In Vitro Neurotoxicity Evaluation of Betel Nut Decoction on the Neurons/Astrocytes Co-cultured Cells

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Abstract. The betel nut has attracted great research interest for their potential applications in pharmacy, food and biomedical areas. However, little information of their neurotoxicity is reported. Herein, we performed a comprehensive study on the neurotoxicity of betel nut decoction (BND) by examining the influences of betel nut decoction on the morphology, viability, LDH release, intracellular Ca\textsuperscript{2+} concentration, mitochondrial membrane potential, synaptic plasticity-related proteins (Arc, SYN, β-III-Tubulin), neurotransmitters and glutamate of co-cultured cells. The results showed that the betel nut decoction had general toxic effects on co-cultured cells. The general toxicity indexes showed that 150 μg/mL group increased LDH release, increased intracellular calcium concentration and decreased mitochondrial membrane potential. Moreover, 50 μg/mL betel nut decoction treated with neurons/astrocytes co-cultured cells for 6 h caused neurotoxicity, SYN can be used as a sensitive protein for rapid detection betel nut decoction neurotoxicity. These results are dose related, and should be considered in the development of bio-applications of betel nut, which provides the basis for future studies on the mechanism of drugs-induced neurotoxicity.

Introduction

Betel nut belongs to a family of Palm branch, which is the dried seed of the plant Areca catechu L.[1]. Betel nut has attracted more and more attention in worldwide as a medical and edible plants for human health owing to its broad range of health benefits and pharmacological effects, such as gastrointestinal motility, anti-microbial, and nerve stimulation, etc [2]. However, betel nut also could produce oral, liver, reproductive and neurotoxic side effects. Gilani et al. [3, 4] had reported that betel nut could inhibit the hydrolysis of acetylcholine, then this inhibit effect causes disorders of the nervous system. Similar results were reported by Sorce et al. [5], whose results also indicated that excessive accumulation of reactive oxygen in neuronal cells induced by betel nut. Moreover, with the development of industrialization of betel nut, it is necessary to evaluate the safety of betel nut due to the development of betel nut industrialization, especially its evaluation of neurotoxicity.

Neurotoxicity is usually evaluated by using in vivo animal experiments and in vitro cell experiments [6-8]. In vivo animal experiment evaluation method has the advantage of being able to detect all potential targets and mimic the human body greatly. However, the animal
toxicity experiment has the disadvantage of high cost and long period [9, 10]. In vitro toxicity evaluation method has received extensive attention due to its high reproducibility, short time cycle, and high sensitivity [11-13]. In addition, co-cultured cells models have been widely applied in vitro toxicity assessment. For example, Brown et al. showed that the sensitivity of neurons to glutamate toxicity was increased in Astrocyte/neuron co-cultured cells [14]. Furthermore, some literatures highlighted that neurons/astrocytes co-cultured models provided the better experimental technology platform for the assessment of toxic compounds [15-17].

Notwithstanding, in the drug scene, betel nut decoction has the reputation of being safe, and there are presently no studies regarding their neurotoxicity in a neuronal/astrocyte co-cultured cells. Thus, the aim of this work was to study the in vitro neurotoxicity of the betel nut decoction using the neuronal/astrocyte co-cultured cells. Cytotoxicity was evaluated through the MTT reduction and LDH release assay, as well as intracellular Ca$^{2+}$, mitochondrial membrane potential, synaptic plasticity-related proteins (Arc, SYN, β-III-Tubulin), neurotransmitters and glutamate of co-cultured cells were also evaluated.

Materials and Methods

Materials

SD rats (24 hours newborn rats) were purchased from the Experimental Animal Center of Jiangsu University. All animals were housed in a room with 12 h light/dark cycles and maintained at 25℃, during which they had free access for food and water. The animal production license number (SCXK-(Su) 2013-0011) was provided by Animal Experimental Center of Jiangsu University. Betel nut was purchased from drugstore in Zhenjiang.

Research Method for Neurotoxicity Evaluation in Vitro

The flow chart for the study of method for neurotoxicity evaluation in vitro is shown in Fig. 1. First, neuron and astrocytes cells were prepared for the establishment of co-culture system. The stability of co-culture model was assessed. Then, the general cytotoxicity and neurotoxicity were studied systematically. Finally, the betel nut decoction neurotoxicity evaluation methods in vitro were established.

Figure 1. Research flow diagram for establishment of neurotoxicity evaluation methods in vitro.
Primary Co-cultures of Neurons and Astrocytes

The astrocyte/neurons co-cultured system was used in this study according to the method of Giordano et al. [18] with minor modification. The primary neurons or astrocytes used in the present study were isolated from SD rats. The glial cells were incubated and maintained in a humidified atmosphere at 37°C and 5% CO2. When the fourth generation of glial cells over the bottom area (70%-80%), neurons were added to the astrocyte monolayer for 7 d. The experiment was performed in duplicate.

Preparation of Betel Nut Decoction

The betel nuts were weighed accurately and added 10 times distilled water for soaking 30 min. The solution was cooled down and filtered out the first decoction. The second decoction was obtained in accordance with the above method. The two filtrates were combined and concentrated under reduced pressure at 45°C. Finally, the concentrate was lyophilized to obtain the lyophilized powder.

Cell Viability Assays

The cytotoxicity of the BND was evaluated on neurons/astrocytes co-cultured cells by using the MTT method. The neurons/astrocytes co-cultured cells were plated into 96-well plate (Nunc, Denmark) at a density of 1×10^5 cells/well. The media were replaced with fresh complete medium containing different BND concentrations (25, 50, 100, 125, 150, 175, 200, 400, 800, 1000 μg / mL) in next day. Then the cells were administered with the BND for different culture time (6, 12, 24, 48, 72, 96 h). Afterwards, The medium in each well was removed and cells were washed once with PBS. Then cells were incubated with 1 mg/mL MTT at 37°C for 4 h. After this, the medium was removed and replaced with 150 μL of DMSO. Finally, the absorbance was measured at 590 nm on the ELISA reader (TECAN, Switzerland). The percentage of cell viability was calculated according to following formula (1) [19]:

Cell viability rate (%) = (experimental absorbance/ control absorbance) × 100 (1)

Evaluation of Lactate Dehydrogenase (LDH) Release Assay

Membrane integrity was evaluated by measuring the LDH activity according to the methods of Leguo Zhang et al. [20]. Briefly, different concentrations of BND (25, 50, 100, 150, 200 μg/mL) acts on co-cultured cells for 24 h. The co-cultured cells were centrifuged for 15 min and re-suspended with 150 μL of LDH release reagent for additional 2 h. The content of LDH in supernatant was measured by using a commercial LDH assay kit (Beyotime, China).

Acridine Orange/Ethidium Bromide (AO/EB) Staining

Co-cultured cells were seeded at 1×10^5 cells/ well in 12-well plates and incubated for 24 h. The medium was replaced with different concentrations of BND (25, 50, 100, 150, 200 μg/mL) for 24 h. After 24 h of incubation, 50 μg/mL AO/EB staining solution was added in wells for 5 min in the dark, Morphological changes were observed by using inverted fluorescence microscope (Leica DMI 4000 B, Germany) with a 200 × objective.

Determination of Intracellular Ca^{2+} Levels

Different concentrations of BND acts on co-cultured cells for 24 h. The medium in each well was removed and cells were washed three times with PBS solution. Then, loaded with 5 μmol/L Fluo-3 AM(Beyotime, China) for 40 min at 37°C in the dark. After removing the extracellular...
Fluo-3 AM, the fluorescence intensity of Ca²⁺ in co-cultured cell was determined by a fluorescence microplate reader (Molecular device, USA) at 488 excitation, 525 emission. Images were obtained under an inverted phase contrast fluorescence microscope (Leica DMI 4000 B, Germany).

**Determination of Mitochondrial Membrane Potential**

The mitochondrial membrane potential was determined according to the method of Carmichael et al. [21]. Briefly, after treatment with different concentrations of BND (25, 50, 100, 150, 200 μg/mL) for 24 h, JC-1 (5 μg/mL) staining solution was added to each well and incubated for 20 min at 37°C. The medium was removed and the co-cultured cells were washed with PBS solution in the dark. The fluorescence intensities (FI) were detected by using a fluorescence microplate reader (Molecular device, USA) at 485 nm/538 nm and 525 nm/590 nm (excitation/emission) for detection of the monomers and the aggregates. The mitochondrial membrane potential in each treatment group was calculated as the fluorescence ratio of red to green. Images were obtained under an inverted phase contrast fluorescence microscope (Leica DMI 4000B, Germany).

**Western Blot Analysis**

After treating co-cultured cells as described above, total protein was extracted from cell lysates using RIPA buffer (Beyotime, China). Supernatant protein concentrations were determined with BCA protein assay, and then electroblotted onto PVDF membranes at 200 mA for 90 min at 4°C. Membrane was blocked with 5% nonfat dry milk in PBS to block the non-specific binding. Before overnight, incubated with primary antibodies specific for β-III-Tubulin (1:1000), Arc (1:1000), SYN (1:10000), using β-actin (1:1000) (Cell Signaling Technology company, USA) as loading control. Membrane was then rinsed three times in TBST and was incubated with horseradish peroxidase (HRP)-conjugated secondary antibody (1:5000) (Santa Cruz Biotechnology company USA) at room temperature for 1 h, followed by a final series of rinses in TBST. Bands of protein were visualized by using an enhanced chemiluminescence ECL kit (Biological Industries, Israel) and were quantified by Quantity One software (Bio-Rad).

**Determination of Neurotransmitter NO, Glutamate and Acetylcholine Contents**

The content of NO in astrocytes and neurons primary co-cultures was measured by using NO kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) based on Griess reactions. Total nitrite was measured at 550 nm. The content of glutamate in astrocyte/neuron primary co-cultures was measured by using glutamate kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China). The absorbance values were determined by spectrophotometry method at 340 nm.

ACh level in astrocyte/neuron primary co-cultures was measured by using the method of Hestrin with some modification, which is based on the the red purple color produced by the binding between ferric ion and hydroxamic acid. The intensity of brown ferric complex was read at 540 nm. ACh content was expressed as micromoles permilligram of hippocampus protein (μg/mg Prot).
Statistical Analysis

All results were expressed as mean±SD from at least three independent experiments. Statistical analysis of data was analyzed by one-way analysis of variance (ANOVA), the data of differences between group means was observed from post-hot test. Values of $P<0.05$ were regarded as significant.

Results

Effects of BND on Cell Viability

The cytotoxic effect of BND was determined by MTT assay which is depicted in Fig. 2 (A-B). After exposure to BND within the range of 25-100 μg/mL, no obvious decrease in cell viability was found (Fig.2A), but at high concentration (200-1000 μg/mL), co-cultured cells viability were decreased from 105±5.2% to 57±7.1% significantly. These results are similar to the results reported by Lu Peng et al. [22]. Moreover, after incubation with 150 μg/mL BND on co-cultured cells for 96 h (Fig.2B), the cell viability rate was (77±4.5) %, compared with the control group, the difference was extremely significant ($P<0.01$). Thus, BND at the dose of 150 μg/mL produced the most significant cytotoxicity than the other doses.

Figure 2. Effects of betel nut decoction on cell viability in co-cultured cells (Mean±SD, n=3). A. Effects of betel nut decoction at the dose of 25-1000 μg/mL on cell viability in co-cultured cells for 6-96 h; B. Effects of betel nut decoction at the dose of 100-200 μg/mL on cell viability in co-cultured cells for 6-96 h (*: $P<0.05$ compared with control, **: $P<0.01$ compared with control).

Effects of BND on LDH Release

This dose-dependent cytotoxic effect is further shown by using the lactate dehydrogenase (LDH) assay. As a stable cytoplasmic enzyme present in all types of cells, LDH is released into the cell culture medium through damaged plasma membrane. Its levels are proportional to the number of damaged cells [23]. As shown in Fig. 3, after treatment with 25, 50, 100, 125, 175 and 200 μg/mL BND for 24 h, the results indicated that the LDH content in the supernatant of the cells increased gradually with the increasing of BND concentration, and with a dose-dependent effect. Group 150 μg/mL BND and group 200 μg/mL BND significantly increased compared with the control group ($P<0.01$). These observations confirmed that the
high-dose betel nut decoction destroys the structure of cell membrane and has obvious cytotoxicity.

Figure 3. Effect of betel nut decoction on the release of lactate dehydrogenase (Mean±SD, n=3); *: P<0.05 compared with control, **: P<0.01 compared with control.

Apoptosis Analysis

The apoptosis of co-cultured cells induced by BND was also investigated by using the AO/EB staining. AO/EB is widely used to evaluate the nuclear morphology of apoptotic cells. Acridine orange is a vital dye that will stain both live and dead cells, whereas ethidium bromide will stain only those cells that have lost their membrane integrity. Thus, apoptotic cells are delineated with orange nuclei and live cells appear uniformly green [24]. As shown in Fig.4, co-cultured cells in the control group were plump, presented fusiform and conical in structure. The synapsis, thick and long, was intertwined forming a dense mesh structure. There was no pronounced apoptosis in co-cultured incubated with BND (25, 50, 100 μg/mL) and the co-cultured cells showed uniform green fluorescence (Fig.3B-D). However, when the concentration of BND (Fig.4E-F) increased to 150 and 200 μg/mL, cells were with orange fluorescence appeared. Thus, Neurons/astrocytes co-cultures exhibited typical swelling and marked decrease in the number of cells induced by high dose of BND. These results are similar to the results reported by MB Luskin et al. [25].

Figure 4. Immunofluorescence staining with AO/EB (×200); A. Control; B-F. 25, 50, 100, 150 and 200 μg/mL betel nut decoction treatment groups.
Effects of BND on intracellular Ca$^{2+}$ levels

The effect of BND on the distribution and content of Ca$^{2+}$ in co-cultured cells were shown in Fig. 5I and Fig. 5II. It can be seen that the intracellular Ca$^{2+}$ fluorescence intensity of co-cultured cells was lower in the control group (Fig. 1A). After treatment with different concentrations of BND, when the concentration of BND (Fig. 5E-F) increased to 150 and 200 μg/mL, the intracellular Ca$^{2+}$ concentration was increased significantly. The results were consistent with the results of inverted fluorescence microscopy (Fig. 5II).

![Figure 5. Fluorescence intensity of the intracellular Ca$^{2+}$ in co-cultured cells (Mean±SD, n=3); (I). Distribution of Ca$^{2+}$ in co-cultured cells(×200) (A: Control; B-F: 25, 50, 100, 150 and 200 μg/mL betel nut decoction treatment groups ); *P<0.05 compared with control, **: P<0.01 compared with control.]

Effects of BND on Mitochondrial Membrane Potential (MMP)

The mitochondrial membrane potential was measured by JC-1 staining. Mitochondrial depolarization was happened with a decrease in the red/green fluorescence intensity ratio (Fig. 6). Treated with several concentrations of BND (25, 50, 100, 150, 200 μg/mL) on co-cultured cells. Only BND at the higher concentrations (150 μg/mL) triggered a significant decrease in mitochondrial membrane potential. The results of immunofluorescence staining (Fig. 6I) are consistent with the values in Fig. 6II.

![Figure 6. Effects of BND on mitochondrial membrane potential. (I). Observation of the co-cultured cells stained with JC-1(×200) (A: Control; B-F. 25, 50, 100, 150 and 200 μg/mL betel nut decoction treatment groups); (II). Relative red/green fluorescence production was determined using JC-1 and quantified through fluorescence microplate. (Mean±SD, n=3) ( *: P<0.05 compared with control, **: P<0.01 compared with control).]
Effects of BND on the expressions of β-III Tubulin, Arc, SYN proteins

To investigate the effect of betel nut decoction on levels of SYN, Arc and β-tubulin III expression in primary neuron/astrocyte co-cultures were measured by Western blotting after 1 h, 3 h and 6 h treatment (Fig. 7). There was no significant difference in cultures incubated with 25, 50, 100 and 200 μg/mL areca decoction for 1 h and 3 h, respectively. (Fig.7a-b). Exposure to betel nut decoction for 6 h induced Arc and β-tubulin III expression was not significant difference compared with the control group (Fig.7c). The protein expression of SYN began to decrease when the concentration of betel nut decoction was 50 μg/mL, decreased by 0.36 times ($P<0.05$) compared with the control group. The above results showed that the betel nut decoction (>50 μg/mL) treated co-cultured cells for 6 h had a neurotoxic effect, and SYN is a sensitive protein.

Figure 7. Effects of betel but decoction on neuronal SYN, Arc and β-III-Tubulin proteins expression in co-cultured cells for 1 h, 3 h, 6 h.

Effect of BND on the Content of Neurotransmitter in Co-cultured Cells

We investigated the effect of betel nut decoction(<50 μg/mL) on contents of NO, ACh and glutamic acid in primary neuron/astrocyte co-cultures after 6 h treatment (Table 1). NO and glutamic acid content showed an upward trend, while the ACh content decreased gradually. The results indicated that 50 μg/mL of betel nut decoction promoted the excessive release of NO and glutamate, while ACh was lower than the normal level of the body, breaking the homeostasis and producing neurotoxicity.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Concentration (μg/mL)</th>
<th>NO (μmol/gprot)</th>
<th>ACh (μg/mgprot)</th>
<th>Glutamate (μmol/gprot)</th>
</tr>
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<tr>
<td>Control</td>
<td>0</td>
<td>1.87±0.18</td>
<td>28.32±1.27</td>
<td>20.37±2.32</td>
</tr>
<tr>
<td>Betel nut decoction</td>
<td>15</td>
<td>2.02±0.58</td>
<td>28.29±1.47</td>
<td>22.12±2.13</td>
</tr>
<tr>
<td></td>
<td>25</td>
<td>2.07±0.34</td>
<td>28.1±1.36</td>
<td>22.56±1.85</td>
</tr>
<tr>
<td></td>
<td>35</td>
<td>2.5±0.53</td>
<td>25.31±2.31</td>
<td>25.76±2.62</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>4.8±0.23**</td>
<td>20.13±1.82*</td>
<td>29.7±2.81*</td>
</tr>
</tbody>
</table>

*P<0.05 compared with control, **P<0.01 compared with control
Discussion

In vitro co-cultured model is an increasing commonly used method for neurotoxicity screens and mechanistic studies due to they focus on changes at the cellular and molecular level rather than on whole-animal morbidity [26]. Neurons/astrocytes in vitro co-cultured cells showed relatively good growth status and imitated the vivo environment. This co-cultured model provides the possibility of determination of cell general toxicity and neurotoxicity after treatment with a toxic compound [27]. In the present study, Neurons/astrocytes co-cultured cells were used for evaluating the cytotoxicity of BND. As shown in Fig. 3, BND at the dose of 150 μg/mL produced the most significant cytotoxicity than the other doses. Compared with neurons only, neurons/astrocytes co-cultured cells are more likely to represent the functional integrity of the brain and communicate neurons with other types of cells [28]. Therefore, these co-cultured cells are a suitable model to evaluate the neurotoxicity of BND.

The MTT reduction, LDH release, intracellular Ca2+ levels and mitochondrial membrane potential assays were used to determine the cytotoxicity profile of the BND. As shown in Fig. 2, viability assays only measure one aspect of cellular dysfunction but do not directly indicate the cells survivability [29]. The dose-dependent cytotoxic effect is further shown by using the lactate dehydrogenase (LDH) assay and intracellular Ca2+ levels. The mitochondrial membrane potential is the central parameter by controlling the accumulation of Ca2+ and ATP synthesis. In our results, BND (150 μg/mL) triggered a significant decrease in mitochondrial membrane potential (Fig. 6). BND induced an decrease in mitochondrial membrane potential, which can be seen as mitochondrial hyperpolarization. Similar results were reported by Poppe et al. [30], whose described in hippocampal neurons exposed to staurosporine, and then oxygen glucose deprivation [31]. In our study, Neurons and astrocytes co-culture cells were treated with 150 μg/mL BND, which resulted in co-cultured cells damaging and then caused apoptosis, mainly in the cell morphology changes, increased intracellular calcium and declined mitochondrial membrane potential. However, these results may indicate that the mechanism of BND neurotoxicity needs further clarification.

Tubulin is a marker protein of neurogenesis, mainly in the motor neurons and neurites, which determines the plasticity of the nerve structure during the growth and development of neurons [32]. In our study, the results showed that the fluorescence intensity of β-III-Tubulin and the protein expression were decreased induced by 200 μg/mL BND treatment for 6 h (Fig. 7). Cytoskeleton Protein Arc is a marker of neuronal activity, large amounts of expression products of Arc protein accumulate at activated synaptic sites which induced by extreme environmental factors [33, 34]. In our study, immunoblotting assay showed that the expression of Arc protein was decreased, which indicated that the activated synaptic site was reduced compared with the control group after treatment with 200 μg/mL BND for 6 h. SYN is located in the synaptic vesicle membrane, which involved in the circulation of synaptic vesicles, the release of neurotransmitters and the regeneration of synapses [35]. The number and density of SYN distribution indirectly reflect the number, density and distribution of synapses [36]. In our study, the results showed that the fluorescence intensity of SYN and the protein expression were decreased induced by 50 μg/mL BND treatment for 6 h. Thus, SYN could be used as an early sensitive protein to detect the neurotoxicity of betel nut decoction.

NO plays an important physiological function in the central nervous system, mainly regulating the formation of memory and pain [37, 38]. However, large amounts of NO could cause nerve damage and neurotoxicity. The mechanism of neurotoxicity action is that high
concentrations of NO could react with $O_2^-$ to form nitrous oxide anion (ONOO⁻) and degrade free radicals OH⁻ and NO² [39]. In addition, NO could mediate glutamate and NMDA receptor binding, to promote a large number of intracellular Ca²⁺ and then produce neurotoxicity, which indicated that NO also mediated glutamate neurotoxicity [40]. In our study, the results indicated that 50 μg/mL of BND promoted the excessive release of NO and glutamate, decreased the extend of Ach. Furthermore, these results may indicate that BND caused neurotoxicity on co-cultured cells.

In conclusion, the results showed that BND caused the general toxicity and changed the expression of synaptic plasticity proteins SYN, Arc, β-III-Tubulin in neurons/astrocytes co-cultured cells. Moreover, it also affected the levels of neurotransmitters NO, glutamate and acetylcholine. Interestingly, we screened out the sensitive protein SYN, which could rapidly detect the neurotoxicity of the betel nut decoction. The above results were used to confirm the feasibility of this assessment method, which were used to further clarify the mechanisms of the traditional Chinese medicine and provided new insight for developing the clinical application.

Conflict of Interest
The authors declare that there are no conflicts of interest.

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References


