Journal of Ethnopharmacology 193 (2016) 272–282

Contents lists available at ScienceDirect

Journal of Ethnopharmacology

journal homepage: www.elsevier.com/locate/jep

Kai-Xin-San, a traditional Chinese medicine formula, induces neuronal differentiation of cultured PC12 cells: Modulating neurotransmitter regulation enzymes and potentiating NGF inducing neurite outgrowth

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ARTICLE INFO

Article history:
Received 28 December 2015
Received in revised form
17 April 2016
Accepted 4 August 2016
Available online 12 August 2016

Keywords:
Kai-Xin-San
Neurotransmitter
Differentiation
TCM formulae
Depression
Chinese medicine

ABSTRACT

Ethnopharmacological relevance: Kai-Xin-San, an ancient formula composed of Ginseng Radix et Rhizoma, Polygalae Radix, Acori Tatarinowii Rhizoma and Poria, was frequently applied for major depression disorders for thousands of years. However, its molecular mechanism has not clearly been investigated.
Aim of the study: We aimed to reveal the action mechanism of KXS on anti-depression on inducing neuronal differentiation on PC12 cells.
Materials and methods: A chemically standardized water extract of KXS was applied onto cultured PC12 cells in determining its effect on neurotransmitter regulation enzymes and neurite outgrowth.
Results: Single KXS treatment showed obvious changes in the expression of neurofilament and neurotransmitter regulation enzymes, which in parallel to treatment of nerve growth factor (NGF). Although KXS by itself did not show significant inductive effect on neurite outgrowth of PC12 cells, KXS could potentiate the NGF induced neurite outgrowth. Among the three ratios, K-652 showed the most powerful effect and cAMP-dependent pathway might play the major role.
Conclusions: KXS might exert the anti-depressant-like action of be inducing neuronal differentiation, which supported the clinically usage of this decoction.

1. Introduction

The major depression disorder has attracted more and more attention in the recent years. With the fast working and living rhythm, people incline to experience over stress and suffered from this mental disorder. The core symptoms of major depression disorder includes: duration of depressed mood over two weeks, anhedonia, irritability, difficulties in concentrating and abnormalities in appetite and sleep. The most serious outcome of this disorder is inclination to commit suicide. It was reported that the incidence rate of major depression disorder was up to 17% and amazingly, nearly 1 in 6 people might experience at least a depressive episode in their lives, which brought great discomfort not only to the patients themselves but also to their families, friends and the whole society. Unfortunately, the actual etiology of the major depression disorder is still unclarified. Up to now, unbalance of neurotransmitter regulation was still regarded as the major pathological hypothesis, which held that shortage of dopamine, norepinepherine and serotonin in synapse led to anhedonia. Indeed, current anti-depression drugs, such as imipramine and fluoxetine, both targeted on inhibiting reuptake and degradation of neurotransmitters, However, nearly 50% patients showed no response to these drugs. Furthermore, it was reported that long term use of fluoxetine might enhance the possibility of committing suicide (Cheung et al., 2015).

In addition to the monoamine hypothesis, retarded neurogenesis had been proposed as one of the etiological mechanism of major depression disorder (Sahay and Hen, 2007). It was widely accepted that normal nervous system development evolved from a series of well-orchestrated processes of neural induction, cell proliferation, differentiation, cell migration, survival, and synapse formation. In progress of depression, neurodegeneration and impairments of neural webs and connectivity in limbic brain had been discovered and supported by observations of decreased gray matter volume of hippocampus, frontal and temporal cortices in depressed patients (postmortem) (Krishnan and Nestler, 2008).

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http://dx.doi.org/10.1016/j.jep.2016.08.013
0378-8741/

Among this complex course, neuronal differentiation, in presence of neurite outgrowth, was one of the most important stages because the directed growth of axons was indispensable to synapse formation. Therefore, neuronal differentiation induction had been also employed as a target for development of anti-depressants. Anyway, the new generation of antidepressants should possess more action targets except only regulation of monoamine neurotransmitters. In this situation, tradition Chinese medicine formulae, a complex system with multiple compounds and action targets, had been paid more attention in development of antidepressants.

Traditional Chinese Medicine (TCM) had been used as medicines and daily dietary supplements for thousands of years in China. Usually, TCM was prepared as a formula by a unique method with a specific combination of herbs. According to the needs of patients, the formulae varied in combinations, or in dosages, to acquire the best therapeutic effects while minimized any side effects there might be (Fan and Zhu, 2002). Kai-Xin-San (KXS) was a famous TCM formula for the treatment of mental disorders, which was first described in *Beiji Qianjin Yaofang < Thousand Formulae for Emergency >* by Sun Si-miao of Tang Dynasty in 652 A.D (Wang, 1997). KXS was comprised of four herbs: Ginseng Radix et Rhizome (root and rhizome of *Panax ginseng* C.A. Mey., Renshen, GR), Polygalae Radix (root of *Polygala tenuifolia* Wild., Yuanzhi, PR), Acori Tatarinowii Rhizoma (rhizome of *Acorus tatarinowii* Schott, Shichangpu, ATR), and Poria (sclerotium of *Poria cocos* (Schw.) Wolf, Fuling, PO) and the ratio was 1:1:25:50 (RG: RP: RAT: PO, K-652). Meanwhile, a herbal formula named Ding-Zhi-Wan (DZW-652, D-652) was also described in this book with the ratio of 3:2:2:3. In addition, another herbal formula named KXS-984 (K-984), was recorded in *Yi Xin Fang* written by Nima Yasunori from Japan in 984 A.D. (Song Dynasty). The author recorded an herbal ratio of 1:1:1:2 for K-984 and cited *Beiji Qianjin Yaofang*. No matter the ratio varied, KXS was prescribed to treat a mental disorder with these symptoms: unhappiness, morbid forgetfulness and dizziness, which was very similar to the major depression disorder nowadays. Until now, KXS was still applied for the treatment of depression in clinic and had been reported to relieve the symptoms of depressive disorder, as revealed in animal studies (Dang et al., 2009; Zhu et al., 2012; Cao et al., 2012; Zhou et al., 2012). However, the molecular mechanism of KXS on anti-depression was seldom explored.

Here, we hoped to evaluate the effect of KXS on neuronal differentiation, an important stage of neurogenesis, and PC12 cells were selected as the cell model. Due to the obvious neurite outgrowth induced by nerve growth factor (NGF), PC12 cells were widely applied for evaluation of substance on neuronal differentiation by determining neurite length, which was regarded as the morphological differentiation marker of PC12 cells. Apart from this morphological change, expressions of neurofilaments and neurotransmitter regulation enzymes were also found to change in differentiated PC12 cells, which could be termed as biological markers. Therefore, the effect of KXS on neuronal differentiation would be evaluated by determination of neurite length and expressions of neurofilaments and neurotransmitter regulation enzymes.

2. Methods and materials

2.1. Preparation of extract of KXS decoction

The preparation of three different ratios of KXS was carried out according to the historical record. The details were listed in Table 1. The following dried raw materials: Ginseng Radix et Rhizome (root and rhizome of *P. ginseng*), Polygalae Radix (root of *P. tenuifolia*), Acori Tatarinowii Rhizoma (rhizome of *A. tatarinowii*), and Poria (sclerotium of *P. cocos*)were all purchased from Tianling Company of Chinese herbs in Suzhou China, which were authenticated by Prof. Hui Yan of Nanjing University of Chinese Medicine, according to their morphological characteristics. The methods ofchemical standardization could be referred to the previous published work (Zhu et al., 2010). The representative fingerprint chromatograms and the quantification results of chemical markers from each herb had been included in Supplementary information. The voucher specimens were deposited in the Jiangsu Key Laboratory for High Technology Research of TCM Formula.

Table 1
Historical record of different KXS formulae.

Notation[a]	Record	Ratio			
		GR	PR	ATR	PO
KXS-652	*Beiji Qianjin Yaofang*[b]	1	1	25	50
KXS-984	*Yixin Fang*[c]	1	1	1	2
DZW-652	*Beiji Qianjin Yaofang*[b]	3	2	2	3

GR=Ginseng Radix et Rhizoma; PR=Polygalae Radix; ATR=Acori Tatarinowii Rhizoma; PO=Poria.

[a] The notation of KXS was corresponding to the years of recording.

[b] *Beiji Qianjin Yaofang* was written by Sun Si-miao in 652 A.D, which was re-edited in 1066 A.D (Song Dynasty).

[c] *Yixin Fang* was written by Nima Yasunori in 984 A.D.

2.2. Cell culture

The rat pheochromatocytoma PC12 cell was obtained from ATCC and maintained in culturing medium: DMEM with 4.5 g/L glucose supplemented with 6% fetal bovine serum (FBS), 6% horse serum (HS) and 1% 100 U/mL penicillin and 100 μg/mL streptomycin (P/S) at 37 °C in a water-saturated 7.5% CO_2 incubator. All reagents for cell cultures were purchased from Invitrogen.

2.3. Drug treatment

During the treatment, cultured PC12 cells were changed with medium for 3 h in DMEM supplemented with 1% FBS and 1% HS, and 1% P/S before drug treatment, and then the cultures were treated with KXS extracts and/or other reagents for 48 h. In analyzing the possible signaling pathway, the cells were pre-treated with the protein kinase A inhibitor H89 (2 μM) for 3 h before the exposure to KXS extract and Bt_2-cAMP (1 mM).

2.4. Determination of neurite outgrowth

The effect of KXS on neurite outgrowth was investigated in cultured PC12 cells, which responded to NGF by extending neurite-like processes. PC12 cells (5×10^4 cells/well) were seeded into 6-well plates. After 24 h, the cells were changed with DMEM medium containing 1% FBS, 1% HS and 1% P/S. Series concentrations of KXS extracts (0.1, 0.3, 1, 3, and 10 μg/mL) were treated every 24 h twice. A light microscope equipped with a phase-contrast condenser, 10× objective lens and a digital camera were used to capture the images with the manual setting. For the analyses of neurite presence and neurite length, approximately 100 cells were counted from at least 10 randomly chosen visual fields from each culture. Firstly, the cells were scored as differentiated if one or more neurites longer than cell body diameter were observed. Secondly, the number of differentiated cells was calculated. In this study, NGF (50 ng/mL) was used as a positive control.