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Review or Mini-review

Effects of glyphosate exposure on sperm concentration in rodents: A systematic review and meta-analysis

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ABSTRACT

Background: Correlation between exposure to glyphosate and sperm concentrations is important in reproductive toxicity risk assessment for male reproductive functions. Many studies have focused on reproductive toxicity on glyphosate, however, results are still controversial. We conducted a systematic review of epidemiological studies on the association between glyphosate exposure and sperm concentrations of rodents. The aim of this study is to explore the potential adverse effects of glyphosate on reproductive function of male rodents.

Methods: Systematic and comprehensive literature search was performed in MEDLINE, TOXLINE, Embase, WANFANG and CNKI databases with different combinations of glyphosate exposure and sperm concentration. 8 studies were eventually identified and random-effect model was conducted. Heterogeneity among study results was calculated via chi-square tests. Ten independent experimental datasets from these eight studies were acquired to synthesize the random-effect model.

Results: A decrease in sperm concentrations was found with mean difference of sperm concentrations(MDsperm) $= -2.774 \times 10^6$/sperm/g/testis(95%CI $= -0.969$ to -4.579) in random-effect model after glyphosate exposure. There was also a significant decrease after fitting the random-effect model: MDsperm $= -1.632 \times 10^6$/sperm/g/testis (95%CI $= -0.662$ to -2.601).

Conclusions: The results of meta-analysis support the hypothesis that glyphosate exposure decreased sperm concentration in rodents. Therefore, we conclude that glyphosate is toxic to male rodent's reproductive system.

1. Introduction

Glyphosate [*N*-(phosphonomethyl) glycine] is one of the most broad-spectrum pesticides, and one of the mostly used herbicides in agriculture globally. Glyphosate is the primary active ingredient in Round up® branded herbicides produced by Monsanto. With rapid popularization of transgenic glyphosate-resistant crops, the amount of glyphosate continue to increase worldwide. Glyphosate has increased by about 20% annually. It is not only the world's largest pesticide production and consumption varieties, but also China's fastest growing, highest output, and the largest export varieties of pesticides.

Glyphosate has been applied in many areas and causes serious pollution to soil and nearby ecosystems, and eventually increases the risk to human (Williams et al., 2000). For the past few years, researchers have paid attention to reproductive toxicity induced by glyphosate and Round up®. Exposure to glyphosate has been associated with many adverse effects on male reproductive system in both humans and rodents (de Brito Rodrigues et al., 2016; Williams et al., 2016b). In mammals, particularly rats, glyphosate could alter sperm characteristics including sperm production, and even fetal development (Chan and Mahler, 1992; Gasnier et al., 2009). Glyphosate causes libido and decreases ejaculate volume and sperm concentration in New Zealand rabbits, possibly due to direct cytotoxic effect of glyphosate on spermatogenesis or indirectly through the effect of hypothalamic pituitary testicular axis (Richard et al., 2005). So far, reported male toxic effects and risk assessment include reduced ejaculate volume, testosterone

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concentration, sperm production of the seminiferous epithelium, sperm concentration, sperm morphology, sperm motility and sperm aberration rate. The most frequently reported semen characteristic is sperm concentration in humans and rodents among these measurements.

However, other researchers have found contradictory conclusions. In Wlliams's review, the authors raised questions about reproductive toxicity of glyphosate in animals, and there was no definitive evidence that glyphosate or Roundup herbicide adversely impacted reproductive function (Williams et al., 2000). Besides, in other reproductive toxicity studies in rodents, no adverse effects have been observed: there was no difference in viability and mobility and sperm concentration, only an increase of abnormal sperm morphology at day 87 and day 122 after an acute exposure to glyphosate-based herbicide in both rats and mice experiments (Cassault-Meyer et al., 2014).

Therefore, potential detrimental effects of glyphosate on sperm concentration have been reported by a number of studies, however the results are inconsistent and controversial. In order to systematically evaluate our current knowledge in this field and provide accurate evidence on the influence of glyphosate exposure to male reproductive system, we conduct a systematic review and meta-analysis of several animal experiment studies to assess the effect of glyphosate exposure on rodents' sperm concentration. This is the first meta-analysis to synthesize reproductive effects of glyphosate exposure in a rodent model.

2. Materials and methods

2.1. Literature search strategy

We performed a comprehensive literature search on the association between glyphosate exposure on rodents and change of sperm concentration. The search was conducted in Pub Med, Web of Sciences, MEDLINE, TOXLINE, Embase, CNKI and Wanfang databases from January 1990 up to November 26,2016, with a combination of the following keywords: glyphosate; round up; reproductive toxicity; testicular; testes; sperm reserves; sperm quality sperm concentrations; male; animal; rats; and mice. Besides; we further examined titles and abstracts of all papers obtained to identify other potential articles. This search and evaluation was conducted in November 2016.

2.2. Inclusion criteria

We screened titles and abstracts of the papers from literature search to identify qualified studies. Selection of studies for further reviewing involved a two-phase process: first, title and abstract of a published paper were screened, and when it was unclear for inclusion from its abstract and title, full text were further retrieved and reviewed. The following criteria were used to assess the paper: a) Published in either English or Chinese between January 1990 and November 2016;b) Reported sperm concentrations; c) Reported results of analyses of an RCT; d) Glyphosate should be the only pesticide used in the experiment and cannot be combined with other pesticides or chemicals; e) Only *in vivo* experiment on rat and mouse models; f) In addition, publications with data available for further analysis were also included.

After the search, 56 published papers were selected and reviewed according to the aforementioned criteria. Most of the papers were excluded as they did not use mice or rats as experiment models. Others focused on reproductive toxicity to offspring. Finally, 8 studies on sperm concentration and with quantitative measures (mean value and standard deviation, SD) were included in our meta-analysis.

2.3. Data extraction

Screening of eligible studies was conducted by two independent reviewers to reduce subjective bias and improve reliability. In addition to mean value and corresponding SD of sperm concentration with or without glyphosate exposure, the following information was also extracted: first author, date of publication, experiment animal (rats or mice), strains of rats, strains of mice, animal age and body weight. All selected studies were controlled under standard animal experimental conditions. Some studies reported exact age and bodyweight of the rodents before and after glyphosate exposure, others only provided crude description, such as "mature" forage. According to the sexual cycle of rats, 21-day was regarded as weaned age and 80-day as mature age. Meanwhile, 54-day was considered as the average mature age for male mice.

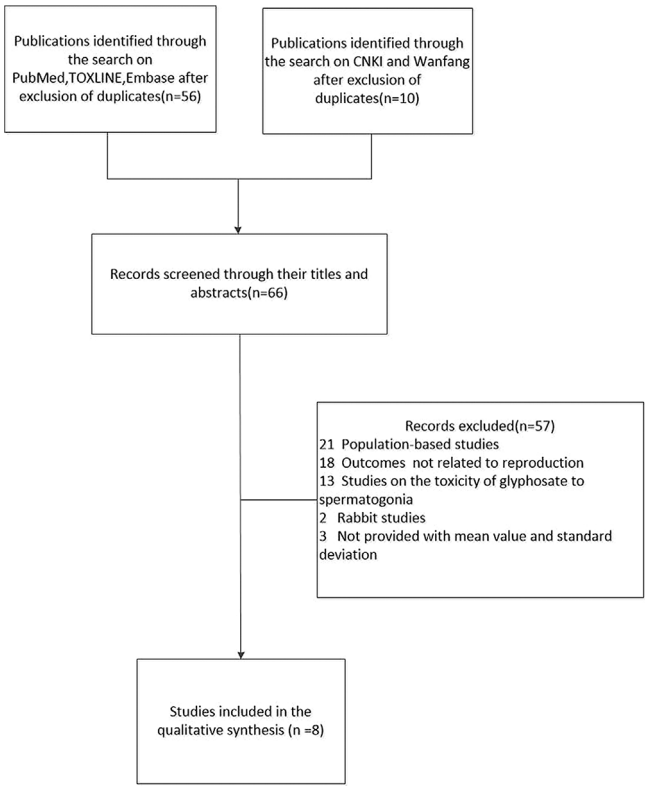

Fig. 1. Process of inclusion of studies in the meta-analysis.

To analyze pooled data, two different approaches were used. First, a fixed-effect model was constructed. Next, data from each study were fitted into a curve equation: changes in the amount of sperm as the dependent variable, and dose effect of glyphosate on rodents as independent variable. Effective dose of glyphosate was multiplied by the body weight (mg), exposure frequency, duration of exposure (day), and exposure dose (mg/kg). We recorded a new value of an independent variable (dose effect of glyphosate), to calculate the dependent variable (amount of sperm), which is the number of sperms by fitting equation. Finally, the fixed-effect model was performed again.

2.4. Statistical analysis

We used STATA software 11.0 (STATA Corp, College Station, TX, USA) for all analyses. Heterogeneity was assessed by the I^2 statistic and Q test with $P < 0.05$ and $I^2 > 50\%$ indicating evidence of heterogeneity. Random-effect model (Der Simonian-Laird method) was used to calculate the pooled effect estimates in the presence or absence of heterogeneity. Sensitivity analysis was conducted by sequentially excluding each study to assess the stability of the results. Begg's test was performed to assess publication bias. All tests were two-tailed and statistical significance was indicated by P values lower than 0.05.

Table 1
Characteristics of Studies Included in the Meta-Analysis of Sperm Concentrations.

Authors	Year	Rats/Mice	Strain	Unexposed Sperm concentrations (10^6/sperm/g testes)			Exposed Sperm concentrations (10^6/sperm/g testes)		
				n	mean	SD	n	mean	SD
Abarikwu	2015	rats	Wister	5	33.5	5	5	19.3	2.2
Eliane Dallegrave	2007	rats	Wister	15	44.2	4.2	15	57.4	6.7
Eliane Dallegrave				15	344.7	30.8	15	257.1*	17.9
Estelle Cassault-Meyer	2014	rats	SD	15	22	3	15	26	2
He Shenzhen	2016	rats	SD	10	8.89	0.98	10	3.72*	0.82
Po C. Chan	1992	rats	F344/N	10	610	36	10	486*	23
Chunmei Li	2016	rats	SD	8	40.72	2.905	8	29.42	1.956
Kang Jufang	2007	mice	KM	5	12.74	1.93	5	2.63	1.12
Kang Jufang				5	12.05	3.48	5	6.28*	3.84
Po C. Chan	1992	mice	B6C3F1	10	1162	44	10	1308	97
Zeng Ming	2010	mice	KM	5	12.54	1.8	5	2.64*	0.62
				5	12.68	2.26	3	6.91*	1.11
				5	12.35	1.57	4	6.27*	0.67

* Significant difference from unexposed, $P < 0.05$.

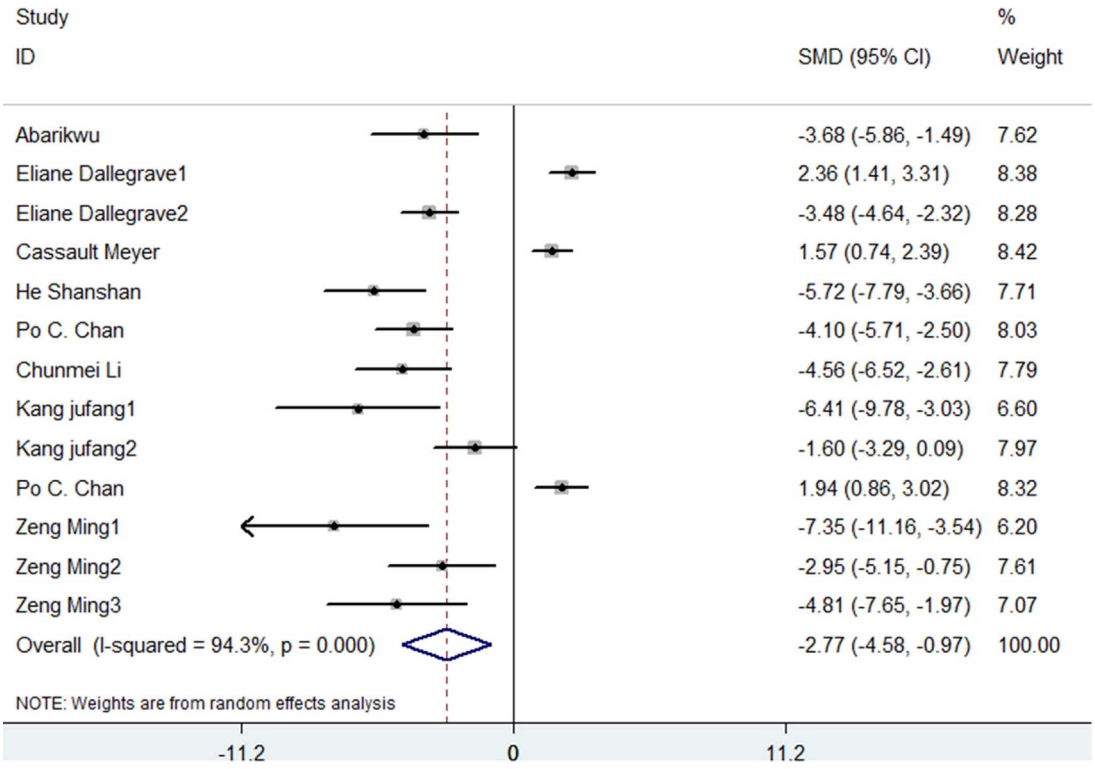

Fig. 2. Forest plot of MDsperm with their 95% CIsperformed by a random-effect model.

3. Results

3.1. Literature search

Search through MEDLINE, TOXLINE, and Embase databases yielded 56 unique articles (Fig. 1). 10 additional publications were identified through CNKI and Wanfang database. After screening their abstracts, titles, and whole article, 57 papers were excluded because the results were based on population (21 studies); outcomes were irrelevant to reproduction (18 studies); and studies focused on toxicity of glyphosate to sperm atogonia (13 studies). A final set of 8 papers on sperm concentration remained and were reviewed. 5 out of these 8 studies revealed significantly reduced sperm concentration after glyphosate exposure.

Several factors could interfere with study results: (1) intraspecific variability, (2) age at the start of experiments, (3) duration of glyphosate exposure, (4) body weight, and (5) other potential biological variations. Of the 8 selected studies, 5 used rats as animal models, and the other 3 used mice. In Dallegrave's study (Dallegrave et al., 2007), two groups of independent rat sperm concentration came from 60-dayage and 140-dayage rats, respectively. Kang's report had same situation (Kang et al., 2008), where two groups of independent mice sperm concentration were measured from 1 week after treatment and 4 weeks after treatment. In another study (Huang and Zeng, 2010), three groups of independent mice sperm concentration were measured 1 week, 4 weeks, and 5weeks after treatment.

Measurements of the included reports are displayed in Table 1. Fig. 2 shows the forest plot of the pooled estimate and 95% confidence interval of reduction in sperm concentrations after glyphosate exposure, calculated from a random-effect model. Sperm concentration

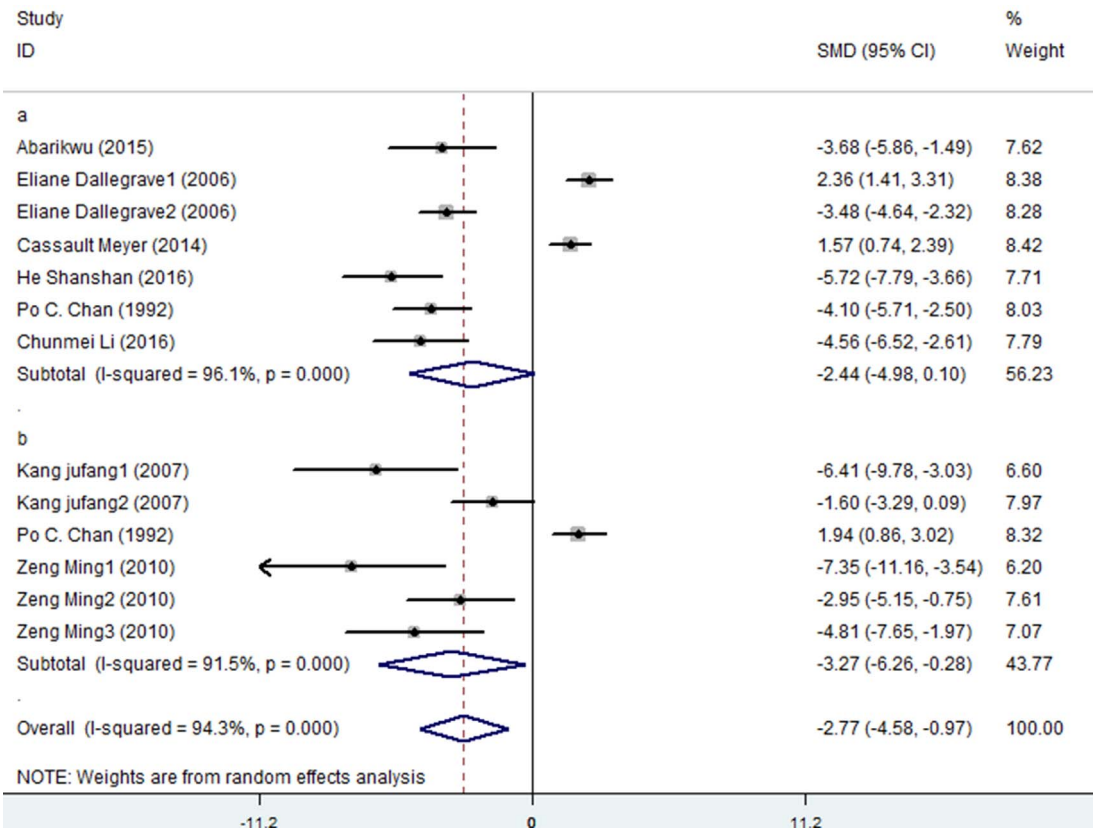

Fig. 3. Forest plot of MDsperm with their 95% CIsperformed by a random-effect model. A, means the group is in rat's animal model; B, means the group is in mice animal model.

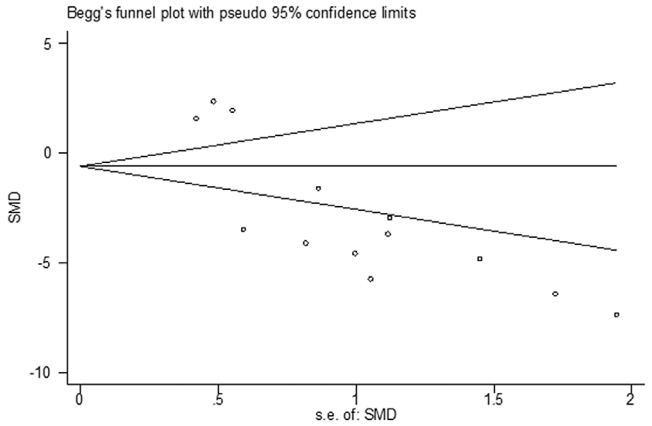

Fig. 4. Funnel plot for MDsperm in the meta-analysis of spermconcentrations.

significantly decreased after exposure to glyphosate, and the pooled mean difference of sperm concentration (MDsperm) was -2.774×10^6/sperm/g/testis (95%CI: −0.969 to −4.579).

Since there was significant heterogeneity between mice and rats, we divided the data into two subgroups accordingly: mice and rats. As shown in Fig. 3, pooled MDsperm of rats was-2.436 $\times 10^6$/sperm/g/testis (95%CI: −4.975 to −0.104),and pooled MDsperm of mice was -3.272×10^6/sperm/g/testis (95%CI: −6.263 to −0.282).

In the meta-analysis of paired testes weight, heterogeneity between the included 13 subgroups was significantly evident, thus a random-effect model was used throughout the analysis and showed by the symmetric funnel plots (Fig. 4). Publication bias was also observed in the meta-analysis of MDsperm. Begg's test showed an intercept of $P = 0.200$ for the meta-analysis of MDsperm, confirming the existence of publication bias.

3.2. Equation fitting

Measurements of the characteristics in the included papers were displayed in Table 2,with more independent experimental data from these studies. Weight was averaged across the data obtained in the papers. Because data utilization rate was not high in the meta-analysis and heterogeneity test results suggested a very large heterogeneity, we used each of the independent experimental data sets in order to fit the corresponding equations and improve the utilization rate of the data based on Table 2.

Total exposure dose was the product of body weight, duration of exposure, frequency of exposure, and dose of exposure. Total glyphosate exposure dose was defined as the independent variable 'X' and the unexposed sperm concentrations and exposed sperm concentrations were defined as the dependent variable 'Y' in the fitted equation. Scatter plot and the fitted curve equation for each dataset were demonstrated in Fig. 5.

According to the exposure model, exposure time and the average body weight of adult rats and mice, the 'X' are re-assigned 100, 600 and 900. Then 3 new Y values were shown in Table 3 after these adjustments. The negative values of the obtained Y should be of no practical importance. We finally computed Y value at X = 100 for meta-analysis again.

Forest plot of the pooled estimate and 95% confidence interval for reduction in sperm concentration in a random-effect model was displayed in Fig. 6. Sperm concentration significantly decreased after exposure to glyphosate, and pooled MDsperm was -1.632×10^6/sperm/g/testis (95%CI: −0.662 to −2.601). Since there was significant heterogeneity between mice and rats, we further divided the data into two subgroups according to different animal models of mice and rats (a and b in Fig. 7), respectively. Pooled MDsperm of rats was -0.53×10^6/sperm/g/testis (95%CI: −1.39 to −0.32),and pooled MDsperm of mice was -3.21×10^6/sperm/g/testis (95%CI: −4.92 to −1.49).

Table 2
Specific characteristics of Studies Included in the Meta-Analysis of Sperm Concentrations.

Authors	Year	Rats/mice	Strain	Body weight (kg)	Ages (day)	Duration of exposure (day)	The frequency of exposure	Exposure dose (mg/kg)	Total exposure dose(mg)	n	Unexposed Sperm concentrations and Exposed Sperm concentrations (10^6/sperm/g testes)
Eliane Dallegrave	2006	rats	Wister	0.2995	65	0		0	0	15	44.2
						60	1[a]	50	898.5	15	53.9
								150	2695.5	15	67.2
								450	8086.5	15	57.4
Eliane Dallegrave[b]	2006	rats	Wister	0.2995	140	0		0	0	15	344.7
						140	1[a]	50	898.5	15	251
								150	2695.5	15	368.7
								450	8086.5	15	257.1
He Shanshan	2016	rats	SD	0.05	30	0	0	0	0	10	8.89
						30	1	250	375	10	4.52
								500	750	10	4.42
								1000	1500	10	3.72
PoC. Chan	1992	rats	F344/N	0.225	136	0	0	0	0		610
						91	1	12500	255937.5	10	561
								25000	511875	10	485
								50000	1023750	10	486
Kang Juan	2007	mice	KM	0.027		0	0	0	0	5	12.74
						5	3[c]	40	3.204	5	6.01
								290	38.715	5	11.04
								580	77.43	5	2.68
								1160	154.86	5	2.63
Kang Jufang	2007	mice	KM	0.027		0	0	0	0	5	12.05
							3	40	3.204	4	6.15
								290	38.715	5	8.45
								580	77.43	5	8.27
								1160	154.86	3	6.28
Po C. Chan	1992	mice	B6C3F1	0.023	143			0	0	10	
						284	1	12500	81650	10	1370
								25000	163300	10	1189
								50000	326600	10	1308
Zeng Ming	2010	mice	KM	0.023	56	0	0	0	0	5	12.54
						5	3	40	3	5	7.66
								290	36.25	5	10.89
								580	72.5	5	2.77
								1160	145	5	2.64
ZengMing[d]				0.023	84			0	0	5	12.86
							3	40	3	4	6.82
								290	36.25	5	9.63
								580	72.5	5	8.13
								1160	145	3	6.91
ZengMing[e]					91		3	0	0	5	12.35
								40	3	5	6.46
								290	36.25	5	8.65
								580	72.5	5	8.31
								1160	145	4	6.27

[a] The frequency of exposure is once a day.
[b] The data from second studies in the Eliane Dallegrave's article is measured in mice 140 days after exposured.
[c] The data from second studies in the Kang Jufang's article were measured after the mice were exposed to normal culture for a period of 4 weeks.
[d] The data from second studies in theZeng Ming's article were measured after the mice were exposed to normal culture for a period of 4 weeks.
[e] The data from second studies in theZeng Ming's article were measured after the mice were exposed to normal culture for a period of 5 weeks.

Heterogeneity among studies, as measured by the Q test and the I^2 statistic, was lower than the first meta-analysis but still remained significant in the random-effect model (I^2 = 80.6%).In mice group, I^2 was 76.3%, while $I^2$was 66.6% in rats group. There was significant heterogeneity in both groups in the random-effect model. According to the sensitivity analysis, sequential removal of a single study did not result in notable changes, and this outcome suggested that the results were stable and robust(Fig. 8).

4. Discussion

There is still controversy on whether glyphosate exposure could cause cancer in humans or animals, as well as other non-cancer diseases (Williams et al., 2016a; Williams et al., 2016b). Recent study has reported that glyphosate could cause weight loss, decreased libido, poor ejaculate volume and low sperm concentrations following a dose-dependent manner in male New Zealand white rabbits, accompanied by increased abnormal and dead sperms (Yousef et al., 1995). Decreasing sperm concentration is the result from a variety of chemical substances on the growth and development of spermatogenic cells and sperm morphology is an indirect index to evaluate the potential adverse effects on sperm genetic material from chemicals. Abnormal sperm rate reflects the reproductive toxicity and potential mutagenicity of the chemical toxicant (O'Shaughnessy, 2014; O'Shaughnessy et al., 2009).

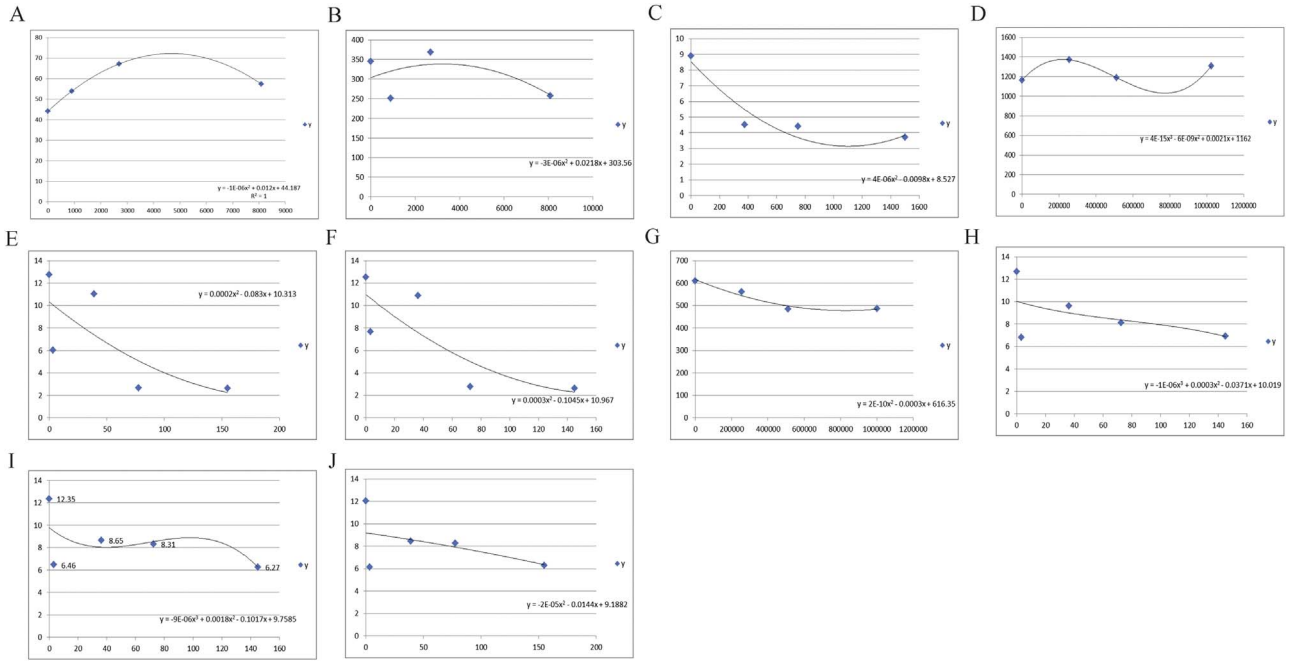

Fig. 5. Fitted curve. A. Eliane et al. 's study 1; B. Eliane et al.'s study 2; C. He et al.'s study; D. Chan et al.'s study 1(rat); E. Kang et al.'s study 1; F. Kang et al. 's study 2; G. Chan et al.'s study 2(mice); H. Zeng et al. 's study 1; I. Zeng et al. 's study 2; J. Zeng et al.'s study 3.

Table 3
Fitting equation and Y value corresponding to X value.

Authors	Strain	fitted equation	X_1 = 100 Y=	X_2 = 600 Y=	X_3 = 900 Y=
Eliane Dallegrave	Rats	$Y1 = -1E\text{-}06 \times^2 + 0.012x + 44.187$	45	51	54
Eliane Dallegrave[a]	Rats	$Y2 = -3E\text{-}06 \times^2 + 0.0218x + 303.56$	306	316	321
He shanshan	Rats	$Y3 = 4E\text{-}06 \times^2 - 0.0098x + 8.527$	8	4	3
PoC. chan	Rats	$Y4 = 2E\text{-}10 \times^2 - 0.0003x + 616.35$	616	616	616
Kang Iufang	Mice	$Y5 = 0.0002 \times^2 - 0.083x + 10.313$	4	33	98
Kang Jufang[b]	Mice	$Y6 = -2E\text{-}05 \times^2 - 0.0144x + 9.1882$	8	−7*	−20*
Po C. Chan	Mice	$Y7 = 4E\text{-}15 \times^3 - 6E\text{-}09 \times^2 + 0.0021x + 1162$	40001162	8640001163	29160001164
ZengMing	Mice	$Y8 = 0.0003 \times^2 - 0.1045x + 10.967$	4	56	160
ZengMing[c]	Mice	$Y9 = -1E\text{-}06 \times^3 + 0.0003 \times^2 - 0.0371x + 10.019$	8	−120*	−509*
ZengMing[d]	Mice	$Y10 = -9E\text{-}06 \times^3 + 0.0018 \times^2 - 0.1017x + 9.7585$	9	−1347*	−5185*

[a] The data from second studies in the Eliane Dallegrave's article is measured in mice 140 days after exposure.
[b] The data from second studies in the Kang Jufang's article were measured after the mice were exposed to normal culture for a period of 4 weeks.
[c] The data from second studies in the Zeng Ming's article were measured after the mice were exposed to normal culture for a period of 4 weeks.
[d] The data from second studies in the Zeng Ming's article were measured after the mice were exposed to normal culture for a period of 5 weeks.
* Significant difference from unexposed, $P < 0.05$.

Serum testosterone level was significantly decreased in rats treated with glyphosate, which may be one of the reasons for the decline of sperm concentration (Sarkar et al., 2003).

Roundup® is a commercial formulation of the herbicide glyphosate and active ingredient in common formula are 120, 240, 360, 480, and 680 g/L. Roundup® has given rise to a considerable amount of reproductive toxicity on male rats including decreased testosterone, lower epithelium height as well as luminal diameter (Romano et al., 2010). Mature rats testicular cells exposed to glyphosate and Roundup® at a lower level have approximately 35% reduction in testosterone concentration *in vitro* (Clair et al., 2012). These results are consistent with our meta-analysis, and we have also found that glyphosate can decrease sperm concentration in both low and high levels. We suggest more studies to explore the explicit dose-response relationship and mechanism of reducing sperm concentration under glyphosate exposure in rodents.

Meta-analysis increases statistical power by combining results from different studies with substantial variation in source population, exposure and outcome assessment and classification, control for confounding, and other key characteristics. The method of curve fitting has the following advantages: (a). All data in each experiment are fully utilized. (b). The effect of confounding factors such as body weight and age can be controlled and reduced by reselecting the same dose of exposure.

However, there are several shortcomings in the meta-analysis as well: (a). The closer the coefficient of determination (R^2) to 1, the better the goodness-of-fit of the equation. However, some R^2in this study are small according to the scatter plots, indicating a suboptimal fit. (b). It is possible that the value of X in the fitted equation is beyond the applicable range of the original total exposure dose.

Besides the technical challenges from meta-analysis, our study have a few other limitations that should be taken into account when interpreting our findings. Heterogeneity of the results obtained from the first meta-analysis was very high even after stratification (rats and mice). Possible reasons for such large heterogeneity might be:(a). A large difference in age and weight of the rodents. (b). Exposure methods were different from study to study. In the acute toxicity experiment, the duration of exposure was short and exposure dose was large, while exposure time was long and exposure dose was low in the chronic toxicity experiment. A common solution is to perform a subgroup

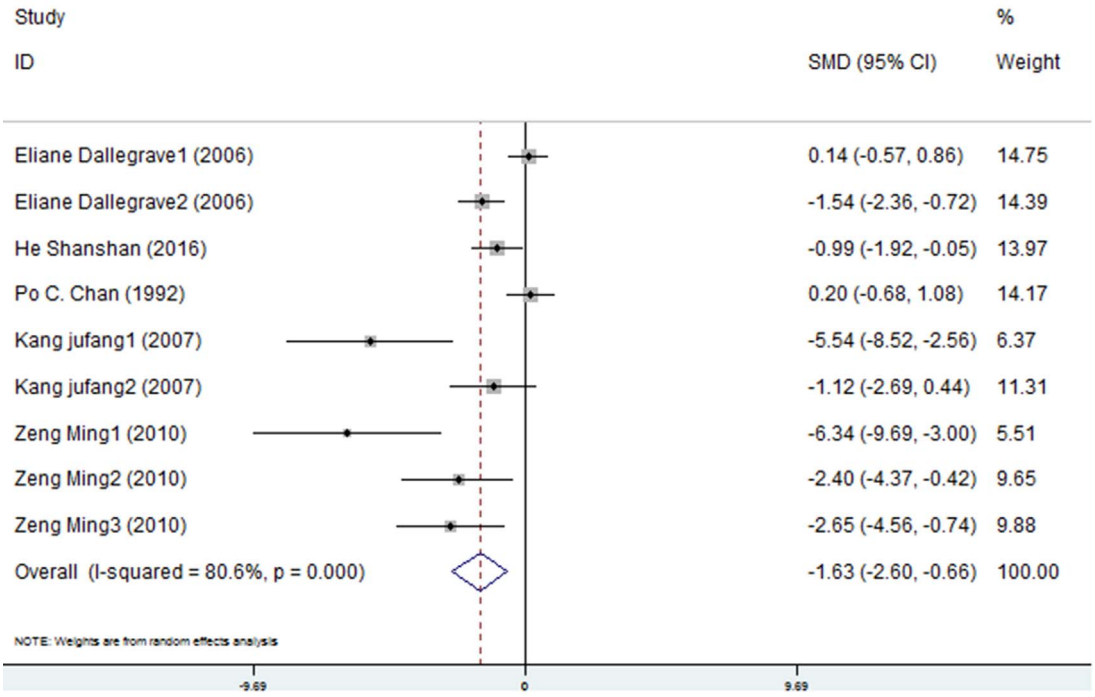

Fig. 6. Forest plot of MDsperm of fitting equationwith their 95% CIsperformed by a random-effect model.

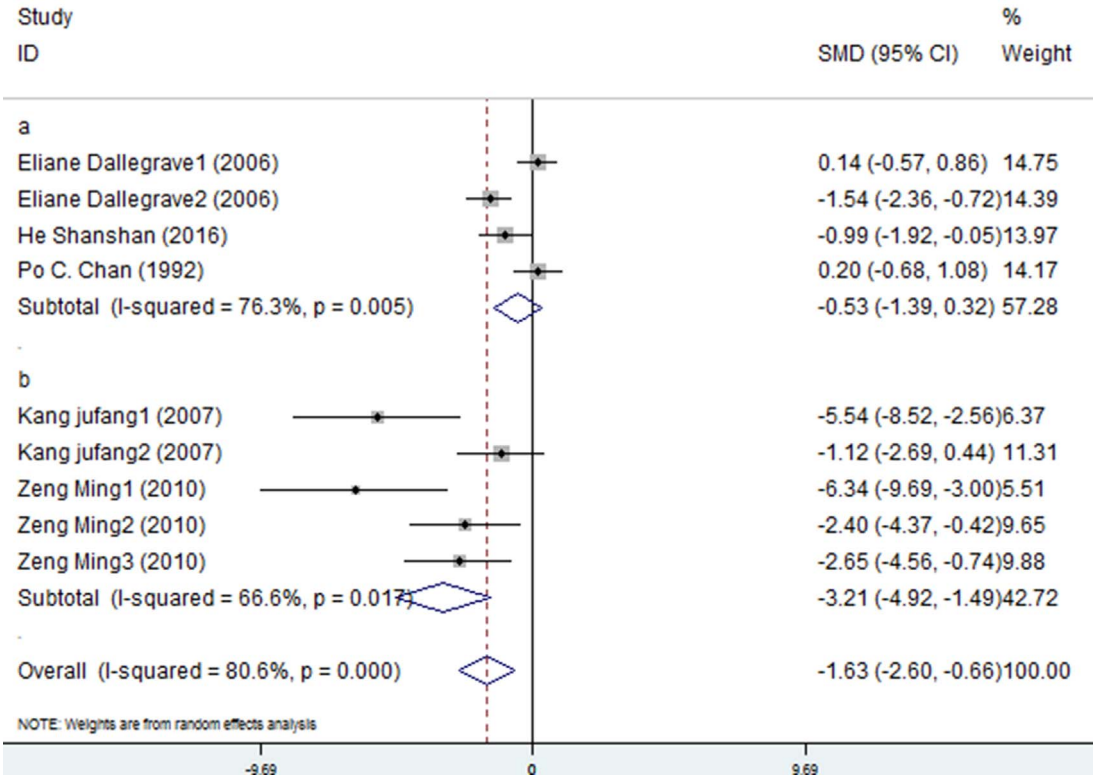

Fig. 7. Forest plot of MDsperm of fitting equation with their 95% CIsperformed by a random-effect model a means the group is in rats animal model's means the group is in mice animal model.

analysis recommended by Oxman and Guyatt (1992). Indeed, combining specific subgroup data across studies might provide further insight into heterogeneity. In subgroup *a* and *b*, heterogeneity both decreased. (c). The papers selected in our meta-analysis were written in only two languages, Chinese and English, and the publication time dated back to no earlier than 1990. Therefore, selection bias might exist as well.

In conclusion, results from our meta-analysis have suggested that exposure to glyphosate caused decrease in sperm concentration in rodents (both mice and rats), and consequently impose adverse effect on

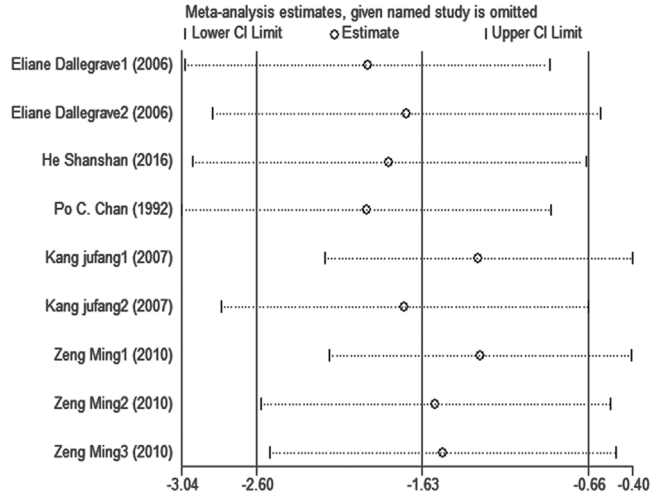

Fig. 8. Sensitivity analysis.

reproductive health. It is desirable to extend the study of glyphosate and its influence on reproductive health in humans and other mammals.

Disclosure statement

The authors declare that there is no financial conflict of interest.

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