Abstract. – Objectives: Efficacy and toxicity of the drug are mainly determined by physicochemical properties and pharmacological effects of its own. In addition, they are also affected by other factors, such as gender, age, genetic character, pathophysiological status, mood states and so on. The paper aims to study whether mood disorder alters drug metabolism process through the pharmacokinetic research on some clinically important anticancer drugs in depression model rats.

Materials and Methods: The depression model rats were built by exposing to chronic unpredictable mild stresses (CUMS) for 8 weeks. 36 female Sprague-Dawley (SD) rats were randomized into model group and control group. In each group, 18 rats were randomized into 2 subgroups: 5-fluorouracil (5-FU) subgroup and cyclophosphamide (CP) subgroup which were given a certain doses of 5-FU and CP. The blood samples were collected at different time points and plasma drug concentration were respectively assayed by high performance liquid chromatography (HPLC) for 5-FU and high performance liquid chromatography-tandem mass spectrometry (HPLC-MS/MS) for CP. Pharmacokinetic parameters were processed with DAS software.

Results: There were significant differences in the pharmacokinetic parameters of 5-FU and CP between in depression model rats group and in the normal control group (p < 0.05), except t1/2 α (p > 0.05) for CP pharmacokinetics in depression model rats group and in the normal control rats group, with the value of those was 0.07 and 0.09 h.

Conclusions: Depression mood disorder might alter drug metabolism process.

Key Words: Chronic unpredictable mild stress, Cyclophosphamide, 5-fluorouracil, Serotonin, Pharmacokinetics.

Introduction

Efficacy and toxicity of the drug are mainly determined by physicochemical properties and pharmacological effects of its own. In addition, they are also affected by other factors such as gender, age, genetic character, pathophysiological status, mood states and so on. Many psychological, social and behavioral factors tend to affect the efficacy of medication and the overall therapeutic outcome. Depressive disorder is the most worthy of attention in the psychosocial factors. This is because that the incidence of depression has been increasing year by year, with the accelerated pace of life and increased work pressure. In an epidemiological survey of more than 5,000 adults over the age of 18 in Beijing and Shanghai, the incidence of depression was 3.6% and 1.8% in 2008. As a matter of fact, it is a common phenomenon in clinical practice that depressive disorder affects the efficacy of drugs. Most cancer patients suffer from depression. A literature review on the effects of various stressors (physical, and environment, and psychological) on the pharmacokinetics of prescribed medications used by the Canadian Forces indicated that psychological stress may significantly alter pharmacokinetics. In this study, we conducted the pharmacokinetic research of some clinically important anticancer drugs in depression model rats to study whether mood disorder alters drug metabolism process. Cyclophosphamide (CP) and 5-fluorouracil (5-FU) were chosen as representatives in pharmacokinetic research because they are most commonly used in cancer treatment in developing China. CP is an alkylating agent, with its metabolites causing alkyl crosslink within and between DNA strands of dividing cells, causing them to apoptosis.
has been widely used in the chemotherapy of a variety of human carcinomas including head and neck, gastrointestinal tract and breast cancer, using various schedules.

Materials and Methods

Establishment of chronic unpredictable mild stress (CUMS)-induced depression model

Female Sprague-Dawley (SD) rats, weighing 180±20 g, 90-day old, were purchased from Laboratory Animal Center of Southern Medical University (Animal license: SCXK 2006-0015). All animal experiments were in compliance with the Use of Laboratory Animals of National Institutes and were approved by the Bioethics Committee of Southern Medical University. Rats were raised in 25°C experimental environment with normal diet and adapted feeding environment for a week before starting experiment. 36 rats were randomized into two groups: model group and control group.

Rats in CUMS-induced depression model group were single cage bred, exposed with random stressor for 8 weeks. The stressor included heat stress (45°C, 5 min), ice water swimming (4°C, 5 min), clip tail (1 min, 1 cm apart from the tail), fasting for 24h, water deprivation for 24h, placing in empty cylinder for 1h, rat cages tilting (45°, 24h), damp padding for 10h, and day/night inversion for 24h. One kind of stressor was randomly assigned daily; however, the same stressor can’t be applied continuously in order to avoid rat’s prediction. Each stressor was applied more than 6 times. In the experimental process, depression model rats were moved into another breeding room to accept the stressor (the lighting levels and temperature of the two room were basically the same), and returned after accepting the stressor. The control group rats were single cage bred without any stressor.

Open-Field Test

The apparatus for the open-field test was a square (76 cm × 76 cm) made of opaque materials which the open-field arena was partitioned into 25 equal-size squares and surrounded by high walls (42 cm). The test was preceded in a quiet room in the morning (8:00 ~ 12:00 am). Each rat was placed in the center of the field and its behavior recorded for 5 min. Four claws climbing square numbers and rearing times were monitored as an index of locomotor activity and exploratory behavior. The open-field was cleaned after each test.

Determination of 5-HT Plasma Level

About 1 ml of blood was collected from each group rat before and after depression model establishment. The anticoagulated blood sample was centrifuged at 3000 × g for 5 min to obtain plasma. The level of 5-HT in plasma was tested by enzyme-linked immunosorbent assay (ELISA) (Shanghai Y-J Biological Co., Shanghai, China).

Dosage Regimen and Plasma Sample Collection

After depression model establishment, 18 rats were randomized into 2 subgroups: 5-FU subgroup and CP subgroup which were given a certain dose of 5-FU (30 mg/kg) and CP (10 mg/kg) iv. The blood samples were collected at different time and plasma drug concentration were respectively assayed by high performance liquid chromatography (HPLC) (Agilent Technologies, Santa Clara, CA, USA) for 5-FU and high performance liquid chromatography-tandem mass spectrometry (HPLC-MS/MS) (Agilent Technologies, Santa Clara, CA, USA) for CP. The other 18 rats in control group were conducted the same experiment.

The time-point for blood sample collection were at: 0.05, 0.083, 0.167, 0.25, 0.5, 1, 2, 4, 6h for 5-FU and 0.083, 0.167, 0.25, 0.5, 1, 2, 4, 6, 8, 10, 12h for CP, respectively.

Determination of 5-FU and CP Concentration in Plasma

A modified HPLC assay was used for 5-FU determination. Briefly, a volume of 100 µl plasma sample was placed in a centrifuge tube. Plasma proteins were precipitated by addition of trichloroacetic acid (10%, 50 µl), and after centrifugation (15000 × g, 5 min) the supernatant (100 µl) was transferred into another centrifuge tube, to which 0.5 M ammonium acetate (pH8, 100 µl) and 1 ml ethyl acetate were added. The sample was vortex mixed vigorously for 1 min, followed by centrifugation at 8000 × g for 5 min. The organic layer was collected and evaporated to dryness at 60°C under a gentle stream of nitrogen. The residue was redissolved in 100 µl mobile phase, and a volume of 20 1 was injected into the
HPLC system. The HPLC system (Agilent, USA) was consisted of a pump, a auto-injector and a UV-detector set at 270 nm, C18 Hypersil ODS column (250 mm × 4.0 mm, 5 μm particle size) with a guard column (15 mm × 4.0 mm), the mobile phase [10 mM acetic acid: acetonitrile, 99:1 (v/v)], with flow-rate of 1.0 ml/min.

A modified HPLC-MS/MS assay was used for CP determination. Briefly, a volume of 50 l of the internal standard (50 µg/ml) and 0.85 ml acetonitrile were added to a centrifuge tube, in which 100 l plasma sample was placed. The sample was vortex-mixed vigorously for 1 min, followed by centrifugation at 15000 × g for 10 min. Then a volume of 1 µl of the supernatant was directly injected into the HPLC-MS/MS system. HPLC-MS/MS instrumentation and conditions for determination of CP were: the LC system was consisted of an Agilent 1200 series G1310A pump, a G1310A degasser, a G1329A auto-sampler and a G1316A adjustable column temperature box. The chromatography of the analytes was performed at 40°C using an Agilent ZORBAX SB-C18 (2.1 mm × 150 mm, 5 μm) column. The flow rate of mobile phase (methanol: 0.1 mol/l ammonium formate, 95: 5 (v/v)) was 0.4 ml/min. The optimized conditions of MS/MS with electrospray were as follows: ion spray source temperature at 350°C, nebulizer (NEB) gas at 10 L/min, ionspray voltage (IS) at 4000V . For CP dissociating potential (DP) was at 115V and collision energy (CE) was 20 units, for ifosfamide dissociating potential (DP) was at 115V and collision energy (CE) was 21 units. The mass spectrometer was interfaced to a computer workstation running Aria® OS software and Analyst software (Version B.01.03 Applied Biosystems, Agilent Technologies, Santa Clara, CA, USA) for data acquisition and processing. Data acquisition was performed via multiple-reaction monitoring (MRM). The ions representing the [M+H]^+ species for both the sample and internal standard were selected in MS1 and dissociated (collision-induced) with nitrogen gas to form specific product ions, which were subsequently monitored by MS2. The precursor-to-product ion transitions monitored for CP and ifosfamide were m/z 260.9 139.9 and m/z 260.9 153.8, respectively.

Level and Activity of Cytochrome P450
Control and CUMS-induced rats were fasted for 24h and killed by cervical dislocation before removal of the liver. The liver was excised, rinsed with ice-cold normal saline (0.9% NaCl, w/v), weighed for 1g and homogenized in a 0.05M Tris-0.25M sucrose buffer (pH 7.5). The homogenate was centrifuged at 10000 × g at 4°C for 30 min and the supernatant was further centrifuged at 105000 × g at 4°C for 60 min. The pellet was reconstituted with 0.05M Tris-0.25M sucrose buffer (pH 7.5). The cytochrome P450 level of the liver microsomes was determined by Carbon monoxide differential spectra, and the cytochrome P450 activity of the liver microsomes was determined by fluorescence quantitative assay with fluorescence quantitative kit, which was purchased from Shanghai Gen-Med (Pudong, Shanghai, China).

Statistical Analysis
Statistical analysis was performed with the SPSS 16.0 (SPSS Inc., Chicago, IL, USA) and DAS2.1 software (Drug and Statistics, Shanghai University of Traditional Chinese Medicine, Shanghai, China). Data were expressed as mean ± SD. p < 0.05 was considered statistically significant.

Results

Validation of CUMS-Induced Depression Model
The locomotion and exploratory behavior scores of rats in depression model group and control group before and after 8 wks' model establishment were monitored through open-field test. Within each group, the locomotion and exploratory scores of rats in depression model group before and after model establishment were from 78.50±6.54 to 15.10±4.46 (p < 0.01), from 15.80±2.66 to 4.70±1.70 (p < 0.01), respectively which were approximately 80% and 70% decrease. However, there were no significant change for the locomotion and exploratory scores in rats of control group before and at the end of 8 wks, which were from 76.70±5.98 to 71.8±8.88 (p > 0.05), from 14.80±2.66 to 15.40±5.23 (p > 0.05), respectively. Meanwhile between the groups, there was no significant difference for locomotion and exploratory scores before the model establishment but significant at the end of 8 wks (p < 0.01) (Figure 1).

5-HT plasma level was measured by ELISA. The levels of rats in depression model group before and after 8 wks' model establishment were from
6.68±1.58 to 1.95±0.62 ng/ml (p < 0.01), and in control group were from 5.92±1.45 to 5.57±1.24 ng/ml (p > 0.05). There was a significant decrease of 5-HT in depression model group before and at the 8 wks end, but no significant difference in control group. Meanwhile, significant difference occurred for 5-HT levels between the model group and the control group at the 8 wks end (1.95±0.62 vs 5.57±1.24 ng/ml) (p < 0.01) (Figure 2).

**Analysis Methods for 5-FU and CP Plasma Determination**

5-FU plasma concentration was determined by HPLC. The assay validation was summarized in

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**Figure 1.** Results of open-field test (n =18 for each group). **A,** The locomotion score of rats in depression model group and in control group before and after 8 weeks’ model establishment. **B,** The exploratory score of rats in depression model group and in control group before and after 8 weeks’ model establishment.

**Figure 2.** 5-HT plasma levels in rats of depression group and in control group (n=18 for each group).
Pharmacokinetics of 5-fluorouracil and cyclophosphamide in depression rats

Table I. Recovery, precision and accuracy for the determination of 5-FU in rat’s plasma by HPLC.

<table>
<thead>
<tr>
<th>Nominal concentration (µg/ml)</th>
<th>Recovery (%)</th>
<th>Measured concentration (µg/ml)</th>
<th>Accuracy (%)</th>
<th>Intra-day precision (RSD %)</th>
<th>Inter-day precision (RSD %)</th>
</tr>
</thead>
<tbody>
<tr>
<td>25</td>
<td>74.37 ± 0.49</td>
<td>26.37 ± 2.93</td>
<td>105.46</td>
<td>0.67</td>
<td>0.48</td>
</tr>
<tr>
<td>10</td>
<td>77.21 ± 0.62</td>
<td>9.73 ± 1.10</td>
<td>97.32</td>
<td>0.65</td>
<td>1.05</td>
</tr>
<tr>
<td>5</td>
<td>79.96 ± 0.88</td>
<td>4.91 ± 0.85</td>
<td>98.13</td>
<td>0.54</td>
<td>3.27</td>
</tr>
</tbody>
</table>

Data are based on analysis of six replicates (n = 6) on three separate days.

Figure 3 and Table I. CP plasma concentration was determined by HPLC-MS/MS. The assay validation for CP was summarized in Figure 4, Figure 5 and Table II with a stable recovery and good accuracy.

**Pharmacokinetics of 5-FU and CP**

The plasma concentration-time data of 5-FU and CP were calculated as two-compartment model with DAS 2.1 software (Drug and Statistics, China) (Figure 6, Table III).

The plasma concentration-time curves after a single iv injection of 5-FU in depression model group and in control group were presented in Figure 6A. There were significant differences in all pharmacokinetic parameters of 5-FU between two groups (p < 0.05). The maximum concentration (C_max) and the area under the curve (AUC) of 5-FU in control group and in depression group were significantly decreased from 46.74 µg/ml and 26.85 µg/ml·h to 38.34 µg/ml and 20.36 g/ml·h, respectively, which were approximately 18% and 24% decrease. The total plasma clearance (CL) was 1.10 and 1.52 L/h in control group and depression group respectively, which was approximately a 27% increase. Meanwhile, the absorption half-life (t_1/2) and the elimination half-life (t_1/2) were shortened from 0.27 and 4.14 h to 0.18 and 1.10 h, and the mean residence time (MRT) was shortened by 70%. The plasma concentration-time curves after a single iv injection of CP in depression model group and in control group were presented in Figure 6B. Similarly, there were significant differences in mostly pharmacokinetic parameters of CP between two groups (p < 0.05), except t_1/2 (p > 0.05). The t_1/2 of CP in control group and depression group was 0.09 and 0.07 h, but t_1/2 was 1.60 and 0.98 h.

![Figure 3. HPLC chromatograms of 5-FU: A, Blank plasma. B, A calibration standard containing 10 µg/ml 5-FU. C, A rat plasma sample 6h after a single iv dose of 5-FU injection with 30 mg/kg.](image)
which was shortened by 38%. The other changes were similar as those of 5-FU.

**Level and Activity of Cytochrome P450**

The CYP450 level was the total amount of enzymes in 1mg protein of the liver microsome. The activity of 7-ethoxycoumarin-O-deacthylase was measured and delegated as CYP450 activity with 7-ethoxycoumarin as the reaction substrate. The average level of CYP450 in the liver microsomes of control and CUMS-induced rats were 0.043±0.013 and 0.108±0.019 mol/mg, and the average activity were 0.393±0.119 and 0.652±0.132 mol/mg/min. There were significant differences for CYP450 level and activity in depression and control group (p < 0.05) (Figure 7).
Pharmacokinetics of 5-fluorouracil and cyclophosphamide in depression rats

Table II. Recovery, matrix effect, precision and accuracy for the determination of CP in rat’s plasma by HPLC/MS/MS.

<table>
<thead>
<tr>
<th>Nominal concentration [µg/ml]</th>
<th>Recovery (%)</th>
<th>Measured concentration [µg/ml]</th>
<th>Accuracy (%)</th>
<th>Matrix effect (%)</th>
<th>Intra-day precision [RSD%]</th>
<th>Inter-day precision [RSD%]</th>
</tr>
</thead>
<tbody>
<tr>
<td>20</td>
<td>65.89 ± 1.05</td>
<td>19.859 ± 0.341</td>
<td>99.30</td>
<td>92.48</td>
<td>1.793</td>
<td>1.715</td>
</tr>
<tr>
<td>5</td>
<td>60.9 ± 0.81</td>
<td>5.009 ± 0.040</td>
<td>100.19</td>
<td>93.71</td>
<td>0.817</td>
<td>0.803</td>
</tr>
<tr>
<td>0.1</td>
<td>67.51 ± 1.21</td>
<td>0.095 ± 0.003</td>
<td>94.69</td>
<td>87.98</td>
<td>3.043</td>
<td>3.608</td>
</tr>
</tbody>
</table>

Data are based on analysis of six replicates (n=6) on three separate days.

Table III. The main pharmacokinetic parameters of 5-FU and CP.

* p < 0.05 compared with control group in 5-FU.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>5-FU</th>
<th>CP</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Depression group</td>
<td>Control group</td>
</tr>
<tr>
<td>Cmax (µg/ml)</td>
<td>38.34 ± 3.13*</td>
<td>46.74 ± 3.46</td>
</tr>
<tr>
<td>t1/2α (h)</td>
<td>0.18 ± 0.09*</td>
<td>0.27 ± 0.05</td>
</tr>
<tr>
<td>t1/2β (h)</td>
<td>1.10 ± 0.43*</td>
<td>4.14 ± 3.48</td>
</tr>
<tr>
<td>AUC0→∞ (µg/ml·h)</td>
<td>20.36 ± 1.82*</td>
<td>26.85 ± 2.68</td>
</tr>
<tr>
<td>MRT (h)</td>
<td>0.80 ± 0.22*</td>
<td>2.68 ± 2.26</td>
</tr>
<tr>
<td>CL (L/h)</td>
<td>1.45 ± 0.09*</td>
<td>1.10 ± 0.09</td>
</tr>
</tbody>
</table>

Figure 6. Plasma concentration-time curves of 5-FU and CP of rats between depression group (2 subgroups, each subgroup n = 9) and control group (2 subgroups, each subgroup n = 9) receiving a single iv dose of 5-FU (30 mg/kg) and CP (10 mg/kg).
Discussion

An ideal animal model should be able to simulate the development process of diseases and the change of pathophysiology. Chronic unpredictable mild stress (CUMS), a well-validated animal model, has been used widely for studying clinical depression as well as evaluating antidepressant effects for many years\(^\text{10,19}\). Traditional animal depression models, such as limited behavior model\(^\text{20}\) and forced-swimming model\(^\text{21}\), are facilitated to make animals tolerate due to their predictability and repeated stimulation of the same intensity. CUMS model, which depressive state is similar with change of psychomotor and loss of interest or pleasure in the clinical diagnosis of depression, has avoided these defects and widely approved by scientists. Rats subjected to CUMS showed a decrease in consumption of sucrose solution, immune system and the hypothalamus-pituitary-adrenal (HPA) axis abnormality and degradation of sexual behavior\(^\text{22}\), and changes in plasma 5-HT and NE levels\(^\text{23,24}\). CUMS-induced depression model was also verified by open-field test and determination of 5-HT plasma levels in this experiment. The results showed that there were significant differences before and after depression model establishment, but there was no significant difference in control group (Figure 1). And the 5-HT plasma levels showed that there was a decrease in of the depression model group before and after model establishment (6.68±1.58 and 1.95±0.62 ng/ml, \(p < 0.05\)), and there was no change in control group (5.92±1.45 and 5.57±1.24 ng/ml, \(p > 0.05\)) (Figure 2). The decrease of 5-HT levels was corresponded with the material basis of depression neurobiology which the small molecule neurotransmitter 5-HT level of brain and plasma in depression patients was lower than in normal patients. Female rats was adopted in this animal model, since female rats are more vulnerable in an animal model of depression with less brain regional 5-HT concentration particularly in the frontal cortex than males\(^\text{20}\).

HPLC was applied for determination of 5-FU plasma concentration. The recovery was stable, and the accuracy was good. Intra- and inter-day precision (RSD %) were less than 3.5. CP plasma concentration was determined by HPLC-MS/MS. Its intra- and inter-day precision (RSD %) were less than 4.0, and the accuracy was almost close to 100% similar with 5-FU. CP exhibited the slight matrix suppression effect, with 87-94% responses in the matrix containing samples. None of the values were considered statistically significant and overall the effects seen were <15% from 100% target (no matrix effect). Although the recovery was low (less than 70%), CP plasma pretreatment using a simple acetonitrile precipitation of plasma proteins was quick and simple, and could meet minimum level of detection in plasma samples with the limit of quantitation of 0.05 µg/ml. The results presented in table 1 and 2 proved that two methods were suitable for the determination for 5-FU and CP.

There were significant decrease in all pharmacokinetic parameters of 5-FU between two groups (\(p < 0.05\)). Similarly, there were significant differences in most pharmacokinetic para-
meters of CP between two groups (p < 0.05), except t1/2 (p > 0.05). The results above suggested that pharmacokinetics of anticancer drugs 5-FU and CP were altered in depression model rats. We speculated that this alter may be mediated by drug-metabolizing enzymes.

A study indicated that mRNA expression of nerve growth factor (NGF) was significantly lower in the hippocampus, and phospholipase A2 was higher in the hypothalamus of depression model rats25. The withdrawal of rats in the neonatal period from their mothers make the mothers more susceptible to the development of neurochemical alterations, with decreased activities of hippocampal Na+, K+-ATPase and antioxidant enzymes, which could be related to depressive features26,27. Indeed, depressive disorder could alter the expression and activity of some enzymes in tissues of depression model rats, but whether it has an impact on drug-metabolizing enzymes is not clear. Dihydropyrimidine dehydrogenase (DPD) is the rate-limiting enzyme of 5-FU metabolism in the liver, more than 80% of 5-FU is transformed to dihydro-fluorouracil by DPD. The efficacy and toxicity of 5-FU are closely related to DPD, patients with DPD deficiency may occur lethal toxicity28. Many factors would affect the level of DPD. There were some relevance between individual differences of DPD and depression in the previous study29. CP is metabolized to a number of metabolites, particularly 4-hydroxy-cyclophosphamide, through P450-mediated pathways involving CYP2B6, CYP2C9, and CYP3A429. Study of CYP2D6 gene polymorphism found that there was CYP2D6 gene polymorphism in 100 patients with depression. It was prone to adverse reactions occurring in patients who are the poor metabolizer-type of CYP2D630. Studies showed that the genotype and phenotype of CYP2D6 had individual differences which are related to personality traits, and the hydroxylation capacity of CYP2D6 is associated with the level of anxiety and the degree of socialization31,32. Our experiment also showed the total content and activity of cytochrome P450 enzyme of liver of rats in depression model group were higher than those of control group (Figure 7A and B).

Conclusions

Based on these findings, we may concluded that in depression model rats, the pharmacokinetics of 5-FU and CP were affected compared with those in control group.


