Combined toxicity and toxicity persistence of antidepressants citalopram and mirtazapine to zooplankton *Daphnia magna*

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Abstract
Citalopram (CTP) and mirtazapine (MTP) are two typical psychoactive drugs used for the depression treatment. As emerging pollutants, CTP and MTP have raised concern because of their harmful effect on aquatic organisms. Therefore, the ecotoxicological risk of these two pollutants to aquatic organisms should be given more attention. In this study, the effects of CTP and MTP on the feeding rate, heartbeat, nutritional enzymes, and their related gene expression of *D. magna* were investigated under single and binary mixture pollutant exposure. Subsequently, the recovery of exposed *D. magna* was studied to assess the toxic persistence of those pollutants. After 24-h exposure, the ingestion rate decreased by 34.2% and 21.5%, in the group of 1.45 mg/L CTP (C-H) and binary mixture with high concentration (Mix-H), respectively. After 24-h recovery, the feeding rate of *D. magna* was stimulated by a compensatory response. Over the exposure period, the heartbeat rate of *D. magna* increased significantly in the groups of CTP, MTP, and their binary mixture with low concentration (Mix-L), and then, their heartbeat rate was recovered during the recovery period. The activity of α-amylase (AMS) and trypsin were significantly changed in most of the exposed daphnia, both during the exposure and recovery period. CTP/MTP exposure stimulated the expression of the AMS gene. MTP and Mix-H exposure inhibited the expression of the trypsin gene and the other groups stimulated its expression. After 24-h recovery, the stimulating or inhibitory effects were alleviated. There were different responses between gene expression and enzyme activity. In conclusion, our results highlighted the toxic effects at high concentrations of single and mixed pollution of CTP and MTP on the feeding rate, heartbeat, AMS and trypsin enzyme activity, and expression of related genes of *D. magna* to assess the environment risk of them.

Keywords Antidepressants · Zooplankton · Mixture toxicity · Exposure and recovery · Aquatic risk assessment

Introduction
The use of antidepressants has been increasing considerably since 2000, and they are inevitably released into the environments where they may cause serious environmental pollution (Sehonova et al. 2018). Given that existing wastewater treatment technology insufficiently removes psychotropic molecules (Removal rate: 25–90%) (Ahmed et al. 2021; Gros et al. 2020; Rathi et al. 2021), psychotropics may be detected in locally very high concentrations in the vicinity of wastewater treatment plants receiving effluents of pharmaceutical enterprises or hospitals (Bueno et al. 2012). Meanwhile, in the depression treatment, CTP and MTP are often used in combination (Zhuang and Hospital 2019) and could be found in the same environmental compartments, such as sediments, surface water, and groundwater (Proctor et al. 2021; Silva et al. 2015). Golovko et al. (2020) reported that the average concentrations of CTP and MTP in Lake Ekoln...
in Sweden were 0.59 and 1.1 ng/L, respectively. Therefore, the study of the risk of psychotropic drugs to aquatic organisms is very necessary.

The aquatic environmental risks of CTP and MTP have increasingly received attention. For example, Bachour et al. (2020) observed a significant decrease in swimming activity of zebrafish exposed to CTP with a concentration of 373 μg/L. The inhibition rate of acetyl cholinesterase (AChE) activity was 73% of D. magna exposed to CTP with a concentration of 1 g/L (Yang et al. 2017). CTP is a selective 5-hydroxytryptamine (5-HT) re-uptake inhibitor that blocks selective serotonin re-uptake transporters (SERTs), while MTP may enhance noradrenergic and serotonergic neurotransmission (Salomone et al. 2011). However, knowledge about the potential impacts of CTP and MTP pollution on aquatic organisms is still limited.

As the model organism, D. magna is sensitive to pollutants in the aquatic environment. Toxicological studies suggested that the feeding rate and heartbeat of D. magna are useful to assess the sub-lethal effects of pollutants, and heartbeat might be considered a promising sensor of effects induced by stressful factors in the aquatic environment (Bownik 2017; Liang et al. 2017). Thus, to deepen the understanding of potential toxicity of CTP and MTP, their individual and combined toxicity on the feeding rate, heart rate, nutritional enzymes activity, and related gene expression of D. magna were thoroughly studied in this study during exposure and recovery periods. There were three research objectives in this study: (1) the effects of CTP and MTP on the feeding rate and heartbeat of D. magna were studied under single and mixed pollutants exposure, (2) the recovery of D. magna after exposure was studied to evaluate the toxicity persistence of CTP and MTP, and (3) the potential toxic mechanism of CTP and MTP was studied by monitoring the digestive enzymes activity and expression of related genes of D. magna. The findings will be useful to evaluate the potential risks of CTP and MTP in aquatic ecosystems.

Materials and methods

Chemicals

Citalopram (CTP; CAS: 59729–33-8) was purchased from Sichuan Kelun Pharmaceutical Co., Ltd (China), and Mirtazapine (MTP; CAS: 85650–52-8) was purchased from N.V. Organon (Netherlands). High-performance liquid chromatography (HPLC, SOD-M 20A, Shimadzu, Japan) was applied to detect the concentration of CTP and MTP in solution, which is shown in the Supporting Information (SI-S1). The physochemical properties of the target compounds are listed in Table S1. The assay kits for measuring the digestive enzyme activity of α-Amylase (AMS) and trypsin were purchased from Nanjing Jiancheng Bioengineering Institute (China). Trizol reagent was purchased from New Cell & Molecular Biotech Co., Ltd (Suzhou, China). Reverse transcriptase was purchased from Vazyme Biotech Co., Ltd (Nanjing, China).

Culture program of Chlorella pyrenoidosa and D. magna

Chlorella pyrenoidosa (C. pyrenoidosa) was obtained from the Institute of Aquatic Biology of the Chinese Academy of Sciences (Wuhan, China), cultured in BG-11, and maintained at a temperature of 25.0 ± 1.0 °C with a light–dark cycle of 16 h: 8 h (4000 Lx). As an inorganic salt, BG-11 (Table S3-4) was the mineral salt medium provided to the microalgae and there was no external carbon source provided to the microalgae. Microalgae grew autotrophically under light conditions. Before the experiment began, C. pyrenoidosa were harvested by centrifuging at 4000 rpm for 10 min. D. magna was cultured in water with medium hardness (pH: 6–9; CaCO₃: 140–250 mg/L) and maintained at the temperature of 20.0 ± 1.0 °C with a light–dark cycle of 16 h: 8 h (4000 Lx). C. pyrenoidosa was used as the food source for D. magna.

Feeding assay

The feeding assay was conducted with 7-day-old D. magna under exposure and recovery for 24 h, respectively. Acute toxicity tests are described in SI-S2. Then, the 1/80 EC50 and 1/20 EC50 of CTP (0.36 and 1.45 mg/L) and MTP (0.25 and 1.03 mg/L) were used for feeding assay and following toxicity assay. In order to avoid the death of D. magna in the groups of the mixture due to excessive concentration of pollutants, the compound concentrations in the mixture groups were determined following the formula: C_mix = (i EC50(CTP) + 1/7 EC50(MTP)) / 2, where i is 20 and 80. The summary of the experimental process is listed in Table 1. Each experimental condition comprised five biological replicates (n = 5) consisting of five 100 mL beakers containing five D. magna each in 60 mL of exposure solution. The initial algal density was 1 × 10⁶ cells/mL to feed D. magna after centrifugal cleaning. To avoid the growth of microalgae, all groups were conducted in the dark condition. The solution before and after 24-h exposure and 24-h recovery in each beaker were shaken fiercely to resuspend the C. pyrenoidosa cells. The algal density was detected by spectrophotometry at 680 nm for calculating the feeding rate (Li et al. 2020). The heartbeat of D. magna was measured by sampling 24 h after exposure and recovery. One D. magna was randomly taken from each beaker (n = 5), and the heartbeat of D. magna was recorded by video under Nikon SMZ1000 stereomicroscope for 2 min and counted later manually.
Measurements of enzyme activity assay

Under the same conditions as the feeding assays, 245 D. magna were cultured at the same time for each group. 200 of them were used for the enzyme activity assessment ($n = 3$), and 45 of them were used for gene expression ($n = 3$). After 24 h of exposure and 24 h of recovery, D. magna was collected and mixed with 0.9% sodium chloride solution in the ratio of 1:9 and then homogenized thoroughly in an ice-water bath (0 °C). Then, the homogenates were centrifuged at 8000 rpm at 4 °C for 10 min, and the supernatant was used to measure the total protein (TP) (mgprot./mL) concentration and the specific activities of AMS (U/mgprot) and trypsin (U/mgprot) according to the instructions of respective assay kits (the units (U) were calculated in µmol/min). In order to maintain activity of enzyme, the supernatant was transferred to $-20 \degree$C immediately after centrifugation. Details for the enzyme activity assays are provided in S3-S5.

Extraction of total RNA and reverse expression

D. magna were performed as experimental animals to detect gene expression under different stress conditions; three replicates were set for each group. D. magna were involved in each replicate for detecting the of the expression of AMS and trypsin. After 24-h exposure and 24-h recovery, D. magna was removed from the culture medium and washed twice with double distilled water, and then transferred to a homogenizer containing Trizol reagent to extract total RNA in the ice-water bath. Nanodrop 2000 was performed to detect the concentration of total RNA (Thermo Fisher Scientific, USA). To obtain cDNA, 2 µg of total RNA was used for reverse expression with HiScript II Reverse Transcriptase (Vazyme, Nanjing, China) following the manufacturer instructions.

The gene expression of D. magna exposed to CTP and MTP was analyzed by real-time quantitative polymerase chain reaction (qPCR). As an internal reference gene, β-actin was used, which was stably expressed throughout the cycles (Lee et al. 2021). The specific primers for the two target genes (trypsin and AMS) were designed at the National Center for Biotechnology Information according to known sequences (D. magna). The primer specificity information is listed in Table S2 (Supporting Information). The expression of these genes was conducted by SYBR Green PCR Master Mix (Vazyme, Nanjing, China) according to the instruction. Cycling parameters were set as follows: one cycle of 95 °C for 30 s, followed by 40 cycles of 95 °C for 10 s and 60 °C for 30 s. Then, the formation of specific products was determined by melting curve analysis. The target gene expression values were calculated by the $2^{-\Delta\Delta C\text{T}}$ method (Livak and Schmittgen 2001). Three independent biological replicates were performed in these experiments.

Data analysis

Statistical analyses were performed by SPSS statistics 26.0 software (IBM, Armonk, New York, USA). The difference between the experimental group and the control group were performed by one-way ANOVA. Shapiro—Wilk test and Levene $F$ were used to test for normality and homoscedasticity, respectively. LSD, Tukey HSD, and Sidak were used as the post-hoc test employed. The correlation between activity of digestive enzymes and their corresponding genes as analyzed by bivariate correlation analysis. The experimental data are present as mean ± standard deviation. *$p<0.05$, **$p<0.01$, and ***$p<0.001$ were set as the significance levels for all calculations. Data visualization was performed using Origin 8.0 software (OriginLab Corporation, Northampton, Massachusetts, USA).

Results and discussion

Feeding inhibition of D. magna caused by psychoactive drugs

The results of the feeding inhibition experiments of D. magna are shown in Fig. 1 A and B, respectively. After 24 h of exposure, the ingestion rate decreased by 34.2%

| Group | Nominal concentration (mg/L) | Actual concentration (mg/L) | Starvation treatment (h) | Exposure time (h) | Starvation treatment (h) | Restores time (h) |
|-------|-------------------------------|-----------------------------|--------------------------|------------------|--------------------------|------------------|
|       | Citalopram (mg/L) | Mirtazapine (mg/L) | Citalopram (mg/L) | Mirtazapine (mg/L) | Citalopram (mg/L) | Mirtazapine (mg/L) | Citalopram (mg/L) | Mirtazapine (mg/L) | Citalopram (mg/L) | Mirtazapine (mg/L) | Citalopram (mg/L) | Mirtazapine (mg/L) |
| Control | 0 | 0 | 0 | 24 | 24 | 24 | 24 |
| Citalopram C-L | 0.36 | 0 | 0.34 | 0 | 24 | 24 | 24 | 24 |
| Citalopram C-H | 1.45 | 0 | 1.29 | 0 | 24 | 24 | 24 | 24 |
| Mirtazapine M-L | 0 | 0.25 | 0 | 0.17 | 24 | 24 | 24 | 24 |
| Mirtazapine M-H | 0 | 1.03 | 0 | 1.02 | 24 | 24 | 24 | 24 |
| Mix (Citalopram and Mirtazapine) Mix-L | 0.18 | 0.125 | 0.18 | 0.08 | 24 | 24 | 24 | 24 |
| Mix (Citalopram and Mirtazapine) Mix-H | 0.72 | 0.52 | 0.54 | 0.44 | 24 | 24 | 24 | 24 |
and 21.5%, in the groups of C-H and Mix-H, which were 

$$(1.56 \pm 0.06) \times 10^5 \text{ cell/ind./h} \quad \text{and} \quad (1.86 \pm 0.12) \times 10^5 \text{ cell/ind./h},$$

respectively. There was no significant decrease in feeding rate in the other exposure groups ($p > 0.05$) (Table S5). Food consumption of *D. magna* decreased when exposed to high concentrations of CTP. Compared with the control, there were no obvious differences in feeding rate when exposed to MTP for 24 h, which showed that *D. magna* was more susceptible to CTP than to MTP. In the binary mixture toxicity experiment, the feeding rate decreased significantly in the group of Mix-H, but it was higher than the group of C-H. It remains to be further verified whether the two compounds act in an additive, synergistic or antagonistic manner. *D. magna* needs the coordination of nervous system to complete food filtering. The presence of antidepressants (fluoxetine) in aquatic environment increased the concentration of serotonin in *D. magna* (Rivetti et al. 2019). The increase of serotonin may decrease the swimming speed of *D. magna* (Heyland et al. 2020). The addition of selective serotonin reuptake inhibitor (SSRI) might disturb the movement behavior of *D. magna* (Campos et al. 2012). In this study, when exposed to 1.45 mg/L of CTP, the ingestion rate of exposed daphnia was significantly reduced. It might be related to the loss of coordination caused by the stimulation of neuroactive substances (CTP) to the motion of the *D. magna*. This was similar to results from study of Alkimin et al. (2020), that the neuroactive substance (chlorpromazine) caused the lack of coordination and inhibition of feeding rate of *D. magna*.

After 24 h of recovery, the ingestion rate increased by 54.8% and 63.9%, in the group of M-L and C-H, respectively. This might be due to the mechanism of overcompensation (Liu et al. 2019; Lv et al. 2018). The feeding inhibition reversed into hormesis from during the recovery period. Our previous study (Liu et al. 2019) observed the same phenomenon when the *D. magna* were exposed to Bisphenol analogues (BPs). This overcompensation after initial disruption in homeostasis helps organisms to restore their nutritional status and enhance self-protection mechanisms (Liu et al. 2018).

**Heart rate**

Figures 2A and B show the effects of CTP and MTP on heartbeats of *D. magna* after 24 h of exposure and 24 h of recovery, respectively. Generally, in the 24-h exposure period, the heartbeats of *D. magna* significantly increased in the exposed groups ($p < 0.01$) except for the Mix-H group. For MTP, compared with low-level exposure, high-level exposure increased the *D. magna* heartbeats. On the contrary, the heartbeats of *D. magna* decreased with the high concentration of CTP and mixed drugs. Those may be related to the higher sensitivity of *D. magna* to CTP. However, those results were consistent with the findings of Liang et al. (2017), in which heartbeat was significantly stimulated by low-concentration perfluorooctane sulfonate (PFOS) and inhibited by high-concentration PFOS (the change from a low-dose stimulation to a high-dose inhibition). The heartbeats in the mixture groups were lower than in the single treatment groups, which suggested that the mixed toxicity of the compounds revealed a synergistic toxicity.

After recovery for 24 h, there were no obvious differences in heartbeat between control group and exposed groups. It was interesting to note that the heart rate decreased slightly with the increasing concentration of MTP, CTP, and their mixture after 24 h of recovery. The heart rate of *D. magna* was disturbed after exposure to high concentrations of pollutants, but whether the disturbance decreased with time needs further studies.
Enzyme activity

The digestive tract is one of the primary sites of toxicant uptake in *D. magna*. Trypsin and α-amylase are synthesized in the digestive F cell of crustaceans located in the alimentary tract (Lehnert and Johnson 2002). The effects of CTP and MTP on the digestive enzymes (AMS and trypsin) of *D. magna* are shown in Fig. 3. AMS is involved in fibrin and starch digestion in *D. magna*, while trypsin is implicated in protein digestion (Huang et al. 2017; Perera et al. 2012). Both of them are the typical digestive enzymes (Houde et al. 2013). Figures 3A and B show the AMS activity after 24 h of exposure and 24 h of recovery, respectively. Compared with the control, the AMS activities of exposed daphnia increased significantly in Group MTP and Mix-H after 24 h of exposure (*p* < 0.01), the increase in activities of AMS could be an adaptation to maximize utilization of the limited amount of food ingested (Seyoum et al. 2021). Those in Group CTP decreased significantly (*p* < 0.01), CTP is a member of the serotonin reuptake inhibitor, which inhibits the reuptake of serotonin in the synapses and increases the amount of serotonin in the intrasynaptic space (Campos et al. 2012). However, serotonin can control the secretion of crustacean hyperglycemic hormone, which controls the mobilization of glucose (Sathyanandam et al. 2008; Santos et al. 2001). Digestive enzymes are involved in carbohydrate metabolism. Whether the change of digestive enzyme is related to the increase of serotonin needs further research. The previous study also found that the AMS activity decreased significantly in a concentration dependent manner after exposure to azithromycin, and the concentration–response relationship was present (Li et al. 2020). There was no significant difference in the AMS activities between Mix-L and control group (*p* > 0.05), which indicated that the different effects on the activity of AMS might be related to different pollutants like CTP and MTP. After recovery for 24 h, the AMS activities of exposed daphnia increased significantly in Group M-L and M-H, while decreased significantly in Group C-H, Mix-L, and Mix-H (*p* < 0.05) (Table S6). There was no significant change in Group C-L (*p* > 0.05). It indicated that the digestive system of *D. magna* was injured by contaminants, as the AMS activities were not well recovered.

Figures 3C and D show that the activities of trypsin after 24-h exposure and 24-h recovery, respectively. After 24-h exposure, the trypsin activities of exposed daphnia increased significantly in Group M-L and Mix-L (*p* < 0.05), which suggested that tissue protein may undergo proteolysis. Protein was reported to serve as an alternate source of energy under extreme stress conditions (Suryavanshi et al. 2009). Stimulating the activity of trypsin might be an adaptive response of *D. magna* to extreme conditions (Dai et al. 2014). There was no significant difference in trypsin activities between Group M-H, CTP, and Mix-H and control group (*p* > 0.05). The activities of trypsin were negatively correlated with the concentrations in the MTP and mixed groups. However, the activities of trypsin were positively correlated with the concentration in CTP, which might be related to the assimilation processes (Lv et al. 2017). After 24-h recovery, the activity of trypsin was decreased significantly in Group C-L and increased significantly in Group Mix-H (*p* < 0.05), and there was no significant change in the rest of the exposed groups (*p* > 0.05).

Gene expression of *D. magna*

It was found that pollutants could affect the feeding and nutrition-related enzyme activities of *D. magna*. The nutritional enzyme genes were used as exploratory data to further analyze the possible cause of feeding rate and heartbeat of *D. magna* caused by pollutants. Houde et al. (2013) used transcriptome tools to identify five significant differentials in genomic expression related to metabolic function, including...
amylase and trypsin. Therefore, gene expression involved in digestive enzyme synthesis was selected for evaluation based on the changes of amylase and trypsin to further explain the possible mode of action of pollutants to *D. magna*. Figure 4A shows the significantly up-regulated AMS gene expression in all exposed groups (*p* < 0.001). Compared with MTP group, the upregulation of AMS in the mixed group decreased significantly. This indicated that CTP, MTP, and their mixture stimulated the expression of AMS genes during exposure period. SSRI affected the activity of heart and lung ventilation in crustaceans, caused tachycardia in crustaceans, and increased ventilation rate (Robert et al. 2019). It has been reported that SSRIs increased the oxygen consumption rate and aerobic catabolism of *D. magna* and decreased the carbohydrate level of adult *D. magna* (Campos et al. 2012). The increase of aerobic catabolism was accompanied by the increase of digestion, which was manifested by the increase of digestive enzyme activity or the increase of digestive enzyme quantity (Li et al. 2014). The upregulation of digestive genes might be regarded as a compensation mechanism for reduced carbohydrate reserves (Soetaert et al. 2007). This was consistent with the results of the Houde et al. (2013), in which the expression of AMS genes was stimulated in the groups added with hexachlorocyclopentadiene (HCCPD). However, this phenomenon was contrary to the results of Zhao et al. (2019), in which the expression levels of AMS gene were inhibited in the groups with BDE-47, BDE-99, and their mixture. Those studies indicated that the different effects on the expression of AMS gene may be related to different pollutants. After 24 h of recovery, the negative effects of CTP/MTP on *D. magna* were alleviated, but it requires further studies to validate whether those negative effects could be completely removed.

In Fig. 3A, the activities of AMS increased significantly in MTP exposed daphnia after 24 h of exposure, and the gene expression of AMS was upregulated significantly (Fig. 4A). However, there was no significant correlation between gene expression and enzyme activity of AMS (*R*² = 0.1753, *p* = 0.266 > 0.05; Fig. 5A). The activity of AMS of *D. magna* was decreased significantly in Group Mix-L and Mix-H after 24-h recovery, and the gene expression levels of AMS in those groups were decreased significantly (*p* = 0–0.01 < 0.01) (Fig. 4B). Correlation analysis was conducted with enzyme activity and gene expression, and the results showed no significant correlation between both (*R*² = 0.0002, *p* = 0.787 > 0.05; Fig. 5B). No significant correlation was found between the gene expression level and the enzyme activity in trypsin. Houde et al. (2013) found that...
Fig. 4 Relative gene expression of α-amylase and trypsin of D. magna in different treatment groups after exposure period and recovery period. A α-amylase after exposure period, B α-amylase after recovery period, C trypsin after exposure period, and D trypsin after recovery period. Gene transcription below 1 represented down-regulation and above 1 represented up-regulation. All data represented means ± SD (The grouping scheme is shown in Table 1. All data represented means ± SD, *: weakly significant (p < 0.05); **: significant (p < 0.01); ***: highly significant (p < 0.001), n = 3).

Fig. 5 Relationship between gene expression and enzyme activity in exposure daphnia. A 24 h-AMS, B 48 h-AMS, C 24 h-trypsin and D 48 h-trypsin. Gene expression and enzyme activity are listed in X-axis and Y-axis, respectively. Regression lines and $R^2$ value are shown in each picture for evaluating the relevance of gene expression and enzyme activity. $p > 0.05$ means no significant relationship between gene expression and enzyme activity.
there was no significant correlation between enzyme activity and gene expression, which was contrary to the results of Schwarzenberger and Fink (2018). Gene expression is the basis of organism’s response to external pressure. However, the relationship between gene expression and related enzyme activity is complex. The lack of correlation between enzyme activity and gene expression might be related to the time required for gene expression and many different factors involved in gene expression process, which increased the uncertainty of the expression process (Ashouri and Farshbaf Pourabad 2021). The inhibition of enzyme activity may stimulate enzyme gene expression and the increase of enzyme quantity, which was due to the compensation of organism.

**Conclusion**

In this study, the toxicity of CTP, MTP, and their mixtures on the feeding rate and bodily functions of *D. magna* was investigated. The aquatic toxicity of the two psychotrophic drugs from two stages of exposure and recovery was evaluated. After exposure, the feeding rate of *D. magna* was significantly inhibited in the Group C-H and Mix-H, and the heart rate increased significantly in all treatment groups. The changes in enzyme activity and gene expression levels of AMS and trypsin indicated that CTP/MTP and their mixture had different effects on the physical function of *D. magna*. However, we observed differences between enzyme activities and gene expression. In the recovery period, an obvious overcompensation effect in the feeding rate could be observed in Group M-L and C-H. There were no obvious differences in heartbeat. Those results demonstrated that there were obvious toxicity effects of psychoactive drugs on *D. magna* under single and mixed environmental stress. Further research is needed to determine the toxic pattern of those compound contaminations and investigate the potential mechanisms of their toxicity.

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**Author contribution** Yunfeng Ma and Dong Xu carried out experiments, analyzed experimental results, and wrote the paper; Chenyang Li and Shu Wei developed the methodology; Ruixin Guo analyzed experimental results; Yang Li developed the methodology; Jianqiu Li and Shu Wei developed the methodology; Ruixin Guo analyzed experimental results; and Yanhua Liu developed the methodology.

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**Declarations**

**Ethics approval and consent to participate** Not applicable.

**Consent for publication** Not applicable.

**Competing interests** The authors declare no competing interests.

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