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Feasibility of serum liver enzymes as markers of microcystin-LR-induced liver injury：A systematic review and meta-analysis

Yu Fu [1,2#], Yao Guo[1#], Zhihui Tian[1], Zhenxing Mao[1*]

1. Department of Epidemiology and Biostatistics, College of Public Health, Zhengzhou University, Zhengzhou, 450001, Henan, PR China.
2. Department of Pharmacy, Henan Cancer Hospital, The Affiliated Cancer Hospital of Zhengzhou University, Zhengzhou, 450003, Henan, PR China.

Abstract: Microcystin-LR (MC-LR) is a hepatotoxin that can cause liver damage, resulting in overflow of liver enzymes from cells into the serum. However, it is still controversial to consider changes of liver enzymes in serum as biomarkers for the diagnosis of MC-LR-induced liver injury. Therefore, this study aimed to quantitatively evaluate the effects of MC-LR on liver biochemical markers in mice through a systematic review and meta-analysis. This will be used to clarify the feasibility of serum liver enzymes as biomarkers of MC-LR-induced liver injury. Literature was screened using the PRISMA process in the Scopus, Web of Science, and PubMed databases. The Cochrane Collaboration Tool was used to evaluate the quality of the publication. And the data was analyzed with Review Manager 5.3 and Stata 12.0. Four studies with 143 subjects were included from the 3819 identified papers. The analysis results showed that serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) concentrations were significantly increased at the oral dose of 1/2-1LD_{50} of MC-LR. Subgroup analysis showed that, compared to the low-dose (1/4-1/2LD_{50}) group, serum ALT, AST and lactate dehydrogenase (LDH) concentrations were significantly increased in the high-dose (1/2-1LD_{50}) group of MC-LR exposure with the intraperitoneal injection. The above biochemical indexes were significantly increased several times compared with the control group. MC-LR can increase serum LDH, ALT and AST levels, and a clear dose-response relationship was observed after intraperitoneal injection of MC-LR. These results suggest that ALT, AST and LDH levels in serum are feasible as biomarkers of MC-LR-induced liver injury, which is helpful for early clinical diagnosis and prevention of MC-LR toxicity.

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1. Introduction

As the largest “chemical plant” in the human body, the liver is involved in important biological processes such as metabolism, digestion, and detoxification so it is of great significance for maintaining healthy[1]. Liver damage will lead to the overflow of related enzymes in liver cells into the serum. By detecting the changes of enzymes in the serum, liver damage can be assessed in a timely manner. At present, there is no single index or simple experimental test in the clinic that can fully reflect liver function. The combined detection of multiple indexes can significantly improve the accuracy of the diagnosis of liver dysfunction. In epidemiological investigations, aspartate aminotransferase (AST) and alanine aminotransferase (ALT) are widely used as biochemical markers of liver injury induced by various factors and they are valuable for the clinical diagnosis of many liver diseases[2].

With the intensification of environmental pollution and the global warming trend, the eutrophication of water bodies will be more likely to occur, and the cyanobacteria blooms will become more frequent[3]. Microcystins (MCs) are endotoxins produced by cyanobacteria, which have the structure of a cyclic heptapeptide. They are heat-resistant, stable and easily dissolve in organic solvent and water[4]. More than 279 isomers of MCs have been found. Among these, Microcystin-leucine arginine (MC-LR) is the most studied and most toxic isomer[5]. In 1998, the World Health Organization set a safety threshold of 1 μg/L MC-LR in drinking water. However the content of MC-LR in freshwater from bloom outbreaks could reach 13000 μg/L[6]. Current water cleaning technology

is not able to completely remove MC-LR. Therefore, MC-LR can enter the body through a variety of ways such as ingestion and drinking contact, skin and respiratory tract, posing a certain potential threat to human health[7].

When MC-LR enters the body, the liver is the main target organ. Many studies have proved that the exposure of MC-LR can lead to changes of liver biochemical markers, damage of liver cell structure, necrosis of cells, and even liver hemorrhage in severe cases[8, 9]. Epidemiological studies in Southwest China have shown that liver diseases are closely related to the presence of MC-LR in drinking water by assessing the changes of MC-LR and liver enzyme concentrations in serum[10]. It has been reported that acute exposure to MC-LR can cause hepatotoxicity in humans, with significant increases in biomarkers of liver injury (ALT, AST and γGT)[11]. However, Su et al. found that MC-LR can cause non-alcoholic fatty liver disease (NAFLD) and damage to the liver, but serum ALT and ALP levels did not increase after MC-LR exposure, so it is still controversial to use serum liver enzymes as biomarkers of liver injury induced by MC-LR[12].

At present, there are no established methods to diagnose and evaluate the hepatotoxicity induced by MC-LR. The most logical and easily available diagnostic tools include the currently commonly used biomarkers for liver health to diagnose and evaluate liver damage caused by various injuries. The study of changes in liver biochemical markers is of great significance for early diagnosis and prevention of liver injury caused by MC-LR. Therefore, in the present study, we conducted a comprehensive systematic review and meta-analysis of available randomized controlled trials (RCTs) to accurately explore the relationship between MC-LR and liver biochemical parameters in mice, and to evaluate the overall impact of MC-LR on liver biochemical indexes in mice, aiming to provide a zoological basis for the formulation of clinical biochemical indicators.

2. Methods

In order to make the search as comprehensive as possible, the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) process were used in this study[13]. We used the following keywords to screen in Scopus, Web of Science and PubMed respectively: (MC-LR or Microcystin-leucine arginine or Microcystin-LR) AND (mouse or mice) AND (liver or hepatic). In addition, we searched all references for eligible publications together.

2.1 Research selection

Selected qualified articles by reading the title, abstract, or the full text of the publication. The inclusion criteria of the study were: (1) it must be a randomized controlled trial; (2) the subjects were mice; (3) the intervention factor must be MC-LR; (4) it must have a suitable control group; (5) the results must be expressed by mean and Standard Deviation (SD). The exclusion criteria were: (1) studies on mice less than 6 weeks of age; (2) studies without an appropriate control group; (3) studies without original data; (4) duplicate publication; (5) research that is unrelated to this study. Finally, all the experimental designs for evaluating the effects of MC-LR on liver biochemical markers were considered.

2.2 Data Extraction

Based on the pre-designed information collection content, the data information was extracted from the full text of these articles and put into a summary table. The extracted information included research literature data (first author, year), statistical data of experimental animals (type, gender and total sample size), intervention data (administrative method, drug dose and study duration), method design criteria and reported biochemical markers. Only the final data of the interventions were considered for this work. In some cases, if there was a lack of relevant accurate data, the corresponding author was contacted and the differences were resolved through negotiation.

2.3 Quality assessment of research

Evaluated the methods and quality of selected articles through the Cochrane Collaboration Tool [14], which includes seven areas: (1) Random sequence generation; (2) Allocation concealment; (3) Blinding of participants and researchers; (4) Blinding of outcome assessment; (5) Incomplete outcome data; (6) Selective reporting; (7) Other sources of bias. Each area was further divided into three categories: low risk of bias, high risk of bias and unclear risk of bias. According to the guidelines, when there were more than two low risks, the overall quality of the study was good; when there were two low risks, the overall quality of the study was general; when there were fewer than two low risks, the overall quality of the study was weak[15]. Studies with scores of 3 and higher were generally considered as high-quality study[16].

2.4 Statistical Analysis

All analyses were performed using Review Manager 5.3 and Stata12. The mean and SD of liver biochemical parameters were used to calculate the overall effect size. The heterogeneity between the studies was tested by I^2 index. The heterogeneity levels of each study were low, medium, and high, corresponding to I^2 values of 0-50%, 50-75%, and greater than 75%, respectively. Since randomized controlled trials were conducted in different settings, all analyses were performed using a random effects model. In addition, to detect potential sources of heterogeneity, subgroup analyses of ALT, AST, and LDH were performed based on the treatment method

(intraperitoneal injection or oral) or the MC-LR intervention doses (1/4-1/2LD_{50}, 1/2-1LD_{50}). Sensitivity analysis was performed on the indicators of the effect group. The publication bias was evaluated using Begg's rank correlation test and Egger's regression asymmetry tests. $P<0.05$ was considered statistically significant.

3. Results

3.1 Selection and identification

As of June 15, 2020, 3792 of the original 3819 publications (489 duplicates) obtained through database searches were excluded because they were not related to the current meta-analysis according to our inclusion criteria. After reading the full text of the remaining 27 papers, 23 studies did not meet the required criteria. The final analysis included a total of 4 eligible papers (12 treatment groups). A flowchart of study selection process was shown in Fig 1.

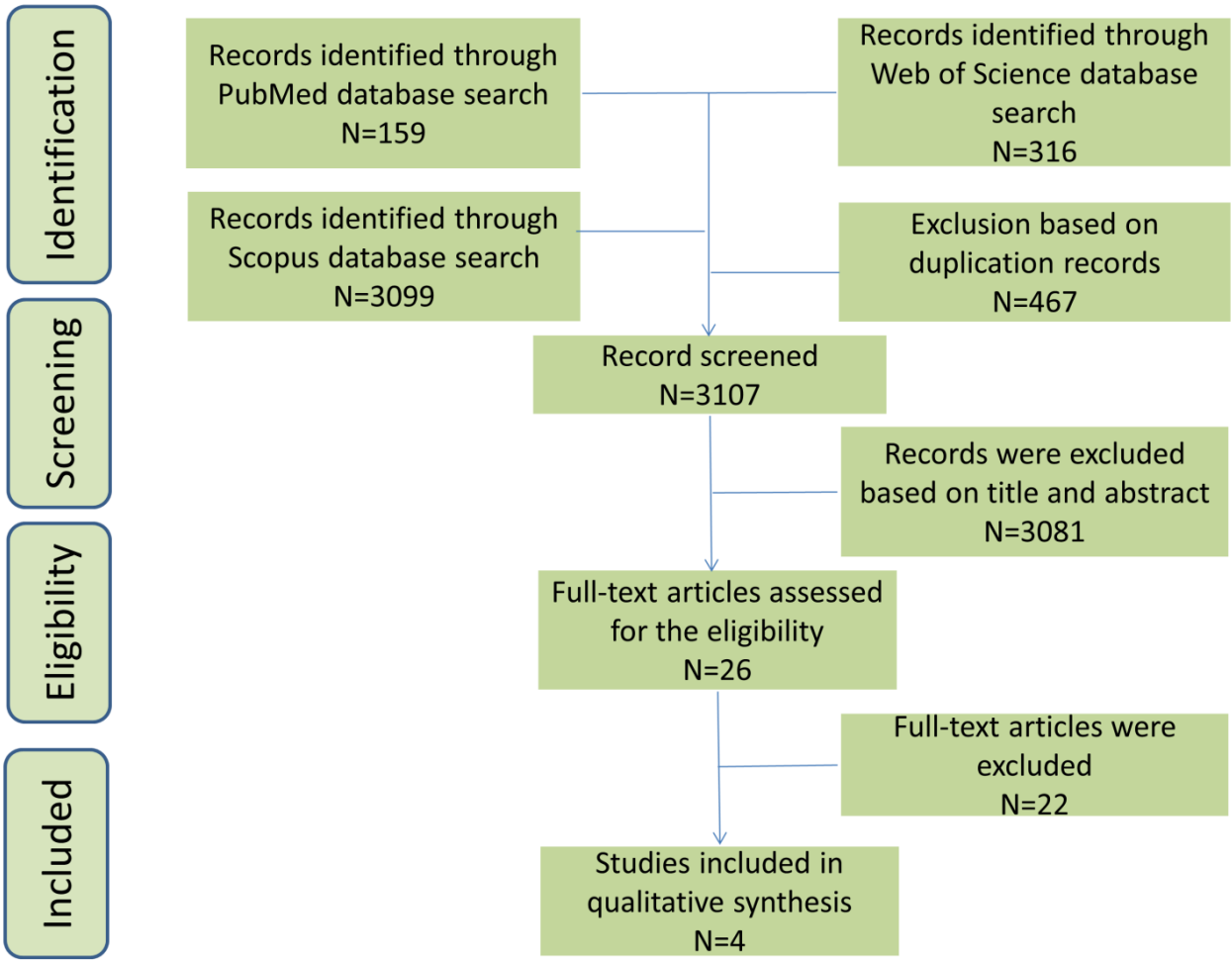

Figure 1. PRISMA flowchart describing systematic literature search and research option

3.2 Characteristics of Studies

This table describes the main features of the studies included in the current meta-analysis (Table 1). Overall, 143 mice from 12 treatment groups were extracted from 4 RCTs, including 75 in the exposure group and 68 in the control group[17-20]. The mice participating in these studies were all adults. All RCTs were designed in parallel studies. The dose of MC-LR was 1/4-1LD_{50} per day. The methods of administration were oral and intraperitoneal injection, and the duration of intervention varied from 1-14 days. In the publication of Rao PV1[20], there were six effect groups (6, 12, 18, 24, 30, 36) by week age for intraperitoneal administration, and two effect groups (6, 36) by week

age for oral administration. It has been pointed out in the article that the indicators needed for this study met the requirements. In the literature of Xianing Huang [19], there were two effect groups (1/4, 1/2LD_{50}) by intervention dose. According to the Cochrane score, all studies were classified as high-quality studies (scores≥3) (Table 2).

Table 1. Description of the studies included in the meta-analysis

Ref.	year	mice variety	sex	sample	methods	RCT design (blinding)	dose	time	Reported biochemical markers
Rao PV1	2004	Mus	male	160	i.p./p.o.	Parallel (double)	LD_{50}	24 h	ALT AST LDH
Xianing Huang	2013	KM	male	21	i.p.	Parallel (double)	1/4LD_{50}, 1/2LD_{50}	7 d	ALT AST ALP LDH
Shawn P. Clark	2007	B6.129-Trp53	male	9	i.p.	Parallel (double)	1/4LD_{50}	14 d	ALT AST
Igor Mrdjen	2018	CD-1	male	20	p.o.	Parallel (double)	1/2LD_{50}	7 d	ALT AST ALP TBIL

Note: i.p.: intraperitoneal p.o.: oral ALT: alanine aminotransferase AST: aspartate aminotransferase LDH: lactate dehydrogenase ALP: alkaline phosphatase ALB: albumin GSH: glutathione TBIL: total biliary red Vegetarian

Table2. Risk of bias assessment for included studies

Domain	Rao PV1	Shawn P. Clark	Xianing Huang	Igor Mrdjen
Random sequence generation (selection bias)	√	√	√	√
Allocation concealment (selection bias)	√	√	√	√
Blinding of participants and personnel (performance bias)	√	√	√	√
Blinding of outcome assessment (detection bias)	×	×	×	×
Incomplete outcome data (attrition bias)	√	√	√	√
Selective reporting (reporting bias)	×	?	?	×
Other sources of bias	?	×	√	√
Score	4	4	5	5
Overall quality	good	good	good	good

3.3 Effect of MC-LR on liver biochemical markers

Combining 4 randomized trials with 12 treatment groups, a significant increase of ALT concentration (mean difference (MD): 255.84 U/L, 95% CI: [217.55, 294.13], I^2 = 96%, P<0.001) and AST concentration (MD: 198.11 U/L, 95% CI: [161.73, 234.49], I^2 = 98%, P<0.001) was observed after exposing to MC-LR relative to control group (Fig 2a and Fig 2b). The change in LDH content (MD: 1911.3 U/L, 95% CI: [1081.5, 2741.2], I^2 = 100%, P<0.001) is shown in Fig 2c. It should be noted that the unit of the value of LDH was * 10 U/L when we made the forest plot, so the correct mean and confidence interval should be expanded ten times, but it did not affect the judgment of other statistical data on the results.

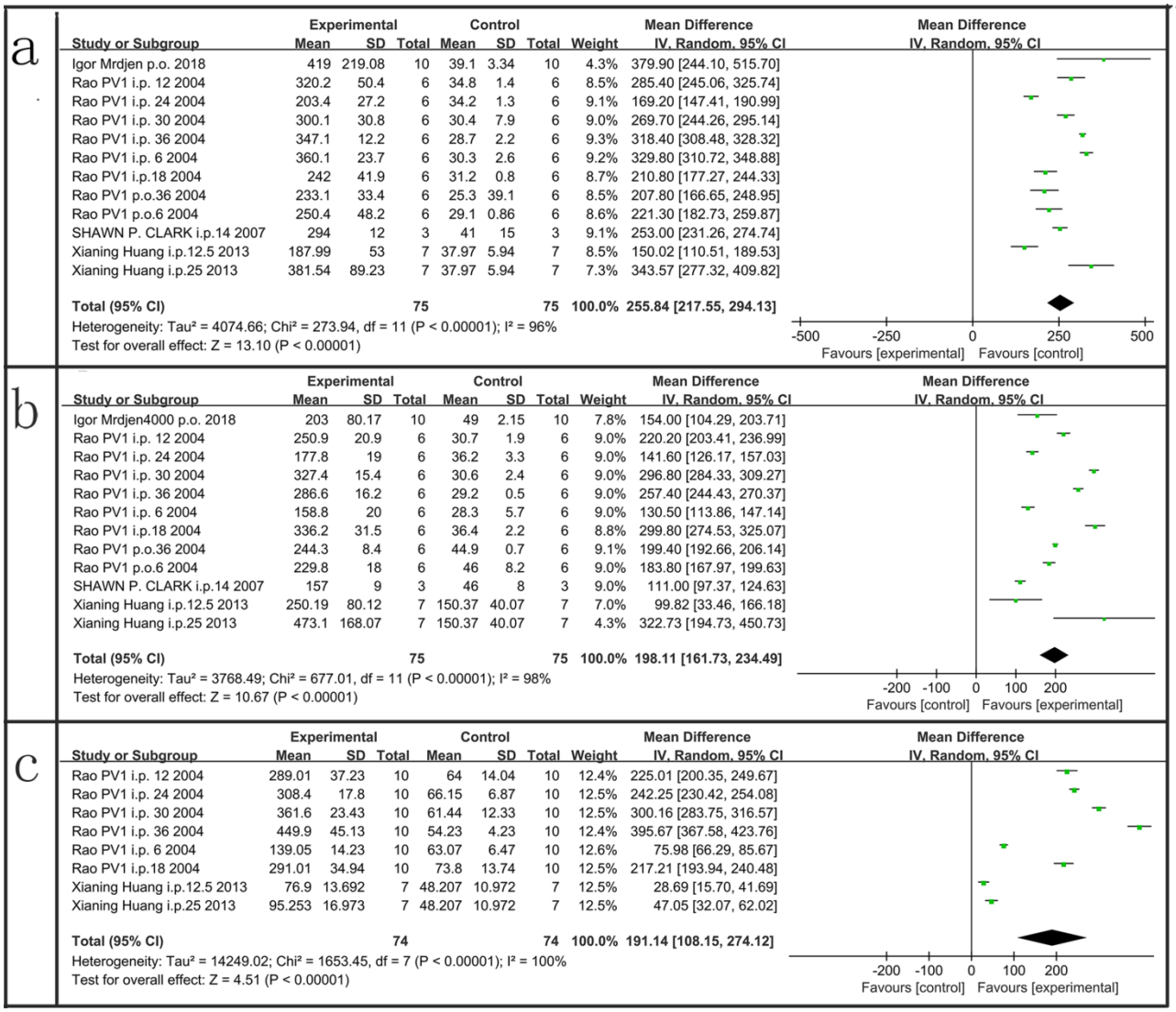

Fig 2. Forest plot of the effect of MC-LR processing on ALT (a), AST (b) and LDH (c)
Note: The size of the box represents the weight of the study, the horizontal line is the 95% confidence interval, and the diamond represents the total effect size

Because both the results of ALT and AST have high heterogeneity and different methods of infection, we performed a subgroup analysis on the methods of infection to explore the source of their heterogeneity. Subgroup analysis for ALT intraperitoneal injection (MD: 257.99 U/L, 95% CI: [214.48, 301.50], I^2 = 97%, P<0.001) and oral (MD: 231.05 U/L, 95% CI: [183.01, 279.09], I^2 = 67%, P<0.001) had a significant elevation (Fig 3a). It should be noted that when the Igor Mrdjen study was removed, the value of I^2 of the oral administration method was 0 without heterogeneity. Subgroup analysis for AST in intraperitoneal injection (MD: 205.33 U/L, 95% CI: [150.50, 260.17], I^2 = 99%, P<0.001) and oral (MD:

188.89 U/L, 95% CI: [171.55, 206.23], I^2 = 67%, P<0.001) also had a significant increase (Fig 3b). When the oral dose of MC-LR ranged from 1/2 LD_{50} to LD_{50}, the serum ALT and AST levels in the MC-LR group were about 9 and 5 times higher than those in the control group, respectively. These results also showed that the heterogeneity of oral administration was significantly reduced, indicating that the difference in the administration mode was one of the sources of the heterogeneity. See Table 3 for details.

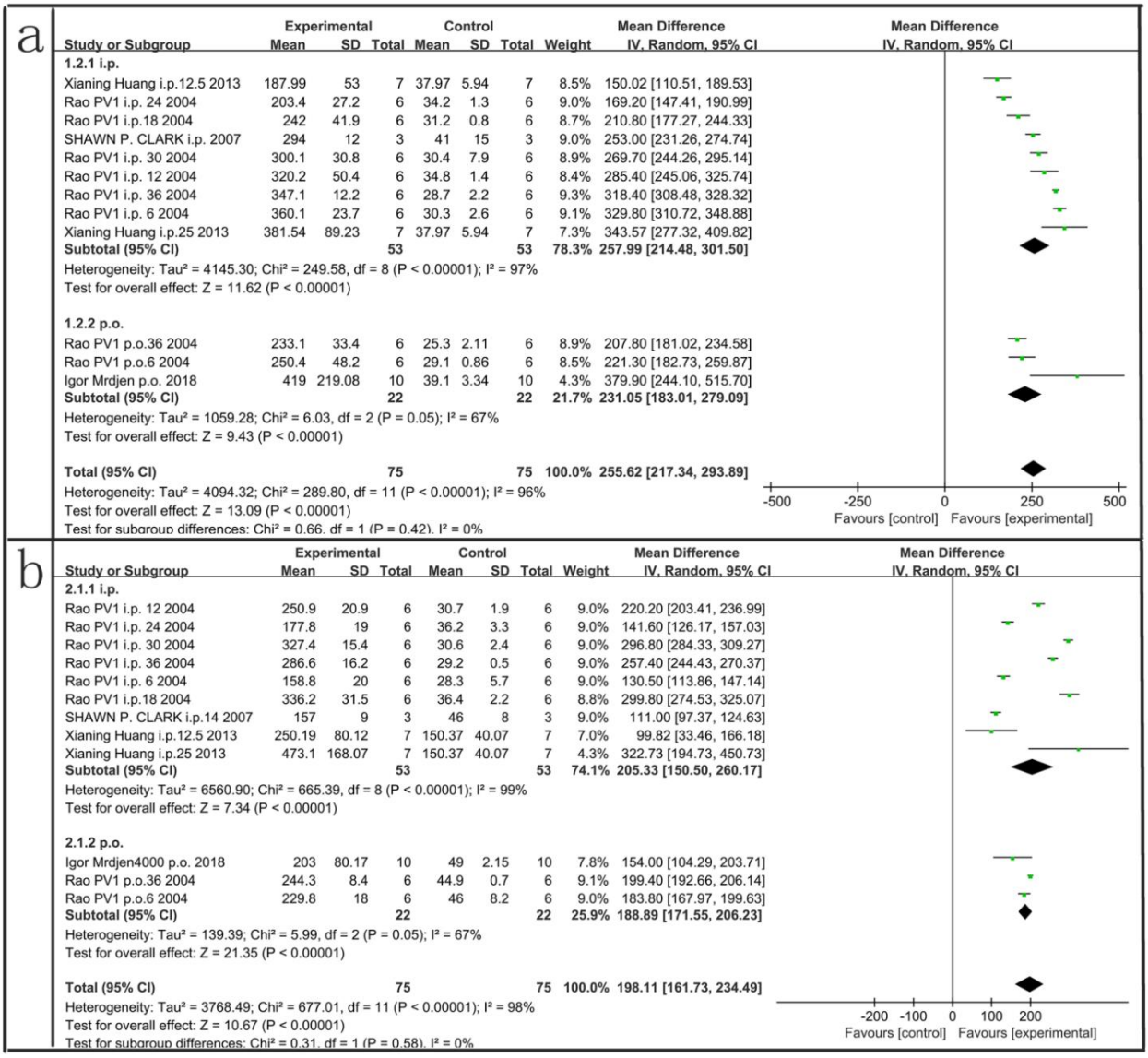

Fig 3. Subgroup analysis of the effects of different treatments on ALT (a) and AST (b)
Note: The size of the box represents the weight of the study, the horizontal line is the 95% confidence interval, and the diamond represents the total effect size.

In addition, to verify whether the differences in dose were also the cause of the heterogeneity under the intraperitoneal injection mode, we made a subgroup analysis on the different doses of intraperitoneal injection. The results of the analysis indicated that the concentrations of ALT, AST and LDH in serum increased more in the high-dose intraperitoneal injection of MC-LR than in low-dose intraperitoneal injection (Fig 4a, b and c). However, the heterogeneity of ALT and AST in different doses was still very high, while the heterogeneity of LDH decreased in 1/4-1/2 LD_{50} dose, and the variation of different doses was significantly different. In the low-dose exposure group, compared with the control group, ALT, AST and LDH in serum increased about 7, 2.5 and 1.5 times respectively. Compared with the control group, the

levels of ALT, AST and LDH in the high dose group were increased by 9, 8 and 5 times respectively. See Table 3 for details.

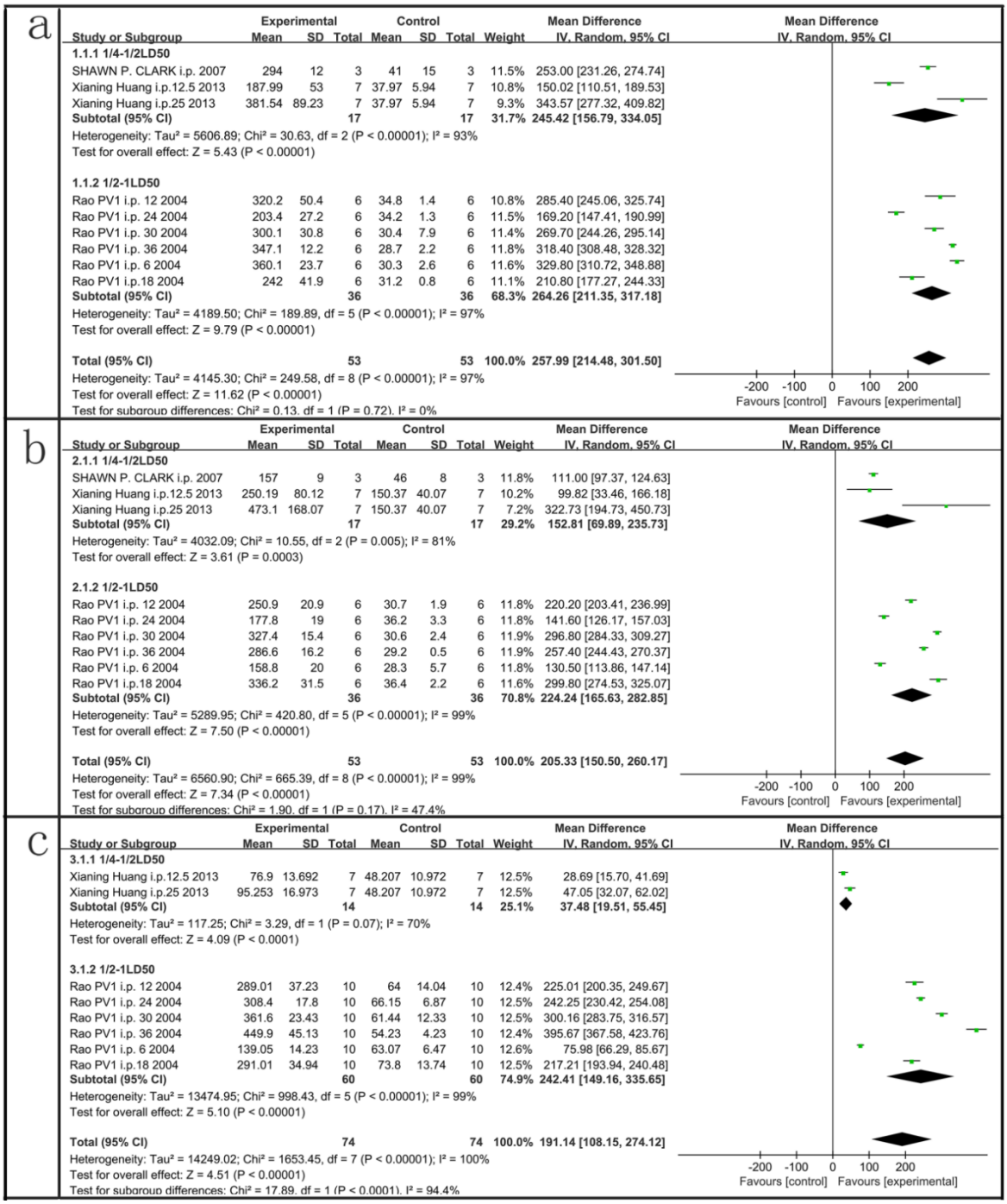

Fig 4. Subgroup analysis of effect of different injection doses on ALT (a), AST (b) and LDH (c)
Note: The size of the box represents the weight of the study, the horizontal line is the 95% confidence interval, and the diamond represents the total effect size

Table 3. Summary of the effects of MC-LR on liver biochemical markers

	Liver biochemical markers	95%CI (U/L)	*P* heterogeneity	I^2 (%)	*P* within group
Routes of treatment					
i.p.	ALT	257.99 [214.48, 301.50]	<0.001	97	<0.001
	AST	205.33 [150.50, 260.17]	<0.001	99	<0.001
p.o.	ALT	231.05 [183.01, 279.09]	0.05	67	<0.01
	AST	188.89 [171.55, 206.23]	0.05	67	<0.001
Dose of MC-LR					
1/4-1/2LD_{50}	ALT	245.42 [156.79, 334.05]	<0.001	97	<0.001
	AST	152.81 [69.89, 235.73]	<0.001	99	<0.001
	LDH	374.8 [195.1, 554.5]	0.07	70	<0.001
1/2-1LD_{50}	ALT	264.26 [211.35, 317.18]	<0.001	97	<0.001
	AST	224.24 [165.63, 282.85]	<0.001	97	<0.001
	LDH	2424.1[1491.6, 3356.5]	<0.001	99	<0.001

3.4 Sensitivity analysis

Each study in the analysis was eliminated in turn, and a new analysis was performed to evaluate the stability of the research results. The results showed that ALT had no heterogeneity when the effect group (Igor Mrdjen) was removed in the oral administration mode (Figure 5).

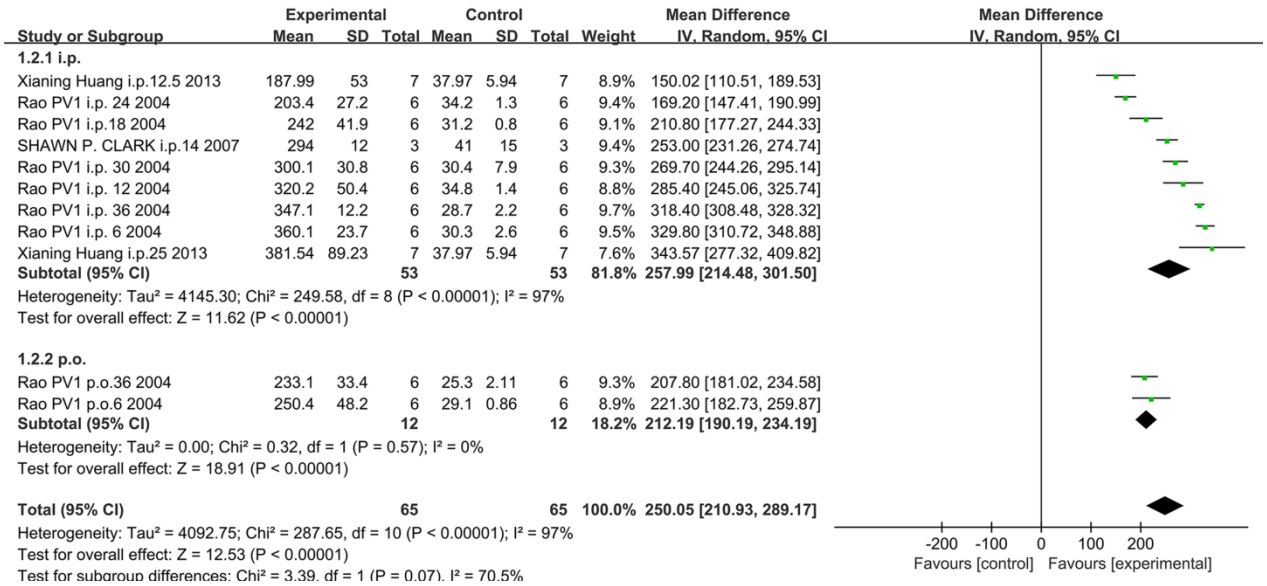

Fig 5. Changes in ALT after removing the effect group Igor Mrdjen

Note: The size of the box represents the weight of the study, the horizontal line is the 95% confidence interval, and the diamond represents the total effect size.

3.5 Publishing bias assessment

We used Begg's rank correlation test and Egger's regression asymmetry tests to evaluate whether there was publishing bias in the process of analysis. The results showed that there was no obvious publishing bias in any of the results.

4. Discussions

MC-LR is a hepatotoxin commonly found in the environment and poses a serious threat to human health. At present, many epidemiological studies have confirmed that MC-LR exposure can cause adverse

effects on the liver. It is noteworthy that MC-LR usually increases liver enzymes levels in the serum along with liver injury[21]. When the liver is damaged by MC-LR, early and correct diagnosis is very important. Changes in liver enzymes are usually the first clinical evidence to be noticed, and elevated serum AST and ALT levels often indicate hepatocyte injury[22, 23]. So it has important clinical significance to reveal the liver injury induced by MC-LR by detecting the changes of liver enzymes in serum.

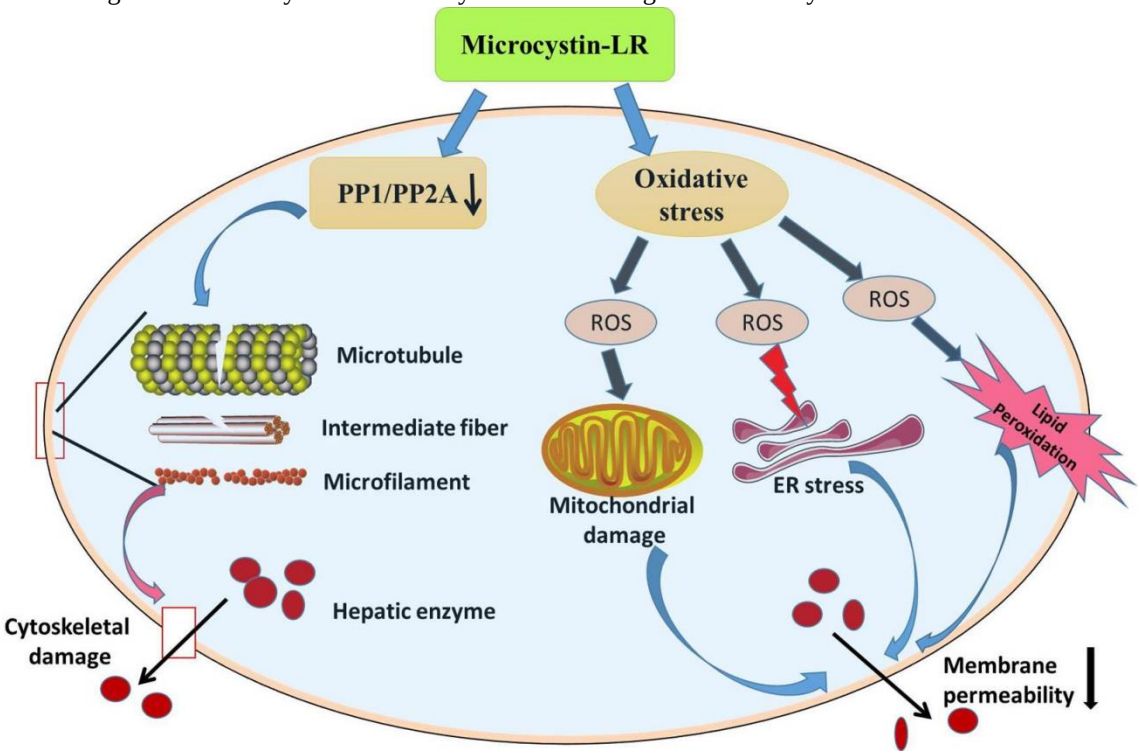

Fig 6. Mechanism of liver enzyme overflowing under the action of MC-LR.

ALT is a kind of cytosolic enzyme that can reflect liver injury[24]. Under normal circumstances, ALT mainly exists in the cytoplasm of the liver and hardly enters the blood. When the permeability of the cell membrane increases, ALT is released, and its concentration in the blood increases[25]. Aspartate aminotransferase (AST) mainly exists in tissue cells. Under normal conditions, the content of AST in serum is relatively low. AST is found in the cytosol and in mitochondria. When hepatocytes are damaged, the permeability of the cell membrane is significantly increased, AST is released into the blood, especially the cytosolic ones[26]. In the present study, the levels of ALT and AST in serum were significantly increased when mice were exposed to MC-LR, whether through intraperitoneal injection or oral administration. And in the same dose group, the change of serum AST level was more significantly altered after MC-LR exposure by the intraperitoneal route compared to the oral route. Moreover, during intraperitoneal injection, the serum levels of ALT and AST increase as the concentration of MC-LR increases. Compared with the 1/4-1/2LD_{50} group, the change of AST level in the 1/2-1LD_{50} group was more obvious than that of the ALT level.

Lactate dehydrogenase (LDH) is a stable cytoplasmic enzyme. It widely exists in human tissues and is abundant in the cytoplasm. Once the cell membrane is damaged, LDH is released out of the cell[27]. When injected intraperitoneally, the serum LDH level of mice in the exposure group was about four times higher than that in the control group. During MC-LR administration, serum levels of LDH increased significantly with the increase of dose. Compared with the corresponding control group, the serum LDH level in the low dose group increased by about 1.5 times, while the serum LDH level in the high dose group increased by about 5 times. LDH tends to signal the presence of ischemic liver injury and albumin allows the assessment of liver function[28].

The mechanism of liver injury induced by MC-LR is very complex. At present, some studies have found that the main toxic mechanisms of MC-LR involve the inhibition of intracellular protein phosphatase, and oxidative stress damage to the cell[29]. In vivo and in vitro studies have shown that MC-LR can directly inhibit the activity of protein phosphatase 2A (PP2A), resulting in the rearrangement or collapse of the three cytoskeleton components (microtubules,

intermediate fibers and microfilaments), and the damage of the cytoskeleton causes hepatocyte injury[30-32]. Another important toxic mechanism of MC-LR is the production of a large number of ROS in cells, which disrupts the antioxidant system, and causes a series of oxidative damage to cells by inducing endoplasmic reticulum stress, mitochondrial stress and lipid peroxidation. These damages can increase cell membrane permeability[33]. Eventually, liver enzymes such as ALT, AST, and LDH in liver cells penetrate the cell membrane and enter the serum, which significantly increases the concentration of transaminase in the serum (Fig 6). Therefore, the serum levels of ALT, AST, and LDH can reflect the degree of damage of liver cells, could assist in determining the severity of liver disease.

Since the lack of studies on liver enzymes in chronic MC-LR poisoning, we mainly analyzed the effects of acute MC-LR poisoning on liver biochemical indexes. The absolute changes of the indicators were described with the mean difference (MD), which had a stronger correlation with the clinical. To our knowledge, this is the first study to assess MC-LR-induced liver injury with changes in liver biochemical indexes by a meta-analysis. It has to be admitted that the present study still has some limitations. The data for meta-analysis is based on relatively a few studies, resulting in high heterogeneity of results, but we consider this still within acceptable limits.

Altogether, our study indicated that MC-LR exposure can increase serum LDH, ALT and AST levels, and a clear dose-response relationship exists after intraperitoneal injection of MC-LR. These results suggest that ALT, AST and LDH levels in serum are feasible as biomarkers of liver injury induced by MC-LR, which will be used for early clinical diagnosis and prevention of MC-LR toxicity.

5. Conclusion

MC-LR can increase serum LDH, ALT and AST levels, and a clear dose-response relationship was observed after intraperitoneal injection of MC-LR.

#These authors contributed equally to this work.

***Corresponding Author:**
Dr. Zhenxing Mao
Department of Epidemiology and Biostatistics, College of Public Health, Zhengzhou University, Zhengzhou, 450001, Henan, PR China.
E-mail: maozhr@gmail.com

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