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Research article

The highly hazardous veterinary drug "maduramicin" and its toxicokinetics in rats

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ABSTRACT

Background: Maduramicin (MAD) is an anticoccidial veterinary drug, but it frequently causes fatal poisonings in poultry, livestock, or humans. However, there is no specific antidote or guidance on first aid for MAD poisoning.

Aim: The aim of the present study is to evaluate the acute toxicity and toxicokinetics of MAD after oral exposure, so as to make a foundation for developing diagnostic and therapeutic protocols for human intoxication.

Methods: Five groups of rats (eight-to-nine-week-old male Wistar rats) were orally administered MAD via gavage at doses of 0, 4.64, 10.0, 21.5, or 46.4 mg/kg bw for only one time. The survival rates of the rats were observed over the following 14 days to assess acute toxicity. To evaluate the toxic effects of MAD, two doses (4.8 mg/kg bw and 10 mg/kg bw) were orally administered via gavage. Biochemical parameters including creatine kinase, lactate dehydrogenase, aspartate aminotransferase, alanine aminotransferase, urea, creatinine, serum myoglobin, and urinary myoglobin were measured. Liver, kidney, heart, and hind limb skeletal muscle samples from severely poisoned rats were obtained for pathological examination. For toxicokinetic analysis, samples of serum, urine, and feces from the 4.8 mg/kg bw dose group were analyzed using high-performance liquid chromatography-tandem mass spectrometry.

Results: The LD50 of MAD in male Wistar rats was determined to be 6.81 mg/kg bw. In the 10 mg/kg bw group, elevated serum urea levels and increased myoglobin levels in both serum and urine indicated renal injury and potential muscle damage. Toxicokinetics in serum revealed that following oral administration of 4.8 mg/kg bw MAD, peak serum concentration of 59.8 ± 8.9 μg/L was achieved at 30.0 ± 13.9 h. MAD exhibited a slow elimination from the blood with an elimination half-life of 72.9 ± 36.8 h and a mean residence time of 79.6 ± 25.5 h. Additionally, fecal excretion of MAD was found to be greater than urinary excretion.

Conclusion: MAD is a highly toxic veterinary drug which requires careful handling. The primary effects of poisoning include kidney injury and suspected rhabdomyolysis. It is excreted very slowly after oral administration. Promoting toxin excretion in individuals poisoned by MAD could potentially serve as an effective treatment method until a specific antidote is identified.

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Abbreviations:

MAD	maduramicin
HPLC-MS/MS	High-performance liquid chromatography-tandem mass spectrometry
bw	body weight
LD50	the median lethal dose
h/hrs	hours
CK	creatine kinase
LDH	lactate dehydrogenase
AST	aspartate aminotransferase
ALT	alanine aminotransferase
UREA	urea
Cr	creatinine
MYO	myoglobin
LOD	the limit of detection
LOQ	the limit of quantification
WHO	World Health Organization

1. Introduction

Maduramicin (MAD) is utilized in veterinary practice as an anticoccidial drug in poultry. It is one of the polyether ionophores, along with salinomycin, monensin, and more than 120 structurally characterized lipid-soluble compounds [1]. They are used to feed ruminants to enhance feed efficiency. Additionally, these substances showed potential for application as anticancer [2–5], antimalarial [6,7], and antiviral [8–10] agent in the treatment of human diseases. Their wide range of biological activities depend on the structural diversity, such as ether rings and the manner of ring closure (Fig. 1) [11]. However, it can also affect normal myocardium and skeletal muscle cells [12,13].

Accordingly, MAD can cause poisoning in animals and even humans and it should be given adequate attention. The most common toxic effects of polyether ionophores are cardiac toxicity, anorexia, body weight reduction, and muscle degeneration, and neuropathy such as myelin degeneration and ataxia [14]. MAD is the most toxic polyether ionophores [15]. As a veterinary drug, misuse or cross-contamination of MAD can cause toxicity in cattle, sheep, and gilts [15,16]. Most notably, the MAD appears as a white powder resembling starch, which could inadvertently lead to poisoning incidents among poultry plant workers due to accidental ingestion. Occupational exposure to MAD can also result in poisoning. As reported by Sharma, a poultry farm worker shared homemade pudding

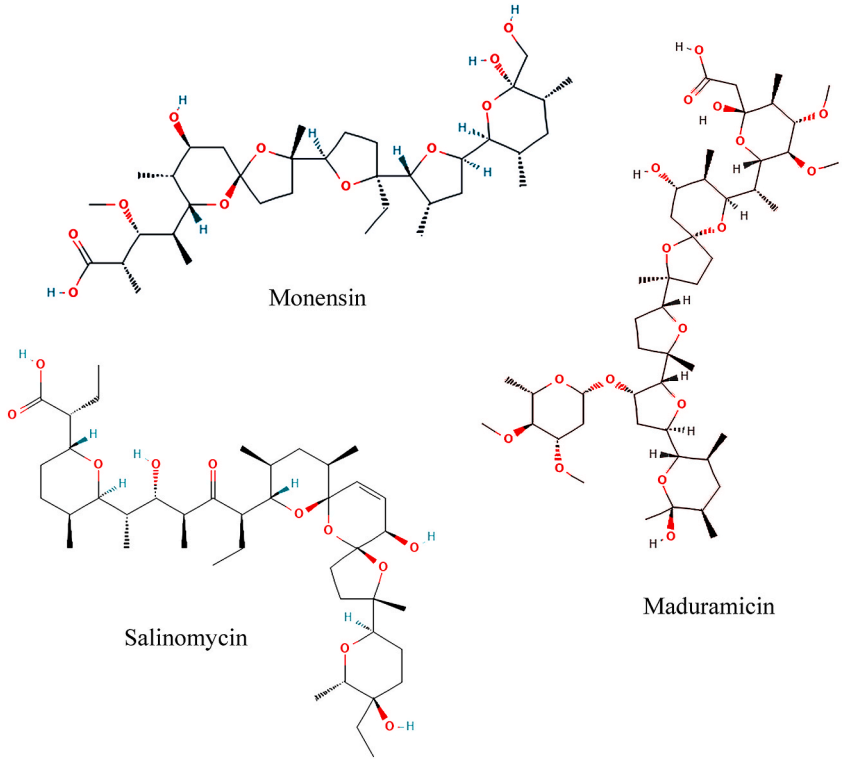

Fig. 1. Chemical structure of three common polyether ionophores.

with six acquaintances, which resulting in poisoning for seven people and subsequent fatality for two individuals. All patients exhibited symptoms of rhabdomyolysis and acute renal failure [17]. Si Min reported that a broiler farm worker developed rhabdomyolysis syndrome and eventually died of respiratory and circulatory failure after handling MAD-mixed chicken feed without gloves. He had eaten dinner without washing his hands [18]. A factory worker suffered rhabdomyolysis with renal failure after exposure to MAD dust on his bare arm during MAD production in the summer [19]. Unfortunately, there is no specific antidote or guidance on first aid for MAD poisoning.

To our knowledge, few acute oral toxicity or toxicokinetic studies of MAD have focused on the treatment of human poisoning. MAD concentrations in the feed and eggs of laying hens was detected to determine the carryover rate of drug from feed to eggs [20]. The pharmacokinetics of lasalocid were compared in broilers and turkeys to determine the risk of consumer exposure to drug residues in eggs [21]. Chronic exposure to drug residues in food and direct exposure to large doses of the original drug cause much different harm to humans. The latter can lead directly to death. Therefore, the aim of this study is to assess the acute toxicity and toxicokinetic pattern of MAD after oral ingestion, so as to make a foundation for developing diagnostic and therapeutic protocols for human intoxication.

2. Materials and methods

2.1. Chemicals

The test substance, maduramicin (molecular formula: $C_{47}H_{83}NO_{17}$; Chemical Abstracts Service (CAS) Reg. No. 61991-54-6, molecular weight: 934.2 g/mol), was prepared at greater than 92.3 % purity by China Institute of Veterinary Drug Control. Test articles were dissolved in dimethyl sulfoxide (DMSO) and then diluted tenfold with ultrapure water, following constant stirring to homogeneity.

2.2. Animals

Eight-to-nine-week-old male Wistar rats, weighing 240–260 g and certified specific pathogen-free, were procured from SiPeiFu Biotechnology Co., LTD (Beijing, China). All rats underwent a 7-day acclimatization period prior to commencing the experiments. The room temperature was maintained at 20–22 °C with a humidity level between 50 and 55 %, following a 12-h light/dark cycle. Rats were housed three per cage on sawdust bedding and provided sterilized tap water and standard diet, except for feed withdrawal the night before gavage and 4 h after dosing. Single gavage was administered at a volume of 1 ml/100 g bw. Animal experiment was conducted strictly in accordance with the Guidelines for Animal Experimentation of the National Institute of Occupational Health and Poison Control, Chinese Center for Disease Control and Prevention (NIOHP, China CDC). The experimental protocol received approval from the institution's Animal Ethics Committee, and the ethics approval number is 2022-NIOHP-IACUC-20.

2.3. Acute oral toxicity study

2.3.1. Experiment design for acute oral toxicity test

Horn's method was used to assess the acute oral toxicity of MAD. After a 7-day acclimatization period, twenty-five rats were randomly divided into five groups of five rats each. For the four experimental groups, rats were once administered by oral gavage at doses of 4.64, 10.0, 21.5, or 46.4 mg/kg bw of MAD solution, respectively. The vehicle control group only received the vehicle solvent (10 % dimethyl sulfoxide) via oral gavage. The general condition, toxic symptoms, and overall welfare of rats were continuously monitored once every hour for the initial day following administration, and subsequently once daily at 9 a.m. for a duration of 14 days. Prior to dosing and on either day 14 after gavage or the day of demise, each animal's body weight was measured. No biological samples were taken from animals in this section to minimize the impact of the experimental manipulation on the health status of the rats. On day 14, all remaining rats were humanely euthanized through exsanguination from the abdominal aorta subsequent to administering the general anesthetic by intraperitoneal injection of 0.1 ml/100 g of tiletamine-zolazepam (Zoletil®50).

2.3.2. Experiment design for toxic effects test

Two doses were selected to compare the characteristics of mild and severe acute poisoning caused by oral ingestion of MAD. Thirty rats were randomly divided into three groups of ten rats each. The first group was administered a dose of 4.8 mg/kg bw of MAD solution, the second group received a dose of 10 mg/kg bw, and the third group received the vehicle solvent (10 % dimethyl sulfoxide). For the 10 mg/kg bw group, in order to extend the observation period, fasting was not implemented prior to gavage administration. For all three group of rats, blood samples were obtained at different time points after dosing (1, 4, 8, 24, 30, 48, 55, and 72 h), and 24-h-interval urine samples were collected at specific time points after dosing (24,48, and 72 h). For agonizing rats in 10 mg/kg dose group, tissue samples were collected for further analyzing. The rat was recognized as agonizing only when it failed to hold on to the railing while it was put on the iron railing and pulled back by its tail. Certainly, it had developed poisoning symptoms such as diarrhea, reduced activity, increased eye secretion, and even bloody eye secretion. Those moribund rats were euthanized through exsanguination from the abdominal aorta after general anesthesia with Zoletil® 50.

2.3.3. Biochemical biomarkers analysis

Blood was collected directly from the rat medial canthus vein into a centrifuge tube and then centrifuged at 2500 r/min for 20 min. The obtained serum was stored at −20 °C for subsequent quantification of MAD or blood biochemistry analysis. The urine sample was

also centrifuged, and the supernatant was stored at −20 °C. The BS-360E automatic biochemistry analyzer (MINDRAY Bio Medical Electronic Limited by Share Ltd, Shenzhen, China) was used to measure serum levels of creatine kinase (CK), lactate dehydrogenase (LDH), and urea (UREA). The corresponding commercial assay kits (CK, GCK460BS; LDH, GLDH460BS; UREA, GS9311S) came from Beijing Strong Biotechnologies, Inc., Beijing, China. The SpectraMax® ABS Plus (Molecular Devices, San Jose, CA, USA) was used to measure serum levels of alanine aminotransferase (ALT), aspartate aminotransferase (AST), creatinine (Cr), myoglobin (MYO) in serum, and MYO in urine. The corresponding commercial assay kits (ALT, C009-2-1; AST, C010-2-1; Cr, C011-2-1; MYO, H150-1-2) came from Nanjing Jiancheng Bioengineering Institute, Nanjing, China. The detailed protocols were carried out according to the manufacturer's instructions.

2.3.4. Immunohistochemistry and histopathology procedures

The liver, kidney, heart, and hind limb skeletal muscle tissues of rat were separated. These organs were washed with physiological saline and subsequently fixed in paraformaldehyde (4 %) for 3 days. Then the specimens were cut into pieces of 5 mm length and 5 mm width in size. The tissue pieces were placed sequentially in different concentration of ethanol for dehydration, with each concentration being immersed for 20 min. They were placed in a solution of ethanol and xylene (1:1, v/v) for 30 min and then in pure xylene twice for transparency. After that, the samples were embedded in paraffin, then sectioned into 4∼6 μm thickness, as well as stained with highly pure hematoxylin and eosin (H&E). Those tissues slides were sealed with neutral resin and observed under a light microscope. The pathological injury score was based on INHAND (International Harmonization of Nomenclature and Diagnostic Criteria for Lesions in Rats and Mice). Paraffin embedding, section, HE staining, microscopic image collection and pathological section analysis were completed by Beijing LAT Science Co., Ltd., Beijing, China.

2.4. Toxicokinetics in serum and excretion pattern of MAD

2.4.1. Experiment design for toxicokinetics study

We employed the approximation of 70 % LD50, which corresponds to a dose of 4.8 mg/kg bw, for conducting toxicokinetic experiments. This dose allowed the chemical to be detected in serum samples from poisoned rats, and the survival rate of rats in this group is 100 % during the 14-day observation period. A total of twenty rats were randomly assigned into two groups consisting of ten rats each. One group was used for the toxicokinetics study of MAD in serum and the other for the study of toxicant excretion patterns in urine and feces. All these animals received a single oral administration of 4.8 mg/kg bw. Notably, no separate blank control group was set up for this section. Pre-gavage samples from the same rats were utilized as blank control samples. Blood samples from the first group were collected from the medial canthus vein one day prior to dosing and at 1, 4, 8, 12, 24, 30, 36, 48, 72, 96, 120, 168, 216, and 384 h after dosing. In the second group, rats were individually housed in metabolic cages to collect urine and feces before dosing and at 24, 48, 72, 96, and 168 h after dosing. Those excreta samples were collected for 24-h intervals.

2.4.2. Determination of MAD by HPLC-MS/MS

Serum and urine samples preparation and preservation were the same as in 2.3.3. To process the feces, a specialized pulverizer was used to break them up, followed by adding 5 ml of methanol-acetonitrile solution (1:1, v/v) per gram of feces before sonication for 30 min. Subsequently, the mixture underwent centrifugation for 20 min at 2500 r/min, and finally, 1 ml of supernatant was retained and stored at −20 °C. For the detection of MAD, stored fecal samples are diluted 20-fold. The serum, urine, and fecal samples were extracted and purified with a methanol-acetonitrile solution (1:1, v/v) before detection. A liquid chromatography system (LC-20AD, Shimadzu, Japan) was adopted for the chromatographic separation. Chromatographic separation was accomplished on a Shim-pack XR-ODS C18 column (50 mm × 4.6 mm i.d., Shimadzu, Japan) with a run time of 8 min. The column temperature was maintained at 40 °C. The flow rate was fixed at 0.6 mL min^{-1} and the injection volume was 5 μL. Mobile phase A (0.1 % formic acid and water) and phase B (0.1 % formic acid and methanol) were freshly prepared. The following gradient elution program was used: 0∼0.5min, 30 % B; 0.5–3min, 90 % B; 3–4.1min, 90 % B; 4.1–7 min, 100 % B; 7–8min, 30%B.

A mass spectrometer (4500MD, SCIEX, USA) was employed to in the present study. The mass spectrometry parameters were set as follows: ion-spray voltage, 5500 V; gas temperature, 500 °C; collision gas, 9.0 psi; curtain gas, 30.0 psi; ion source gas 1, 50.0 psi; ion source gas 2, 60.0 psi. Electrospray ionization positive ion mode (ESI+) and multiple reaction monitoring (MRM) were used for qualitative and quantitative detection.

MAD in serum, urine, and feces was quantitated in the Occupational Disease and Poisoning Department of Beijing Chaoyang Hospital using high-performance liquid chromatography-tandem mass spectrometry (HPLC-MS/MS).

2.5. Statistical analyses

DAS2.0 pharmacokinetic software was utilized to analyze the toxicokinetics of MAD in serum and the excretion of MAD from urine and feces. Other data processing and statistical analysis were conducted using Excel 2016 and SPSS 22.0. The results were presented as mean ± SD (standard deviation). Statistical analysis was performed using one-way ANOVA, followed by a Dunnett-t multiple comparison test. A p-value of less than 0.05 was considered statistically significant.

3. Results

3.1. Acute oral toxicity

The survival rate of rats decreased with increasing dose of MAD. As illustrated in Fig. 2, following a single dose of 46.4 mg/kg bw of MAD, the survival rate within 24 h dropped to 60 %, and to 80 % for the 21.5 mg/kg bw dose. Rats in these groups exhibited sluggish movement, general debility, clear fluid covering the surface of their eyes, and clear discharge at the corners of their eyes. Furthermore, their hind limb grip strength declined in tests putting on the iron fence and pull back.

By 48 h post-toxin administration, all rats in the 46.4 mg/kg bw group had died, showing epistaxis and bloody discharge from the corners of their eyes, with their hind limbs extended backwards. Among the surviving rats in the 21.5 mg/kg bw group, only head mobility was observed without any limb movements. All rat deaths occurred within the 48-to-72-h period in the 10 mg/kg bw group. However, the survival rates were 100 % for both the 4.64 mg/kg bw dosage group and the vehicle control group within 14 days.

Following Horn's method for acute oral toxicity, the LD50 was calculated based on the number of animals that died in each group and the dose used. The study involved at least four groups of five animals each, with a metric ratio of dose escalation ($\sqrt[3]{10}$). In the present study, starting with a dose of 4.64 mg/kg bw, there was no death in the 4.64 mg/kg bw group, while there were 5 deaths each in the 10, 21.5 and 46.4 mg/kg bw groups, resulting in an LD50 of 6.81 mg/kg bw.

During the survival period, rats in the dosage groups of 46.4 mg/kg bw, 21.5 mg/kg bw, and 10 mg/kg bw exhibited a decrease in body weight (Fig. 3A). In particular, the 10 mg/kg bw dosage group presented the largest reductions in body weight, likely because rats in this group survived longer than those in the 46.4 and 21.5 mg/kg bw groups. On average, each rat lost a total of 81.2 g of body weight over 3 days. In contrast, rats in the 4.64 mg/kg bw dose group initially experienced weight loss but began to recover on the fourth day after intoxication, with gradual increases thereafter.

The primary reason for weight loss in rats was reduced food intake. As shown in Fig. 3B, upon restoration of feed availability, all rats showed a spike in food intake to peak levels (6–24 h after dosing), followed by a decline within 24–48 h. Overall, the control group exhibited higher feed consumption compared to the four experimental groups. Among the experimental groups, there was generally an inverse relationship between feed consumption and gavage dose. Specifically, rats in the 10 mg/kg bw group consumed less food than those in 21.5 mg/kg bw group, which may explain why rats in the former group experienced the greatest weight loss.

3.2. Toxic effects

Mild poisoning was induced by 4.8 mg/kg bw of MAD, resulting in decreased body weight and reduced food intake in rats within this group. Approximately 24 h after gavage, the sole symptom observed was diarrhea. Biochemical analyses depicted in Figs. 4 and 5 showed no statistical difference in all biochemical indicators compared to the control group.

Severe poisoning was induced by 10 mg/kg bw of MAD, resulting in evident clinical manifestations in rats, including diarrhea, limb weakness, and increased eye secretion. All rats in this group died within 96 h after dosing, which is longer than expected 72 h, allowing for an extended observation period and enabling the collection of more blood samples. As depicted in Fig. 4F, urea levels in serum of the 10 mg/kg bw group were significantly higher than those in the control samples from 30 to 55 h, indicating renal injury. In Fig. 4, a slight increase in CK levels was observed in a few serum samples, but there were no statistical differences compared to the vehicle control group. This suggests that minor muscle damage or myocardial injury may have occurred among these rats. However, other biochemical indicators (LDH, ALT, AST, Cr) did not show any time-dependent changes or significant differences between the10 mg/kg bw group and the control group. Additional biomarkers may have been required to definitively rule out myocardial damage.

As shown in Fig. 5, serum MYO levels in severely poisoned rats were elevated at 24 h, and urinary MYO levels during the 8–24h and

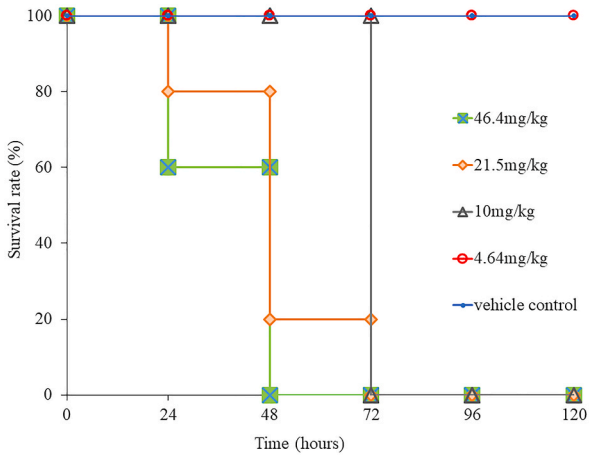

Fig. 2. Survival rate of rats within 120 h after gavage. Note: No mortality was observed after 120 h.

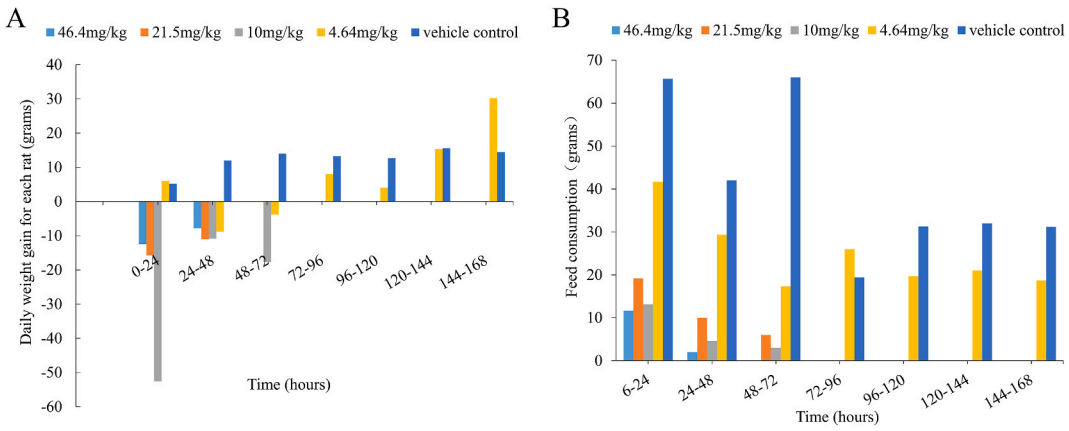

Fig. 3. (A)Effects of MAD gavage with different concentrations on the body weight of rats. (B) Effects of MAD gavage on food intake of rats.

24–48h periods were significantly higher than in the control samples. At the same time, severely poisoned rats exhibited myoglobinuria (Fig. 6). The urine samples from 0 to 24h and 24–48h appeared turbid with a tea-colored hue, while the control samples were translucent and light yellow. By 48–72 h, the urine color had lightened but remained darker than the control samples. Two rats did not produce urine within 24 h of dosing, and two others had decreased urine output. After one week of storage, a substantial amount of sediment was observed at the bottom of both the 0–24h and 24–48h urine samples, likely composed of myoglobin.

Despite the biochemical changes observed in severely poisoned rats (10 mg/kg bw), no significant alterations in organ pathology were noted. Histological examination of the kidney, heart, liver and muscle revealed no discernible pathological changes, even in rats in agony (Fig. 7).

3.3. Toxicokinetics and excretion pattern of MAD

The validation parameters of HPLC-MS/MS were provided by the Occupational Disease and Poisoning Department at Beijing Chaoyang Hospital. The calibration curves exhibited a linear trend within the range of 0.5–100 μg/L ($R^2 \geq 0.9998$) for MAD analysis. The limit of detection (LOD) and limit of quantification (LOQ) were determined as 0.3 μg/L and 1.0 μg/L, respectively. Moreover, the average recovery rate for MAD ranged from 86.4 % to 114.6 %, with relative standard deviations below 10 % observed across different samples within a batch and between three different batches.

The toxicokinetics model for serum fitted well with the two-compartment model. The toxicokinetics data were shown in Table 1, the peak concentrations were reached approximately 30.0 ± 13.9 h after administration, which is the time when maximum exposure in vivo occurs. The maximum concentration of MAD in serum amounted to 59.8 ± 8.9 μg/L. The total serum clearances of MAD were 0.08 ± 0.03 L/h. The elimination half-live was 72.9 ± 36.8 h indicating a slow disappearance of MAD from blood. This was confirmed by the high value calculated for the mean residence time (MRT) which was 79.6 ± 25.5 h. The apparent volume of distribution after oral dose was 105.2 L, suggesting significant diffusion of MAD within a specific organ or across a large area of tissue.

As depicted in Fig. 8A, MAD was detectable in serum 1 h after oral administration, with concentrations peaking at 30 h. The concentration vs time curve generally exhibited a unimodal distribution, with a smaller secondary peak observed at 48 h. The concentration of MAD in serum at 384 h post-administration was relatively close to the lowest limit of detection (LOD).As illustrated in Fig. 8B, a large fraction of MAD was excreted in feces within the first 48 h, followed by a gradual decrease in excretion over time. Remarkably, a significant amount of MAD was still detectable in feces from 144 to 168 h. In comparison, the excretion of MAD in urine was much lower. The MAD concentration in urine vs time curve showed two peaks around 24–48h and 72–96h. It is similar to the elimination pattern observed in serum, but at a slower rate in urine.

4. Discussion

The current study describes the toxicity of MAD and its toxicokinetics characteristic. The acute oral LD50 for MAD in rats was determined to be 6.81 mg/kg bw. The poisoning event primarily affects the kidney and muscle tissues of rats. The metabolic profile of MAD obtained from this study could serve as a valuable reference for diagnosis, medical intervention, and further investigation into disease mechanisms.

4.1. Acute oral toxicity

In this study, the acute oral LD50 of MAD for rats was 6.81 mg/kg bw, which is comparable to the LD50 found in broilers (5.53 mg/kg bw) [22]. According to WHO Recommended Classification of Pesticides by Hazard and Guidelines to Classification (2019 edition), oral toxicity in rats with LD50 between 5 mg/kg bw and 50 mg/kg bw is classified as "highly hazardous" [23]. Additionally, based on

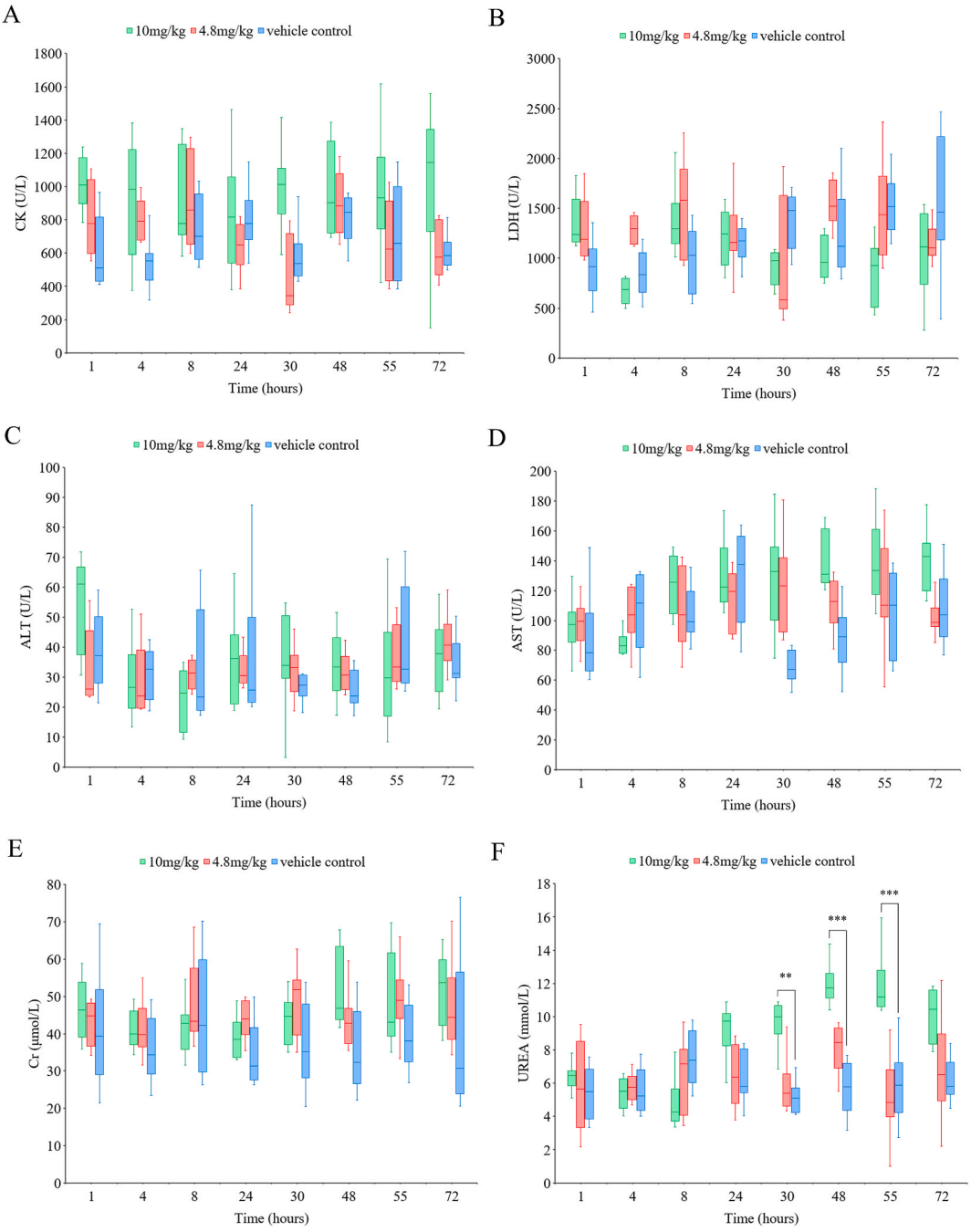

Fig. 4. Biochemistry analyses remind renal injury and possible minor muscle damage for rats in 10 mg/kg bw group. (A)Analysis of serum levels of creatine kinase (CK, U/L). (B) Serum levels of lactate dehydrogenase (LDH, U/L). (C) Serum levels of alanine aminotransferase (ALT, U/L). (D) Serum levels of aspartate aminotransferase (AST, U/L). (E) Serum levels of creatinine (Cr, μmol/L). (F) Serum levels of urea (UREA, mmol/L). (n = 10, **p < 0.05, ***P < 0.01, ANOVA Dunnett-t test).

the User's Manual for the IPCS Health and Safety Guides, an oral LD50 in rats less than 25 mg/kg is classified as "very toxic" [24]. Hence, our study confirms that MAD is a highly toxic chemical. The Commission of the European Communities Agriculture approved the use of MAD at a level of 5 mg/kg for chickens, provided there is a withdrawal period of 5 days before slaughter [25]. Similarly, in China, the approved dose of MAD for chickens is also 5 mg/kg bw [26]. This dosage has been deemed safe during the initial seven days of administration. However, prolonged use beyond 14 days has been found to induce toxic effects in chickens [27]. For non-target animals, MAD can be particularly harmful. Liang Yunxia reported an LD50 of 0.7 mg/kg bw for rabbits [28]. Shimshoni reported a lethal dose of 0.41 mg/kg bw for pigs when fed MAD over 7 consecutive days [16].

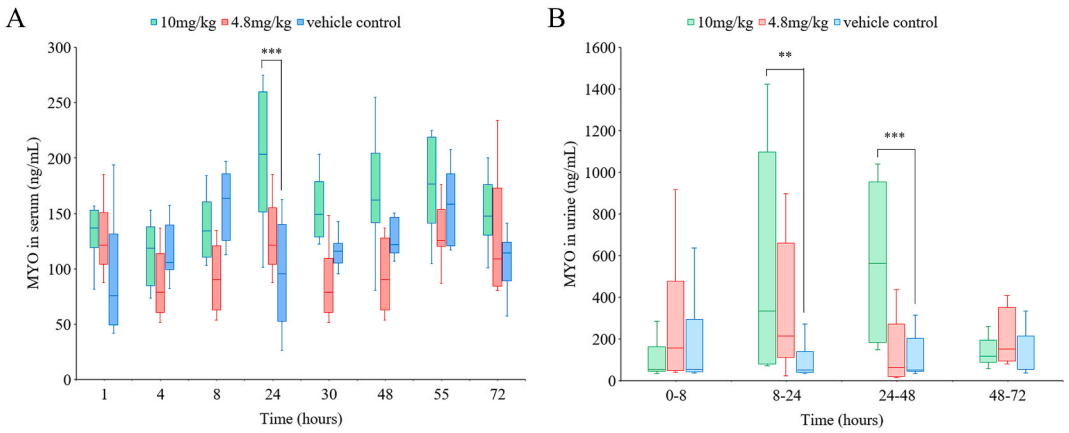

Fig. 5. Increased MYO levels remind possible muscle damage of rats. (A) Analysis of serum levels of myoglobin (MYO, ng/ml). (B)Analysis of urine levels of myoglobin (MYO, ng/ml). (n = 10, **p < 0.05, ***p < 0.01, ANOVA Dunnett-t test).

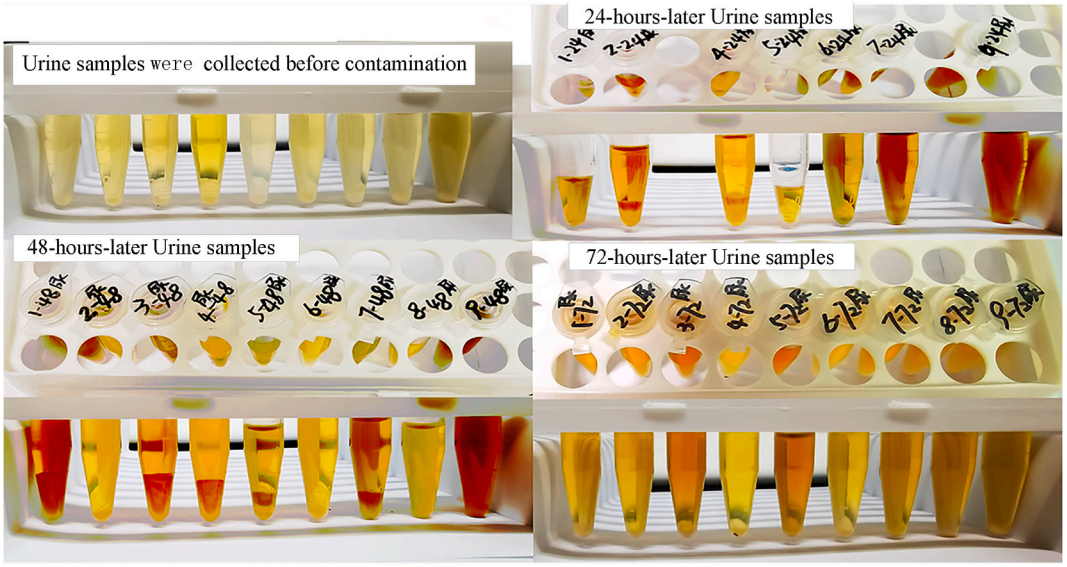

Fig. 6. The photographs of urine of rats in the 10 mg/kg bw dose group before gavage (the upper left picture) and after administration (the top-right picture was 24 h urine, the left-down is 48 h, and the right-down is 72 h).

4.2. Toxic effects

4.2.1. Similar symptoms with human intoxication

In this study, we observed poisoned rats exhibiting decreased muscle power, kidney damage and rapid death. Those symptoms were similar to human cases of MAD intoxication [17,29]. Previous research also noted that affected animals displayed weight loss and reduced food intake, consistent with our findings [17]. Other studies have documented that MAD can lead to conditions such as rhabdomyolysis, myocardial dysfunction, and liver dysfunction [18,29,30]. Studies at the molecular biological level have indicated that MAD induces apoptosis and necrosis in rat myocardial cells [13,31]. Notably, in our study, the poisoned rats did not develop rhabdomyolysis, as indicated by the current diagnostic consensus, which requires a 5-fold increase in CK values [32–34].

4.2.2. Acute kidney injury and suspected rhabdomyolysis

Sharma reported that patients poisoned with MAD have developed renal failure as a complication of rhabdomyolysis [17]. In our research, rats exhibited acute kidney injury, characterized by elevated serum urea levels, oliguria, anuria, and the presence of precipitates in urine. Concurrently, elevated serum myoglobin and myoglobinuria indicated that the kidney injury resulted from myoglobin leakage from skeletal muscle [35–37], resembling the pathophysiology observed in rhabdomyolysis-induced acute kidney injury [38]. Although rhabdomyolysis was not diagnosed in our study, myoglobin released from muscle is known to be the primary contributor to renal damage in rhabdomyolysis [39].

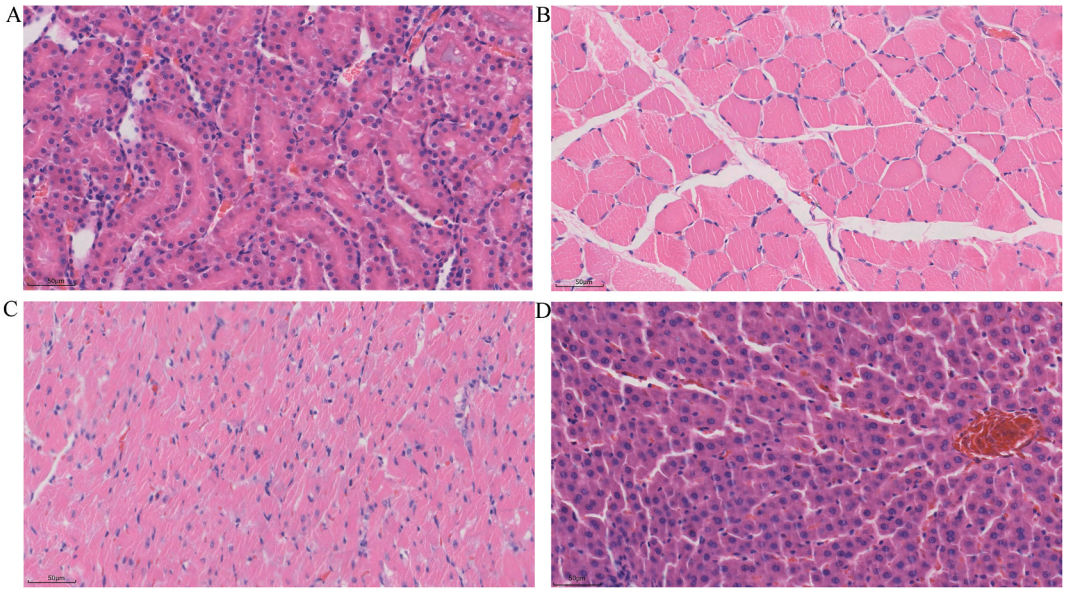

Fig. 7. H&E staining pathological examination of organs of severely poisoned rats in the brink of death. (A) kidney, (B) skeletal muscle, (C) heart, and (D) liver. (40X).

Table 1
Toxicokinetic parameters of MAD in serum after a single oral gavage in rats.

Parameters	Value
Single oral dose of MAD (mg/kg bw)	4.8
C_{max} (μg·L^{-1})	59.8 ± 8.9
T_{max} (h)	30.0 ± 13.9
$T_{1/2\ Elim}$ (h)	72.9 ± 36.8
CL (L·h^{-1})	0.08 ± 0.03
$AUC_{0\to t}$ (μg·L^{-1}·h)	5561.9 ± 1587.4
$AUC_{0\to\infty}$(μg·L^{-1}·h)	6014.7 ± 1768.8
$MRT_{0\to t}$ (h)	79.6 ± 25.5
$MRT_{0\to\infty}$ (h)	101.4 ± 36.5
Vd (L/kg)	105.2 ± 46.6

Note: C_{max} is concentration maxima of MAD in serum. T_{max} is time of occurrence of maxima concentration of MAD in serum. $T_{1/2Elim}$ is terminal elimination half-life. CL is total serum clearance. AUC is area under serum concentration-time curve. MRT is mean retention time of MAD in serum. Vd is the apparent volumes of distribution.

4.2.3. Potential skeletal muscle or myocardial injury

In our study, we observed elevated myoglobin levels in serum and urine. However, no pathological abnormalities were observed in skeletal muscle or myocardial tissue. Unlike human poisoning resulting from accidental ingestion of MAD, experimental rats exhibited potentially enhanced and fast recovery abilities. Muscle damage in animals likely occurred only after repeated exposure to MAD. For instance, calves showed enlarged livers and abnormal myocardium upon necropsy after consuming MAD contaminated feed for several days consecutively [40]. Histopathological examination of poisoned gilts revealed necrotic skeletal muscle and myocardial myofibers, along with necrotic tubular epithelial cells after 7 days of contaminated feed consumption [16]. In 15 outbreaks of livestock poisoning in South Africa, cattle and sheep developed cardiac lesions after being fed broilers litter for 20–40 days, where MAD had been incorporated into the broilers feed [41].

4.2.4. Potential association of haff disease with human exposure to MAD

In recent years, MAD and other ionophore antibiotics have been implicated as potential triggers for Haff disease, a syndrome characterized by unexplained myalgia and rhabdomyolysis in individuals who have consumed seafood within 24 h [42]. MAD, when administered to chickens via feed, has been shown to be primarily excreted through feces [25]. Poultry feces are commonly used in aquaculture feed to save costs [43,44], potentially leading to significant MAD residue in aquatic products. Additionally, MAD concentrations ranging from 4.8 to 31.7 ng/L have been detected in surface water samples collected from a wastewater treatment plant in northwestern Spain [45], highlighting the threat posed by this contaminant to the aquaculture industry. Consequently, it is plausible

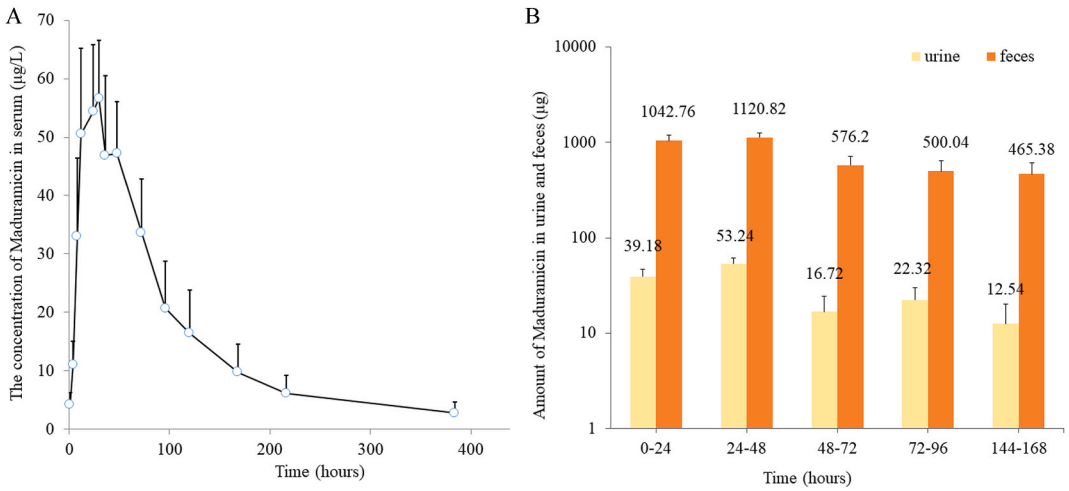

Fig. 8. The toxicokinetics of MAD in serum, urine, and feces after single oral administration of 4.8 mg/kg bw. (A) Serum concentration of MAD vs time curve. (B) The average excretion of MAD in the urine and feces.

that humans may be exposed to MAD and other ionophore antibiotics through consumption of contaminated seafood. As our understanding of the acute toxicity of MAD advances, future research may validate the hypothesis linking Haff disease to ionophore antibiotics.

4.3. Toxicokinetics and excretion pattern

There is little information on the toxicokinetics behavior of MAD after controlled dosing. In this study, after the single oral dose of 4.8 mg/kg bw, MAD was both absorbed (T_{max} = 30 h) and eliminated slowly ($T_{1/2Elim}$ = 72.9 h). The low total serum clearance (CL = 0.08 L h^{-1}) is also adequate to explain the slow elimination of MAD in the rat. This was confirmed by the high value calculated for the mean residence time ($MRT_{0\rightarrow t}$ = 79.6 h, $MRT_{0\rightarrow\infty}$ = 101.4 h). The bioavailability of MAD was relatively high, as indicated by the large area under serum concentration-time curve (AUC0→t = 5561.9 μg L-1 h, AUC0→∞ = 6014.7 μg L-1 h).

Interpreting the time-dependent physiological and behavioral responses of animals is challenging, as only diarrhea was observed in rats at a dose of 4.8 mg/kg bw. The apparent volumes of distribution (Vd = 105.2 L/kg) were large, suggesting extensive distribution throughout body fluids or accumulation in specific organs. While the total amount of drug excreted in feces was not quantified in this study, a significantly higher amount of MAD was excreted via feces compared to urine following oral administration in rats. This suggests that MAD may accumulate in the gastrointestinal tract. Similarly, the high concentration of MAD found in poultry feces suggests significant gastrointestinal retention in fowl [25,41].

The toxicokinetics and excretion pattern of MAD can provide valuable insights for developing diagnostic and first-aid protocols in case of MAD poisoning, especially for poison removal strategies. The study results indicate that MAD was eliminated slowly from the blood over a period of up to two weeks following oral exposure. This underscores the diagnostic significance of detecting the toxin in blood within the first two weeks after suspected MAD poisoning. Importantly, peak serum concentration occurred at 30 h, suggesting that prompt implementation of blood purification therapies between 17 and 43 h after ingestion may eradicate the toxin and reduce internal exposure. Such therapies have the potential to alleviate poisoning symptoms and extend overall survival time [46]. The initial peak concentration observed at 30 h likely indicates absorption in the small intestine, while a smaller peak at 48 h may indicate enterohepatic circulation [47]. Moreover, the significantly greater excretion of MAD via feces compared to urine suggests that gastric lavage and catharsis could aid in eliminating poison from the body and reducing absorption in the small intestine and enterohepatic circulation [47,48]. Future experiments should validate this hypothesis.

There are some limitations in this study. First, in the two high dose groups (46.4 mg/kg bw and 21.5 mg/kg bw), where rats died within 48 h, we could not determine the cause of death or identify the locations of lesions. Additionally, we observed that rats in the 21.5 mg/kg bw group consumed more feed but had a shorter survival time compared to those in the 10 mg/kg bw group, but the reasons for this were not assessed. Second, for MAD poisoning, it remains unclear whether the kidney injury results from myoglobin, MAD, or a combination of both factors.

5. Conclusion

MAD is a highly toxic veterinary drug. Animal husbandry professionals should exercise extreme caution to prevent accidental ingestion. The primary effects of poisoning include kidney injury and suspected rhabdomyolysis. In cases of suspected poisoning, conducting a blood test for MAD within two weeks is crucial for diagnosis. MAD is excreted very slowly after oral administration. Until a specific antidote is identified, expediting the elimination of the toxin could be an effective initial treatment for MAD poisoning.

CRediT authorship contribution statement

Bowen Cheng: Writing – original draft, Software, Project administration, Methodology, Investigation. **Huarui Zhang:** Resources, Methodology. **Wenjin Zhao:** Supervision, Methodology, Conceptualization. **Shaofeng Jiang:** Validation, Methodology, Data curation. **Zhijun Wu:** Writing – review & editing, Methodology, Conceptualization. **Huiling Li:** Software, Resources, Methodology. **Shuai Liu:** Writing – review & editing, Supervision, Methodology. **Hongshun Zhang:** Writing – review & editing, Supervision, Methodology.

Declaration of conflict interest

All authors declare no conflicts of interest.

Data availability statement

Data will be made available on request.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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