The Establishment of the Method of Cell Biochromatography and Analysis of the Active Ingredients from TongQiaoHuoXue Decoction Acting on the Neurocytes

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Abstract

A novel strategy for screening active components in traditional Chinese medicines (TCM) using living cells and HPLC and GC analysis is proposed. The hypothesis is that when cells are incubated with the extract of Tongqiao Huoxue Decoction (TQHXD), a famous ancient prescription in TCM, the potential active components in the TQHXD should selectively combine with the cells, and the cell-combining components would be detectable in the extract of denatured cells. The identities of the cell-combining components could be determined by HPLC and GC analysis. Using the proposed approach, two characteristic active ingredients binding to the membrane of the PC12 cells were indicated. In the fingerprint of HPLC, there are two characteristic peaks. One active ingredient with its retention time was at around 70 min had been identified as muscone by HPLC, GC, which came from Moschus herb, the other active ingredient may come from the Allium fistulosum, its structure needs further research. Also, the protective effect of muscone on PC12 cells induced by Oxygen and glucose deprivation (OGD) had been explored. These results show that the pretreatment with muscone on PC12 cells observably increased cell viability, reduced the release of lactate dehydrogenase (LDH) and cell apoptosis. Combined with the pharmacodynamic study of muscone on neuroprotective effect, it could be identified as one of the effective components in TQHXD.

Key words cell biochromatography; Tong-Qiao-Huo-Xue Decoction; active component; PC12 cell; muscone

Tongqiao Huoxue Decoction (TQHXD) is a classical herbal formula of traditional Chinese medicines (TCM), established by Qingren Wang, the Qing Dynasty of China and comprising seven crude herbs: Moschus, Carthamus tinctorius, Rhizoma chuanxiong, Semen pruni persicae, Ziziphus jujuba, Radix Paeoniae Rubra and Allium fistulosum. It can promote blood circulation by removing blood stasis, induce resuscitation by dredging the channel which plays an important role in blood stasis in systemic blood vessels, especially in the cephalic and facial blood vessels. It has both preventative and therapeutic effects on diseases such as stroke (cerebral circulation insufficiency, cerebral infarction). Our previous studies showed that TQHXD had an evident neuro protective effect.

In our previous experiment, fingerprints of Tongqiao Huoxue Decoction by HPLC had been done. But the effective substance of TQHXD on neuroprotective effect still needs to be investigated. When using traditional methods to screen active ingredients of TCM, there are many limitations due to the complexity and diversity of ingredients of TCM. This is why the study of bioactive components has been the aporia and focus of the TCM research. The emergence of biochromatography provides new idea and method for finding the active ingredients of TCM.

Biochromatography, born in the 1980s, is the combination of chromatographic separation and life science technology. The biologically active macromolecules such as enzymes, receptors, carrier proteins and even the active cell membrane can be fixed in the carriers of chromatography. The stationary phase containing living cells can distinctly and selectively combine with the active ingredients of TCM, rule out the interference of non-active ingredients. Thus can we separate and retain the active ingredients of TCM by using this stationary phase.

In the method of cell biochromatography, derived from the biochromatography, living cells from the humans or animals replaced detached receptors from the cells as the stationary phase. Therefore, the selective combining process between the drugs and targets (such as receptors, ion channel, enzymes, etc.) can be simulated in vitro, and the retention behavior of drugs could also be investigated to determine pharmacological or physiological significance. Cell membrane has many target sites, which are defined as biological macromolecules. Drug molecules can combine with these target sites and activate biological information system, then produce biological effects. Using living cells as the stationary phase to fix the components of TCM can maintain the integrity of the structure and function of the cell membrane. In this study, the appropriate concentration of the water extract of TQHXD which was precipitated with alcohol acting on the PC12 cells has been confirmed by 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-2-H-tetrazolium bromide (MTT) assay. Then PC12 cells have been cultivated with the extract of TQHXD for one hour, the active chemical composition from the extracts of TQHXD are fully bound to the receptor molecules on the surface of cells under imitated physical environment. The unconjugated substances are washed down by the phosphate buffer solution (PBS) of pH 7.4 several times, PBS of pH 7.4 is then collected respectively and called eluent sample which

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contained ingredients that do not combine with the targets on membrane of PC12 cells. PC12 cells are then treated with PBS (pH 4), which causes inactivation of targets on membrane and the active components are released from membrane into PBS (pH 4), which is named as dissociation solution.

To further determine herbal origin of the active ingredients, fingerprints of dissociation solution, the last eluent sample, the blank control sample, reference substance, TQHXD and respectively single materia medica in TQHXD are compared with each other by chromatographic analysis such as HPLC, GC, etc., then the effective ingredients of TQHXD have been analyzed, thus we define the origin of effective compounds from which kinds of materia medica of formula, then the protective effect of the active ingredient which binding with cell had been explored. Combining with pharmacodynamics study on neuroprotective effects of these ingredients, we can confirm the active compounds of TQHXD and provide the basis for the quality standards control of TQHXD.

Experimental

Plant Materials and Reagents TQHXD was composed of Moschus (0.15 g), Semen pruni persicae (9 g), Carthamus tinctorius (9 g), Ziziphus jujube (5 g), Radix Paeoniae Rubra (9 g), Rhizoma chuanxiong (3 g), Allium fistulosum (3 g). These herbs were provided by He Yi Tang Plants Co., Ltd., (Hefei, China). Their species were kindly authenticated by Prof. Shoujin Liu (Professor of Pharmacognosy, Anhui University of Chinese Medicine). Muscone (purity ≥ 99%) was purchased from Macklin Bio-Tech Co., Ltd. (Shanghai, China). Nimodipine was purchased from Bayer healthcare Co., Ltd. (Leverkusen, Germany). DMSO, acridine orange/ethidium bromide (AO/EB) kit, fluorescein isothiocyanate Annexin V (FITC)/propidium iodide (PI) apoptosis detection kit were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). RPMI-1640 and fetal bovine serum were obtained from Hyclone (Hyclone, Logan, UT, U.S.A.).

Cells PC12 cells line was purchased from the Shanghai Institute of Cell Biology, Chinese Academy of Sciences (Shanghai, China).

Apparatus and Chromatographic Conditions Agilent Series 1100 liquid chromatograph (Agilent Technologies, U.S.A.), equipped with a dual pump, a Rhodyne 7125i injection valve with a 20 µL sample loop and a diode array detector (DAD) detector, data were acquired and processed by an HP Chemstation. A C18 column (Phenomex luna, 4.6 mm × 250 mm, 5 µm).

Preparation of Standard Solution Work solutions of muscone samples and rude Moschus herb had been prepared by solution in methanol and the final concentrations were 2.0 and 1.0 mg/mL respectively by appropriate dilution.

Preparation of TQHXD Sample Semen pruni persicae 90 g, Radix Paeoniae Rubra 30 g, Rhizoma chuanxiong 30 g, Carthamus tinctorius 90 g, Ziziphus jujube 50 g and Allium fistulosum 30 g mixed together were decocted in water for 2 h twice and filtered. After the confluent solutions pump vacuum, 95% ethanol was added into it until the concentration of ethanol was below to 75%. Then pump vacuumed again, 1.5 g Moschus was added to the solutions to form the extract of TQHXD with concentration of 1.6 g/mL (equivalent to dry weight of raw materials) as stock solutions. Then the extracted TQHXD was diluted with the cell-culture medium before filtration on a 0.22 µm Millipore filter.

Survival Rate of PC12 Cells Treated by TQHXD Stock Solution PC12 cells were seeded in 96-well culture plates at 1×10^4 cells per in logarithmic growth phase. Starved cells were treated with TQHXD of 50, 10, 5, 1, 0.1 and 0.01 mg/mL for 12, 24 and 48 h. The survival rate of PC12 cells was assayed by MTT assay to determine the appropriate concentration of TQHXD acting on the PC12 cells.

Establishment of Method of Cell Biochromatography The effective ingredients binding to the targets on membrane of PC12 cells, PC12 cells grown to 80% confluence were treated with TQHXD stock solutions (1 mg/mL) in incubator for 1 h at temperature of 37°C.

The Number of Times of PBS (pH 7.4) Eluting the Unconjugated Substances with Targets of PC12 Cells After 1 h incubation, PC12 cells were collected at 800 rpm for 5 min. The supernatant was discarded and the cells were dispersed with PBS (pH 7.4) and centrifuged again. The supernatant was considered as the first eluent sample. After the fifth elution, ingredients which do not bind to the targets on membrane of PC12 cells were washed into eluents, while the active ingredients combined with membrane of PC12 cells would not be washed down. Eluents were filtered for analysis by HPLC, the eluent without the change of the fingerprint peak was defined as the last eluent sample.

Inactivation of the Targets of PC12 Cells by PBS of pH 4 After the last elution, PC12 cells were blown until suspended, then collected in the polypropylene tube and dissociated with PBS (pH 4), the tube was placed in a water bath at 37°C under constant agitation within 1 h. The cells and medium culture were then centrifuged at 3200 rpm for 8 min. The supernatant which is called disassociation solution sample was prepared at −70°C overnight and frozenly concentrated.

Preparation of the Blank Control Sample The blank control solution sample was dealt with the same procedure by using medium1640 instead of TQHXD.

Disposal of Disassociation Solution Sample before Measurement with HPLC and GC Disassociation solution sample was prepared at −70°C overnight and frozenly concentrated. After that, the concentrate was transferred into a separatory funnel and extracted by ethyl acetate for four times. Four batches of extracts were combined, evaporated and dissolved in methanol (2 mL). The sample was then added to the solid phase extraction (SPE) column cartridge which was preconditioned and equilibrated by sequential washings with methanol–0.05% phosphate and water–0.05% phosphate. After the sample is loaded, the cartridge was washed with methanol–0.05% phosphate. The elute of methanol–0.05% phosphate was collected into a test tube and condensed to 2 mL under a gentle flow of nitrogen at room temperature and passed through a 0.22 µm Millipore filter and stored at 4°C which was defined as the disassociation solution sample.

Confirmation of the Source of Active Ingredients of TQHXD Which Bound to PC12 Cells The fingerprints of dissociation solution sample, the last eluent sample, the blank control sample, reference substance, TQHXD and respectively single materia medica in TQHXD were compared with each other by chromatographic analysis such as HPLC, GC, ac-
The percent value was measured at 490 nm with a micro plate reader.

Cell viability was calculated as follows: Cell viability (culture plates at an approximate density of 1 × 10^5 cells per well) using MTT assay. PC12 cells were evenly added into 96-well plates at the density of 1 × 10^5 cells per well. After treatment with muscone or NMDP, the supernatant was used for measuring LDH activity when media was collected and centrifuged. Absorbance (A) at 440 nm was collected and centrifuged. Absorbance (A) at 490 nm was measured with a micro plate reader (ELX800; Bio-TEK instruments, Inc., U.S.A.). The LDH value was expressed as percentage of sample

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The standards and samples were analyzed by HPLC chromatography using a gradient mobile phase consisting of 0.05% H₃PO₄ (solvent A) and methanol (solvent B). The protocol was as follows: 0 min 90% A, 15.0 min 72% A, 30.0 min 60% A, 55.0 min 52% A, 70.0 min 46% A, 80.0 min 35% A, and 83.0 min 90% A. Given Moschus whose characteristic ingredient is muscone was the principal herb in TQHXD, and in general, muscone was detected by HPLC using its phenylhydrazine derivatization, so each sample was phenylhydrazine derivatization. Five hundred milligrams 2,4-dinitrophenylhydrazine (DPNH) was added to 30 mL vitriol–water–alcohol (2:3:10, v/v), which was considered as 2,4-dinitrophenylhydrazine solution. Then, this solution was added into muscone standard sample, Moschus medicine and disassociation solution sample for 1 h and crystalline precipitation was washed with water and filtered with reduced pressure until pH was closed to 7. Then the precipitation was dissolved in ethanol which were considered as the phenylhydrazine derivatives of muscone and disassociation solution sample.

Establishment of OGD Model and Determine the Optimal Time Point To mimic cerebral ischemia, the ischemia model of PC12 cell was established according to the Tagliari's B methods in vitro. Briefly, PC12 cells were washed with PBS for 3 times after culture medium was removed, ensure that each cell be the same background before damage. Therefore, glucose-free RPMI1640 was added to prepared cells, finally transferred to an incubator chamber (Thermo, U.S.A., N 2–CO₂, 5% CO₂) for 37°C. Following attachment period, respectively.

Cell Culture and Drug Treatment Cells were cultured for 7 d in an atmosphere of 5% CO₂ at 37°C. Following attachment, PC12 cells were pretreated with nimodipine (NMDP) (final concentration: 10 µM) and different concentrations of muscone (final concentrations: 0.1, 1, 10 µM) for 2 h prior to OGD. Control group was added to the same amount of medium and disassociation solution sample. 17,18) In general, muscone was detected by HPLC using its phenylhydrazine derivatization, so each sample was phenylhydrazine derivatization sample.

Cell viability was detected with MTT assay. PC12 cells were evenly added into 96-well culture plates at an approximate density of 1×10⁵ cells per well. At the end of muscone and NMDP treatment, MTT was added to the medium and incubated for another 4 h at 37°C. Then removed the culture medium, the insoluble dark blue formazan crystal was dissolved with 150 µL DMSO. The absorbance (A) value was measured at 490 nm with a micro plate reader (ELX800; Bio-TEK instruments, Inc., U.S.A.). The percentage of cell viability was calculated as follows: Cell viability (%)=(A₄₉₀, sample−A₄₉₀, blank)/(A₄₉₀, control−A₄₉₀, blank)×100.

Cell Viability Assays PC12 cell viability was detected using MTT assay. PC12 cells were evenly added into 96-well culture plates at an approximate density of 1×10⁵ cells per well. At the end of muscone and NMDP treatment, MTT was added to the medium and incubated for another 4 h at 37°C. Then removed the culture medium, the insoluble dark blue formazan crystal was dissolved with 150 µL DMSO. The absorbance (A) value was measured at 490 nm with a micro plate reader (ELX800; Bio-TEK instruments, Inc.). The LDH value was expressed as percentage relative to control group.

Cell Apoptosis Cell apoptosis was detected by AO/EB staining. The experiment was performed according to the protocols. After the muscone and NMDP treatments, briefly, cells were washed with PBS for 3 times, exposed to AO/EB working solution (100 µg/mL AO and 100 µg/mL EB in PBS) at 4°C for 20 min. Fluorescence images were taken by a fluorescence microscope (Leica Microsystems, German). Herein, PC12 cells apoptotic rate was quantified by AV/PI apoptosis assay kit. PC12 cells were harvested and washed twice with pre-cooled PBS, then incubated with binding buffer containing FITC-AV and PI for 30 min at room temperature in the dark. Samples were determined by flow cytometry (FAC Scan, Becton-Dickinson, U.S.A.) and analyzed by FCS software (BD Biosciences).

Statistical Analysis All data were analyzed with the SPSS statistical software. Statistical analysis of the data was evaluated by one-way ANOVA, followed by a post hoc least significant difference (LSD) test. p<0.05 was considered statistical significance. All data were presented as the mean ± standard deviation (S.D.).

Results

Influence of the Extracts of TQHXD on Proliferation of PC12 Cells To examine if the extracts of TQHXD affects proliferation of the PC12 cells, different concentrations of TQHXD were added to PC12 cells for 12, 24 and 48 h. As shown in Fig. 1, when its concentration exceeded 10 mg crude drug/mL, TQHXD stock solutions showed toxic effects on PC12 cells. While the concentration of TQHXD ranged from 0.1 to 1 mg crude drug/mL, it promoted growth of PC12 cells. TQHXD stock solutions of 0.01 mg crude drug/mL had no significant effect on PC12 cells growth.

Optimization of HPLC Conditions Compared with the acetonitrile–water system, the methanol–water system showed more powerful separation ability and elutive power to TQHXD sample. When phosphate (H₃PO₄) was added to mobile phase, the peak capacities and shapes of all the chromatographic peaks were enhanced remarkably. The optimal solvent system was realized via gradient elution using a mobile phase of 0.05% phosphate (H₃PO₄) in water (A) and methanol (B)
within 83 min. The compounds of each herb in TQHXD belong to different chemical families and present different UV absorption. According to the compound 3D-plot graph showed the absorption wavelength range, combined with the best absorption wavelength of component in single medical herb in prescription, based on our previous study, we ultimately determine the detection wavelength was 230, 254, 280 and 320 nm.

**Optimization of the Elution Times**

To eliminate the influence of non-combined ingredients, PBS (pH 7.4) was used to elute for several times and each eluate were detected by HPLC. As shown in the supplementary Fig. 1, comparing the HPLC chromatograms of the first eluent sample, the forth eluent sample and the fifth eluent sample, there was no combined ingredients in fifth eluent. Thus, the influence of non-combined ingredients was removed utterly after TQHXD was eluted by PBS (pH 7.4) for five times.

Therefore, after five elutions, the cells were collected and treated with PBS (pH 4) within 1 h and PBS (pH 4) was collected as dissociation solution. Then the active ingredients combined with PC12 membrane were collected by changing the pH of PBS from 7.4 to 4.

**The Optimal Treatment of Dissociation Solution**

Because of less volume of the collected sample of dissociation solution and a small quantity of ingredients in it as well as its unclear chemical property, we tried a variety of methods in processing the dissociation solution sample before its detection by HPLC. Concentration by freezing and extraction by ethyl acetate was carried out respectively in treating samples. But results revealed that there were no obvious characteristic peaks in HPLC chromatogram in the frozen concentrated samples.

Characteristic peaks of the dissociation solution samples extracted by ethyl acetate were more sensitivity than frozen concentrated samples. When these two methods were used together, characteristic peaks of dissociation solution samples showed higher sensitivity with little interference. Simultaneously, after freeze concentration and ethyl acetate extraction, dissociation solution samples was then enriched, the chromatographic peak symmetry was improved greatly. Basing on these results, we established the sample extract procedure as described in section “Disposal of Disassociation Solution Sample before Measurement with HPLC and GC.” HPLC chromatograms of dissociation solution treated by different conditions were shown in supplementary Fig. 2.

**Fig. 2. Comparison of HPLC Chromatograms of Dissociation Solution (A), TQHXD Sample (B), Allium fistulosum Sample (C), Fifth Eluent Sample (D) and Control Disassociation Solution Sample (E)**

The retention time of the sample of dissociation solution was same as the TQHXD sample, and chromatographic peak which retention time was 38.3 min also appeared in *Allium fistulosum* sample too, which illustrated that this ingredient may come from *Allium fistulosum*. Detection wavelength: 230, 254, 280, 320 nm. Ellipse indicated peak appear in 38.3 min. Arrows indicate peak appear at around 70 min. (Color figure can be accessed in the online version.)
at around 70 min may be muscone. But this conclusion needs further confirmation. Comparing GC chromatograms of dissociation solution sample, muscone standard solution, rude Moschus herb solution, TQHXD sample and control group of methanol, the same chromatographic peak which retention time was 12.7 min appeared in above samples except control group. At the same time, this result was reproduced in three batches of dissociation solution sample. Therefore, we confirmed this active ingredient in dissociation solution was muscone, which was from Moschus (Fig. 4).

**OGD Time Point and Cells Viability** Firstly, we screened the time point of oxygen and glucose deprivation injury by MTT. PC12 cells were subject to 2, 4, 6h of OGD. After OGD treatment, in 2, 4 and 6h, the cell body became shrunk and round was greater than under control conditions, and the phenomenon is more obvious at 4, 6h (Fig. 5A). As shown in Fig. 5B, the majority of cells in the Control group displayed a uniform, bright green staining, while the cell became reddish or orange staining in 2, 4, 6h by OGD, and a lot of cells became reddish staining in 6h. Cell viability was tested for each time point. MTT assay for cell viability showed that PC12 cells viability decreased significantly in 4 and 6h, although the cells injury is most serious in 6h by OGD, the cell viability reduced to 47%. Ultimately, cell OGD for 4h as the best time point in our research (Fig. 5C).

As shown in Fig. 5D, cell viability remarkably reduced by OGD compared with control group. However we found that cell viability was significantly increased in NMDP group (10 µM), and different concentrations of muscone have tended to dose-dependent, which indicated that muscone could significantly improve cell viability. LDH assays were performed to assess cell cytotoxicity of PC12 cells. The cell LDH release was elevated after OGD exposure, which was reversed by muscone at concentrations of 0.1, 1, and 10 µM in a concentration-dependent manner (Fig. 5E). Taken together, these results suggest that muscone and NMDP has protective effect on PC12 cell injury induced by OGD.

**Effect on the Apoptosis Changes of PC12 Cells** Cell apoptosis plays a crucial role in cerebral ischemia injury. In order to explore the effect of muscone on apoptosis by OGD, AO/EB staining was performed. Under fluorescence microscopy, PC12 cells had remarkably morphological apoptotic change by OGD (reddish or orange staining represents late apoptotic cells and yellow staining represented early apoptotic cells), while PC12 cells treated with muscone or NMDP showed manifest green fluorescence (Fig. 6A). Finally, in order to examine the effect of muscone on PC12 cells apoptosis induced by OGD, AV-FITC/PI double staining was monitored with flow cytometric analysis. As shown in Fig. 6, the proportion of apoptotic cells rose to 21.6% by OGD for 4h, which was significantly higher than control group (6.9%). While, treatment with 0.1, 1, 10 µM muscone reduced the apoptosis rate to 19.3, 18.7 and 14.0%. These results suggested that muscone inhibited apoptosis in PC12 cells injury induced by OGD in a dose-dependent manner.

**Discussion**

For the key issue to be resolved in the modernization of TCM is screening of the effective substance, which has been an important subject of TCM, especially Chinese herbal formula. It is difficult to distinguish which active compounds are effective or ineffective in the separation stage of traditional...
analysis methods. However, the cell membrane can be selectively combined with the effective ingredients and avoid many complicated processes in the body. It is possible to directly find the active compounds and eliminate the interference of the non-active impurity component. Therefore, biochromatography is an important method to analyze and separate the components of TCM, effective compounds analysis was carried out at the same time as component separation, and active components released after target inactivation can be directly analyzed in accordance with requirements of chromatographic, which the requirements of biology is no consideration to lower the requirement of chromatographic analysis technical, or reduce the biological requirements to gratify the requirement of chromatography. It can not only greatly reduce the scope of screening the active ingredients of TCM but also effectively eliminate the interference of invalid ingredient to the results of the analysis. The principle of biochromatography provides new methods and ideas for solving the problems in finding the active ingredient of TCM.

The rat adrenal pheochromacytoma PC12 cells line is widely used as a model neuronal cell line because the cells behave like neural progenitor cells: they proliferate in growth media, but stop proliferating and differentiate into sympathetic neuron-like cells following treatment with nerve growth factor (NGF). PC12 cells are also used to study signal transduction, differentiation, survival and proliferation mechanisms as they respond to many growth factors, neurotrophins and hormones.\(^{23}\) \textit{In vitro} pharmacological studies showed that the serum containing TQHXD had protective effects on cell membrane permeability and proliferation in glutamate-injured PC 12 cells.\(^{24}\) Therefore, the PC12 cells was identified as the target cells of TQHXD in this study.

In this study, a safe concentration range of TQHXD on cells was tested to explore the appropriate concentration range of TQHXD on PC12 cells. MTT assay results showed that the range of 0.01–5 mg crude-herb/mL of TQHXD had no significant toxic effect on PC12 cells. So the appropriate concentration had been confirmed. Then the biological sample preparation method was established and successfully applied to prepare the eluent sample and disassociation solution sample. During the process of building nerve cell biochromatography, our study has investigated various influencing factors as follows:

1. Time of cells incubated with TQHXD: PC12 cells were cultured with TQHXD for 30, 60 and 90 min, respectively. Results showed that the dissociation solution collected after 30min incubation had no obvious chromatographic peak; however, chromatographic peak was better in dissociation solution of 60 min incubation and showed no changes at 90 min, so 60 min incubation time was chosen which could ensure many effective ingredients were bound to the targets on cells.

2. Composition of eluate and elution times of PBS (pH 7.4) eluting the unconjugated substances with targets of PC12 cells. This study was aimed to separate non-combined ingredi-
ents from active ingredients that had bound to effective targets on cell membrane by changing elution factors. Firstly, ingredients that were not specific binding to PC12 cells were washed off with PBS (pH 7.4). Next, due to the use of PBS (pH 4), effective targets in PC12 cells membrane became allosteric and inactive in the acidic conditions which made effective

Fig. 5. OGD Time Point and Cells Viability

(A) PC12 cells morphology in 2, 4, 6 h observed by light microscope, respectively (×100 magnification). With the OGD time prolongs, the cells show different degrees of swelling, swell degree of cells represents cells injuries. (B) Apoptosis morphology was detected using fluorescence microscope through AO/EB staining (×200 magnification). (C) Cell viability detected by MTT. Effect of muscone on PC12 cells injury induced by OGD for 4 h. (D) MTT assay shows that PC12 cells in the model group exhibited decreased viability than control group. The cell viability in muscone groups was remarkably increased compared with the OGD group. (E) LDH assay shows that cytotoxicity increased in treatment with OGD, muscone pretreatment reduced cytotoxicity after OGD injury in a dose-dependent manner. All the experiments were performed in triplicate. Scale bar=100μm (×200 magnification). Values were expressed as mean±S.E.M. (n=6). ##p<0.01 vs. control group; *p<0.05, **p<0.01 vs. model group.

Fig. 6. Effect of Muscone on PC 12 Cells Apoptosis by OGD

Cells apoptosis was detected by AO/EB staining. (A) Control group, OGD group, muscone or NMDP group while the effect in a dose-dependent manner. (B) The proportion of apoptotic PC12 cells assessed by AV-FITC/PI staining. (a) Control group. (b) OGD group. (c) OGD+0.1μM muscone. (d) OGD+1μM muscone. (e) OGD+10μM muscone. (f) OGD+10μM NMDP. (C) Graphic representations of the apoptotic rate of each group. Scale bar=100μm (×200 magnification). The data are represented as means±S.D. for three independent experiments. ##p<0.01 vs. control group; *p<0.05, **p<0.01 vs. model group.
substances lose binding with targets of PC12 cells, then entered into PBS of pH 4 which was collected and called dissociation solution. Repeated tests proved that five times was the most appropriate elution condition. These treatments not only excluded the interference of non-specific binding components, but also demonstrated the specific combination of each effective substance with targets on PC12 cells membrane.

(3) Treatment on dissociation solution: Due to a small quantity of effective substances contained in collected dissociation solution and their unclear physical and chemical properties, a variety of treatments of screening effective substances were carried out to reach optimization condition for detection before analysis dissociation solution. The results showed that active ingredients could be enriched by ethyl acetate extraction. In order to reduce the interference of base material, SPE was used to enrich the effective substances in dissociation solution, and to improve the analytical sensitivity to effective substances. Combining the methods of freeze concentration, ethyl acetate extraction and SPE solid phase extraction to treat the dissociation solution, the peak shape, peak number, and peak area of active ingredients in dissociation solution was superior to single treatment. In addition, by investigating repeatability of nerve cell biochromatography and stability of dissociation solution, it proved that nerve cell biochromatography was a stable and repeatable method which can be used to screen bioactive ingredients in TCM.

Using this method, two characteristic active ingredients binding to the membrane of the PC12 cells were indicated, their retention time was 38.3 min and at around 70 min respectively. After comprehensive comparison of HPLC chromatograms of dissociation solution and each single herb, the effective substance which retention time was at around 70 min was verified to be muscone by HPLC and GC. The effective substance which retention time was 38.3 min possibly derived from the Allium fistulosum and its structure remains to be further identified.

Muscone (structural formula shown in Supplementary Fig. 3) is one of the active ingredients of Moschus, also the protective effect of muscone on PC12 cells injury induced by OGD had been explored. We found that the treatment with muscone on PC12 cells could observably increase cell viability, reduce the release of LDH and cell apoptosis. Our previous experiment could observably increase cell viability, reduce the effect of muscone on PC12 cells injury induced by OGD. These results could be provided a specific basis for the establishment of the quality standard of TQHXD.

Conclusion
There is an effect component of TQHXD that could be specifically combined with a PC12 cell effect target, the component analysis was muscone through cell biochromatography and derived from Moschus herbs, muscone could significantly improved PC12 cells injury induced by OGD. These results could be provided a specific basis for the establishment of the quality standard of TQHXD.

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Conflict of Interest The authors declare no conflict of interest.

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