sungnam, Kyunggi-do, Korea).

Reagents

Dulbecco's modified eagle medium (DMEM), Hank's balanced salt solution (HBSS) and trypsin were purchased from Gibco Lab. (Grand Island, NY). Penicillin-streptomycin and amphotericin B were products from Sigma Chemical Co. (St. Louis, MO). Horse serum and collagen were obtained from Hyclone (Logan, UT) and Calbiochem Co. (La Jolla, CA), respectively. [^{3}H]-Leucine, [^{3}H]-uridine, [^{3}H]-thymidine and aquasol were purchased

from NEN (Wilmington, DE). All other chemicals and organic solvents were obtained from Sigma Chemical Co. and Duksan Company (Seoul, Korea), respectively.

Extraction and fractionation

Fruits of *Lycium chinensis* were extracted three times with methanol at 80℃ under reflux. The extracts were combined and concentrated *in vacuo* at 40℃ to give brown residue. The residue was suspended in distilled water and subsequently extracted in the order of chloroform, ether, and buthanol to examine bioactivities of each fraction and further isolate the bioactive components.

Cell culture and treatment

Primary cultures of chicken brain cells were prepared from cerebral hemesphere of 10-day-old chicken embryos[7]. Dissociated cells were plated at a density of 1×10^5 cells/ml/dish (35×10 mm). Cultures were grown to confluency in a medium containing 87.5% of DMEM, 2.5% of chicken embryo extract, 10% of horse serum, penicillin 10,000 IU/100 m*l* and streptomycin 10,000 μg/100 m*l* at 37℃ in a humidified atmosphere of 95% air/5% CO_2. The medium was changed every third day. Chicken embryo extract was prepared from 10-day-old chicken embryos[8], except that the brain and spinal cord were excluded. Also, in order to obtain vivid effects of the test samples on brain cells, brain cells were cultured with a deficient medium excluded of chicken embryo extract from the standard medium to induce the abnormal condition. For the treatment of test samples, fresh standard medium or fresh deficient medium containing various concentrations of test samples were changed every third day.

Estimation of survival rate of neuronal cells

The number of living neuronal cells bearing neurites was determined using a phase contrasted invertoscope at a magnification of 200 x. A count was performed on five microscopic fields per dish. The number of neuronal cells bearing neurite of at least two cell diameters in length was counted. The initial number of phase bright neuronal cells was counted after two hours in culture and the number of surviving neuronal cells was counted after 24 hours in culture.

Assays for the PDHC

The activity of PDHC in cultured chicken embryonic brain cells was measured by the spectrophotometric method[9]. The formation of NADH at 25℃ in the presence of 0.6 mM *p*-iodonitrotetrazolium violet, 0.1 mg/m*l* lipoamide dehydrogenase, 2.5 mM NAD, 0.2 mM thiamine pyrophosphate, 0.1 mM coenzyme A, 0.3 mM dithiothreitol, 5 mM pyruvate, 1 mM $MgCl_2$, the cultured cell homogenate and 1 mg/m*l* of BSA in 0.05 M potassium phosphate buffer (pH 7.8) was measured at the wavelength of 500 nm.

Incorporation of [^3H]-leucine, [^3H]-uridine and [^3H]-thymidine into protein, RNA and DNA

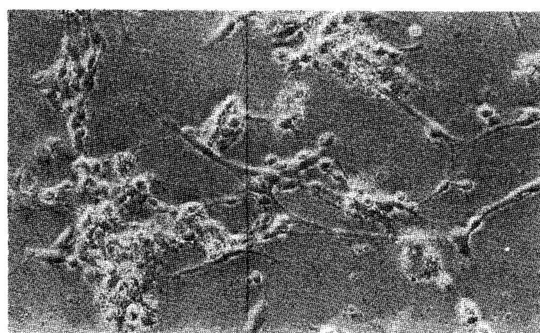
Cultures of brain cells were plated at a density of 1.0×10^6 cells/m*l* in 35 mm culture dish and incubated for 48 hours. For the final two hour of incubation, the cells were pulsed with 1 μCi of [^{3}H]-leucine (140 Ci/mmol), [^{3}H]-uridine (50 Ci/mmol) and [^{3}H]-thymidine (6.7 Ci/mmol), respectively. After washing three times with 1 m*l* of HBSS, cells were fixed with 1 m*l* of ice-cold, 10% trichloroacetic acid (TCA) for 10 min and washed 3 times with 1 m*l* of HBSS and then treated with a mixture of ethanol : ether (3 : 1, v/v) to remove residual TCA. Then, the cells were solubilized in 0.5 m*l* of 1N NaOH for 30 min at room temperature and neutralized with equal volume of 1N HCl. The radioactivity of an aliquot of solubilized cell solution was measured using a liquid scintillation counter to evaluate the incorporation of [^{3}H]-leucine to protein, [^{3}H]-uridine to RNA and [^{3}H]-thymidine to DNA[10].

Statistical analysis

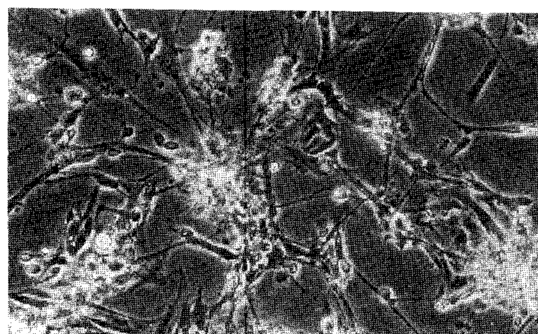
The statistical significance of the data was evaluated by "ANOVA" test.

RESULTS AND DISCUSSION

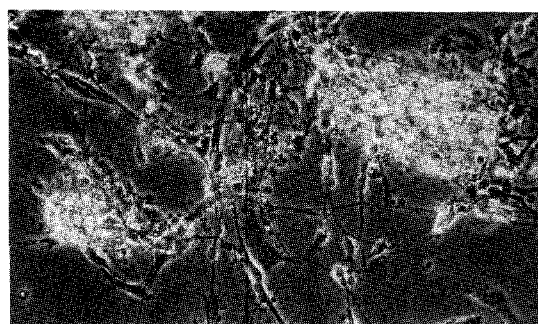
In the course of our study for the screening of bioactive natural products which act on the central nervous system, it was observed under the microscope that the total methanol extract of Lycii Fructus had a marked effect on the survival and outgrowth of neuronal cells which were purposely induced to abnormal state by culturing in the deficient me-



(A)



(B)



(C)

Fig. 1. Primary cultured chicken embryonic brain cells. (×200).
A. Chicken embryonic brain cells were cultured for 48 hours with a deficient medium; B. Chicken embryonic brain cells were cultured for 48 hours with a standard medium; C. After 2 hour culture of chicken embryonic brain cells with a deficient medium, 100 μg/m*l* of the total methanol extract of Lycii Fructus was added to the cells and incubated for 46 hours further.

dium. This circumstantial evidence provoked us to further study on Lycii Fructus.

Table I. Effect of the total methanol extract of Lycii Fructus on the activity of PDHC in chicken embryonic brain cells cultured with a deficient medium

Concentration (μg/m*l*)	Activity of PDHC	
	Activity (nmol/min/mg protein)	Relative activity (%)
0	40.53± 2.83	100
10	48.86± 2.77	121
20	61.59± 4.23*	152
30	71.72± 5.42**	177
50	52.42± 3.09	129
70	51.45± 1.34	127
100	53.20± 6.30	132

The values are means± SE.
Significantly different with respect to control: $p<0.05$*; $p<0.01$**

Table II. Effects of Lycii Fructus on the survival rate of chicken embryonic brain cells cultured with a deficient medium

Fraction	Concentration (μg/m*l*)	Survival rate
Control[a]		18.7± 0.7
Control[b]		38.3± 1.8
Total methanol extract	10	17.1± 0.5
	50	26.1± 2.5*
	100	26.4± 3.5*
Chloroform fraction	10	18.1± 2.8
	50	24.9± 2.5
	100	25.9± 2.9*
Ether fraction	10	21.5± 0.6
	50	17.0± 0.3
	100	18.8± 4.1
Buthanol fraction	10	24.9± 2.9
	50	30.1± 2.7**
	100	27.2± 0.6**
Aqueous fraction	10	23.8± 1.3
	50	29.1± 4.5**
	100	35.4± 0.7***

Control[a]: in the absence of the chicken embryo extract
Control[b]: in the presence of the chicken embryo extract
Survival rate are means± SE.
Significantly different with respect to control: $p<0.05$*; $p<0.01$**, $p<0.001$***

Table III. Effects of Lycii Fructus on the syntheses of protein, RNA and DNA in chicken embryonic brain cells cultured with a deficient medium

Fraction	Concentration (μg/ml)	Protein [^{3}H]-Leucine incorporation (dpm/1×10^6 cells)	RNA [^{3}H]-Uridine incorporation (dpm/1×10^6 cells)	DNA [^{3}H]-Thymidine incorporation (dpm/1×10^6 cells)
Control		2,187± 121	12,868± 302	18,409± 2,701
Total methanol extract	100	2,829± 170*	15,902± 1,054	–
Chloroform fraction	100	2,354± 38	16,885± 2,776	20,004± 797
Ether fraction	100	2,164± 20	–	–
Buthanol fraction	100	2,823± 130*	21,886± 1,040**	17,965± 591
Aqueous fraction	100	2,926± 145*	18,294± 187*	18,497± 1,122

The values are means± SE.
Significantly different with respect to control: $p<0.05$*; $p<0.01$**

It was noticed that the intact brain cells which were cultured with a standard medium seemed inadequate for the investigation of effects of natural products on brain cells from our previous study[11,12]. Therefore, the malfunction and restriction on the growth of the brain cells were purposely induced by culturing with a deficient medium which did not contain chicken embryo extract, an essential component for its growth and differentiation. In the absence of the chicken embryo extract, the number of living neuronal cells was markedly decreased and the development of neurite outgrowth from the neuronal cells appear to be noticeably inhibited. However, the inhibition was greatly recovered by the addition of the total methanol extract of Lycii Fructus. At the concentration of 100 μg/ml of the total methanol extract, the development of neurite outgrowth was markedly stimulated and the number of survived neuronal cells were significantly increased in the deficient medium for the culture period of 48 hours (Fig. 1).

To support the above microscopic observation, the activities of PDHC which is known to involve the biosynthesis of acetyl Co A, the precursor of acetylcholin, one of the important neurotransmitter[13] and the level of this enzyme was significantly lowered in Alzheimer's disease[14,15] in brain cells were measured by the addition of the total methanol extract of Lycii Fructus at the concentration range from 10 μg/ml to 100 μg/ml (Table I). The activity of PDHC increased to 1.8 times by the addition of 30 μg/ml of the total methanol extract. Based on these promising results, the methanol extract was subjected to further solvent fractionation by the polarity to isolate components having activities.

To seek the active fraction, the effect of each fraction on the survival rate of the neuronal cells was determined (Table II). The number of the neuronal cells was counted 2 hours after plating, and then 10, 50 and 100 μg/ml of each fraction of Lycii Fructus was added to the deficient culture medium, respectively. After 24 hours of incubation, the number of the neuronal cells bearing neurites was counted again. The survival rate of the neuronal cells was significantly increased by the addition of 100 μg/ml concentration of the buthanol or aqueous fraction. Especially, it is interesting to note that at the concentration of 100 μg/ml of the aqueous fraction, the survival rate of the neuronal cells in the deficient medium reached 92% of the cells in the standard medium.

In order to elucidate the mode of action of the buthanol and aqueous fractions of Lycii Fructus on the brain cells, the incorporation of [^{3}H]-leucine, [^{3}H]-uridine and [^{3}H]-thymidine into protein, RNA and DNA, respectively, was determined at the concentration of 100 μg/ml of the fractions (Table III). The addition of the buthanol or aqueous fraction to the brain cells in the deficient medium significantly increased the syntheses of protein and RNA, but not synthesis of DNA. Upon these results, the buthanol and aqueous fractions of Lycii Fructus may act on neurons which are known to non-dividing cells.

During the isolation of bioactive components from Lycii fructus by activity-guided fractionation,

the interesting results were obtained; the aqueous and buthanol fractions of Lycii Fructus are bioactive fractions and they may act on neurons in brain cells. The isolation and characterization of the bioactive components from the buthanol and aqueous fractions of Lycii Fructus are being performed.

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