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Bi-directional regulation of TGF-β/Smad pathway by arsenic: A systemic review and meta-analysis of in vivo and in vitro studies

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ABSTRACT

Background: Arsenic exposure can cause fibrosis of organs including the liver, heart and lung. It was reported that TGF-β/Smad pathway played a crucial role in the process of fibrosis. However, the mechanism of arsenic-induced fibrosis through TGF-β/Smad signaling pathway has remained controversial.
Objective: A systematic review and meta-analysis was performed to clarify the relationship between arsenic and TGF-β/Smad pathway, providing a theoretical basis of fibrosis process caused by arsenic.
Methods: A meta-analysis was used to reveal a correlation between arsenic and fibrosis markers of TGF-β/Smad pathway, including 47 articles of both in vivo and in vitro studies. (Standardized Mean Difference) SMD was employed to compare and analyze the combined effects. When I^2 > was 50%, random effect model was selected and subgroup analysis was used to explore the source of heterogeneity.
Results: Arsenic exposure up-regulated the expression of TGF-β1, p-Smad2/3, α-SMA, Collagen1/3 and FN. The dose-response relationship showed that low dose (≤5 μmol/L) arsenic exposure up-regulated the expression of TGF-β1, whereas high doses had a tendency to down-regulate that of TGF-β1. Subgroup analysis showed that low or short-term arsenic exposure induced the expression of TGF-β1 and fibrosis markers.
Conclusion: The results indicated that arsenic activates the TGF-β/Smad pathway and induced fibrosis. The mechanism is related to the up-regulation of NADPH oxidase and ROS accumulation. However, high-dose arsenic exposure may inhibit this pathway.

1. Introduction

Arsenic is a known toxic metal. During recent years, in comparison to others, China and India have become the most affected countries with serious diseases and the largest number of victims seen in hospitals due to arsenic poisoning [1]. Inorganic arsenic is more toxic than the organic one which is widely distributed in water, air and soil [2]. Exposure to inorganic arsenic can cause fibrosis in multiple organs [3,4]. At present, the molecular mechanism of arsenic-induced fibrosis is still unclear and needs further study.

Arsenic exposure stimulates the production of ROS; including superoxide anion, hydrogen peroxide, hydroxyl radicals and nitric oxide, which induce the expression of inflammatory and pro-fibrotic factors in the body [5]. NADPH oxidase is regarded as the main source of reactive oxygen species in the body. Arsenic can induce ROS production through NADPH oxidase (NOX2, NOX4, p47phox), which stimulates the transfer of α-SMA into the nucleus to induce extracellular matrix (ECM) synthesis. On the one hand, if arsenic exposure induces ROS production and is regarded as an important factor in arsenic-induced renal fibrosis [6]. While on the other, we found that low concentrations of arsenic (30 ppm) in male Sprague–Dawley rats induced ROS production and NADPH oxidase (NOX2, p47phox and NOX4) activities, which in turn up-regulated TGF-β1, Collagen1 and expression of α-SMA mRNA thus inducing fibrosis in liver tissue [7]. We also found out that arsenic activates the expression level of NADPH oxidase (NOX2, NOX4) and accumulation of ROS, which in turn stimulates the expression of pro-inflammatory factors (IL-1β, TNF-α) and pro-fibrotic factors thus leading to oxidative damage to DNA, proteins and lipids [8]. In addition, arsenic also inhibits NOX4 protein expression, decreases the expression of TGF-β1, p-Smad3 and p-Smad2 proteins, which in turn inhibits lung fibrosis [9]. These results are baffling with the unanswered question of how arsenic regulates NADPH oxidase.

TGF-β/Smad signaling pathway is an important pathway regulating fibrosis, along with TGF-β1 as the most effective pro-fibrotic cytokine [10]. TGF-β is mainly regulated by the Smads superfamily. Phosphorylation of Smad2/3 is through the activation of membrane specific

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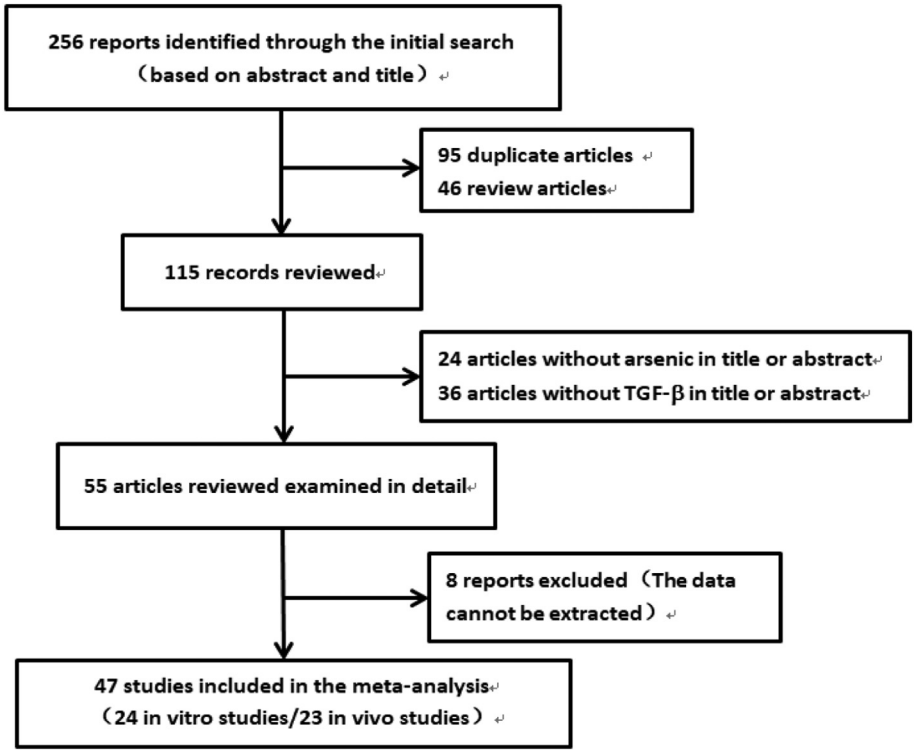

Fig. 1. Flow-chart of the methodology used to identify studies for inclusion in the meta-analysis.

Table 1
Basic characteristics of the included literature for using in vitro studies.

Reference	Country	Cell types	n	Types of arsenic	Concentration (μmol/l)	Time (hour)	Outcome indicators
Z. H. Lv[a] [30]	China	HL-60	3	AS_2O_3	> 5	< 48 h	1
J. W. Zhang[a] [31]	China	SGC-7901	3	AS_2O_3	≤5	≥48 h	1
C. L. Yu[a] [32]	China	ECA109	3	AS_2O_3	≤5	≥48 h	1
G. S. Jiang [33]	China	K562	3	AS_2O_3	≤5	≥48 h	1.7
L. Wang[a] [34]	China	HL-60	7	AS_2O_3	> 5	≥48 h	1
M. Nanjundan [27]	America	HEY cells	3	AS_2O_3	> 5	< 48 h	1.2.3.4
Y. Li[a] [35]	China	HL-60cells	3	AS_2O_3	≤5	< 48 h	1
H. S. Huang [36]	China	HepG2	3	AS_2O_3	≤5	< 48 h	3.4
T. Hirano [37]	Japan	PBMCs	4	AS_2O_3	≤5	≥48 h	1
B. F. Yang [28]	China	CFs	3	AS_2O_3	≤5	< 48 h	1.6.12
K. H. Bi[a] [38]	China	K562	3	AS_2O_3	≤5	≥48 h	1
P. Allison [14]	America	IME cells	3	$NaAsO_2$	≤5	< 48 h	1.2.3
W. F. Chu [12]	China	NRCFs	3	AS_2O_3	> 5	< 48 h	1.2.3.5.6
W. Yue [39]	China	C6 cells	5	AS_2O_3	≤5	< 48 h	1
S. N. Liu [40]	China	SV-HUC-1	3	$NaAsO_2$	≤5	< 48 h	1
X. J. Yang [17]	China	PC-3/LNCaP	3	AS_2O_3	≤5	< 48 h	2.3.13
J. P. Zhang [18]	China	MHCC97H	3	AS_2O_3	> 5	≥48 h	3
W. F. Chu [29]	China	NMCFs	3	AS_2O_3	≤5	< 48 h	1.2.3.8.12
B. F. Yang [41]	China	HAECs	3	AS_2O_3	≤5	≥48 h	9.10.11.12
L. Wang[a] [42]	China	HL-60	3	AS_2O_3	> 5	≥48 h	1
Y. Zhang [19]	China	RA-FLS	3	AS_2O_3	≤5	≥48 h	1
W. F. Chu [43]	China	NMCMs	3	AS_2O_3	≤5	< 48 h	1.6
Y. P. Song[a] [44]	China	PBMNC	5	AS_2O_3	≤5	≥48 h	1
X. H. Du [45]	China	BGC823	3	AS_2O_3	≤5	≥48 h	1

Note: n = Sample size; 1 = Transforming growth factor-β(TGF-β), 2 = p-Smad2, 3 = p-Smad3, 4 = NOX2, 5 = NOX4, 6 = Reactive oxygen species (ROS), 7 = Collagen content, 8 = Type-1 collagen (Collagen1), 9 = Type-3 collagen (Collagen3), 10 = α-smooth muscle actin (α-SMA), 11 = Fibronectin (FN), 12 = Plasminogen activator inhibitor-1 (PAI-1), 13 = Fibrosis.

[a] Published in Chinese.

serine/threonine kinase receptor and then bound to the common type Smad4 to form oligomers, which can transport to the nucleus to initiate the expression of TGF-β1, leading to the levels of fibrosis-related genes, such as α-SMA, Collagen1, FN [11]. ROS produced by reactive nitrogen mediates intracellular TGF-β/Smad signal transduction and promotes the expression of fibrosis factors. We found out that As_2O_3 up-regulated the expression of TGF-β1 and p-Smad2/3 to induce cardiac and smooth muscle cells fibrosis [12]. Under the action of arsenic, the expression of Smad3, Smad4, TβRII and TGF-β significantly increased the induction of hypertension and vascular fiber remodeling [13]. T. D. Camenisch et al. [14] found that arsenic exposure promoted the expression of TGF-β1 and the phosphorylation of Smad2/3, which were the signal transduction protein of TGF-β1, leading to the occurrence of cardiovascular diseases. Whilst, it was also discovered, among rats studies, arsenic

Table 2
Basic characteristics of the included literature for using in vivo studies.

Reference	Country	Species	Age	Weight	n	Types of arsenic	Unit	Route of administration	Outcome indicators
Q. Yang [46]	China	Mice	10 months	–	3	$NaAsO_2$	> 50 mg/L	Drinking water	7.10.11.12
Y. H. Liang[a] [16]	China	Mice	–	200 ± 20 g	5	AS_2O_3	≤50 mg/L	Drinking water	1
F. R. Liu[a] [47]	China	Rats	–	180–200 g	10	$NaAsO_2$	> 50 mg/L	Drinking water	1
L. Li[a] [7]	China	Mice	–	18–20 g	10	$NaAsO_2$	> 50 mg/L	Drinking Water	1.8.12
D. Ghosh [48]	India	Mice	–	120–150 g	5	$NaAsO_2$	≥5 mg/kg	Gavage	1.6
J. C. Xing [49]	China	Rats	8–12 weeks	–	3	AS_2O_3	≥5 mg/kg	Gavage	1
X. Pan [8]	China	Mice	8–12 weeks	180 ± 10 g	10	$NaAsO_2$	≤50 mg/L	Drinking Water	1.2.3.4.5.7.9.11
Z. Q. Qi [50]	China	Rats	8–12 weeks	–	3	AS_2O_3	< 5 mg/kg	Gavage	1
Z. P. Xu[a] [51]	China	Mice	8 weeks	16-20 g	9	AS_2O_3	< 5 mg/kg	Gavage	1.9
Y. Q. Deng[a] [52]	China	Mice	–	18–22 g	3	AS_2O_3	≤50 mg/L	Drinking water	1
B. F. Yang [28]	China	Rats	–	250–350 g	3	AS_2O_3	< 5 mg/kg	Gavage	1.6.13
Z. Q. Qi [53]	China	Guinea pigs	8–12 weeks	20 ± 2 g	3	AS_2O_3	< 5 mg/kg	Gavage	1
G. F. Sun [54]	China	Mice	–	4–50 g	8	DMA	> 50 mg/L	Drinking water	1
Z. L. Yu [15]	China	Rats	6 weeks	180 ± 10 g	10	$NaAsO_2$	≤50 mg/L	Drinking water	2.3.5.6.9.10
X. K. Zhao[a] [55]	China	Rats	–	180–200 g	12	$NaAsO_2$	> 50 mg/L	Drinking water	1.12
J. A. Lasky [9]	America	Mice	–	18–22 g	7	AS_2O_3	≥5 mg/kg	Gavage	2.3.4.5.9.11.13
J. L. Yan [56]	China	Mice	–	20–22 g	3	AS_2O_3	< 5 mg/kg	Drinking water	1.7
A. Visnagri [57]	India	Rats	–	200–250 g	4	$NaAsO_2$	≥5 mg/kg	Gavage	1
S. H. Xi [58]	China	Rats	–	40–50 g	3	DMA	> 50 mg/L	Drinking water	1
P. Ghosh [59]	India	Rats	–	200–230 g	4	$NaAsO_2$	> 50 mg/L	Gavage	1.3
S. N. Sarkar [13]	India	Rats	–	100–120 g	6	$NaAsO_2$	≤50 mg/L	Drinking water	1.3.6.8
W. F. Chu [29]	India	Rats		250–300 g	6	AS_2O_3	< 5 mg/kg	Gavage	1.2.3.6.7.13
B. Li [60]	China	Mice	6–7 weeks	18–22 g	4	$NaAsO_2$	≥5 mg/kg	Gavage	1

Note: n = Sample size; 1 = Transforming growth factor-β(TGF-β), 2 = p-Smad2, 3 = p-Smad3, 4 = NOX2, 5 = NOX4, 6 = Reactive oxygen species(ROS), 7 = Collagen content, 8 = Type-1 collagen(Collagen1), 9 = Type-3 collagen(Collagen3), 10 = α-smooth muscle actin(α-SMA), 11 = Fibronectin(FN), 12 = Plasminogen activator inhibitor-1(PAI-1), 13 = Fibrosis.
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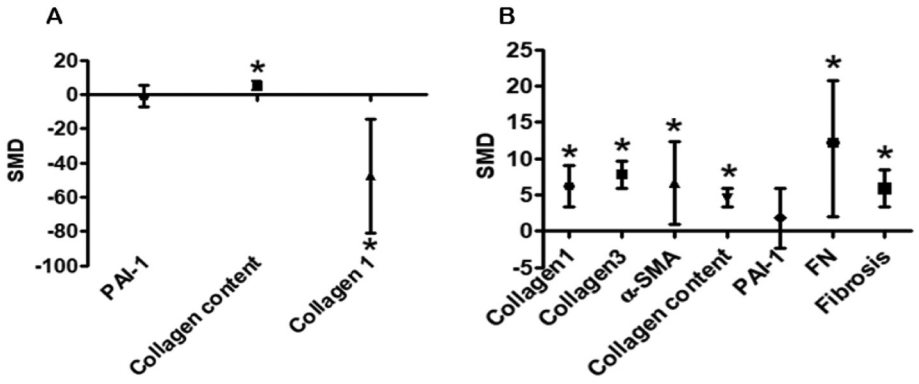


Fig. 2. As_2O_3-induced fibrosis. (A) Effects of arsenic on fibrosis markers in in-vitro studies. (B) Graphpad Prism 6 software was used to evaluate the effects of arsenic on the indicators of fibrosis in vivo. Abbreviations: Collagen1/3, type I and type III collagen; α-SMA,α smooth muscle actin; PAI-1, plasminogen activator inhibitor-1; FN, fibronectin; SMD, standardized mean difference. *$P < 0.05$ versus control group.

exposure increased ROS accumulation, activated the TGF-β/Smad signaling pathway and accelerated the development and progression of renal fibrosis [15]. This indicates that arsenic can activate the TGF-β/Smad pathway and thus induces the expression of fibrosis markers. However, some studies have also reported, that arsenic will down-regulate the synthesis of TGF-β1 and collagen, inhibiting skin inflammation [16]. Studies in normal human lung fibroblasts have shown that arsenic down-regulates the expression of TGF-β1 and the pro-fibrotic factor Collagen3, which antagonizes fibrosis in the lung tissue within the body. Arsenic inhibits the expression of TGF-β1 and p-Smad2/3 proteins, preventing angiogenesis and inducing apoptosis in prostate cancer cells [17]. In addition, AS_2O_3 also inhibits the activation of the TGF-β/Smad2 pathway by regulating microRNA-491 and microRNA-155, thereby down-regulating the expression level of fibrosis markers [18]. It was also discovered during mice studies that arsenic exposure reduced the expression of TGF-β1 and by anti-angiogenesis, protects the synovial tissue of mice [19]. From this standpoint, it is clearly evident that the arsenic regulation of the TGF-β/smad pathway remained controversial. It deserves further study.

The present study aimed to elucidate the regulatory effect of arsenic on TGF-β/Smad signaling pathway, with the meta-analysis method systematically evaluated the researches in vivo and vitro, and then further clarify the specific regulatory mechanism of arsenic-induced fibrosis, providing a theoretical basis for the prevention and treatment of arsenic poisoning.

2. Materials and methods

2.1. Search strategy

The search was conducted in Cochrane, Pubmed, Web of Science, Embase, China National Knowledge Infrastructure (CNKI) and Wanfang database. The deadline was taken as July 14, 2018. Keywords included: arsenic, arsenite, ATO, AS_2O_3, TGF-β, TGF, transforming growth factor beta, Smad2, Smad3, NADPH, NOX2, NOX4, α-SMA, Collagen1, Collagen3, PAI-1, FN, Collagen Content and Fibrosis. The specific strategy implied was: ((arsenic) OR (arsenite) OR (ATO) OR (AS_2O_3)) AND ((TGF-β) OR (TGF) OR (transforming growth factor beta)) AND ((NADPH) OR (NOX2) OR (NOX4) OR (α-SMA) OR (Collagen1) or (Collagen3) OR (PAI-1) OR (FN) OR (Collagen Content) OR (Fibrosis)).

2.2. Inclusion/exclusion criteria

Inclusion criteria: (1) All studies determined according to PICO

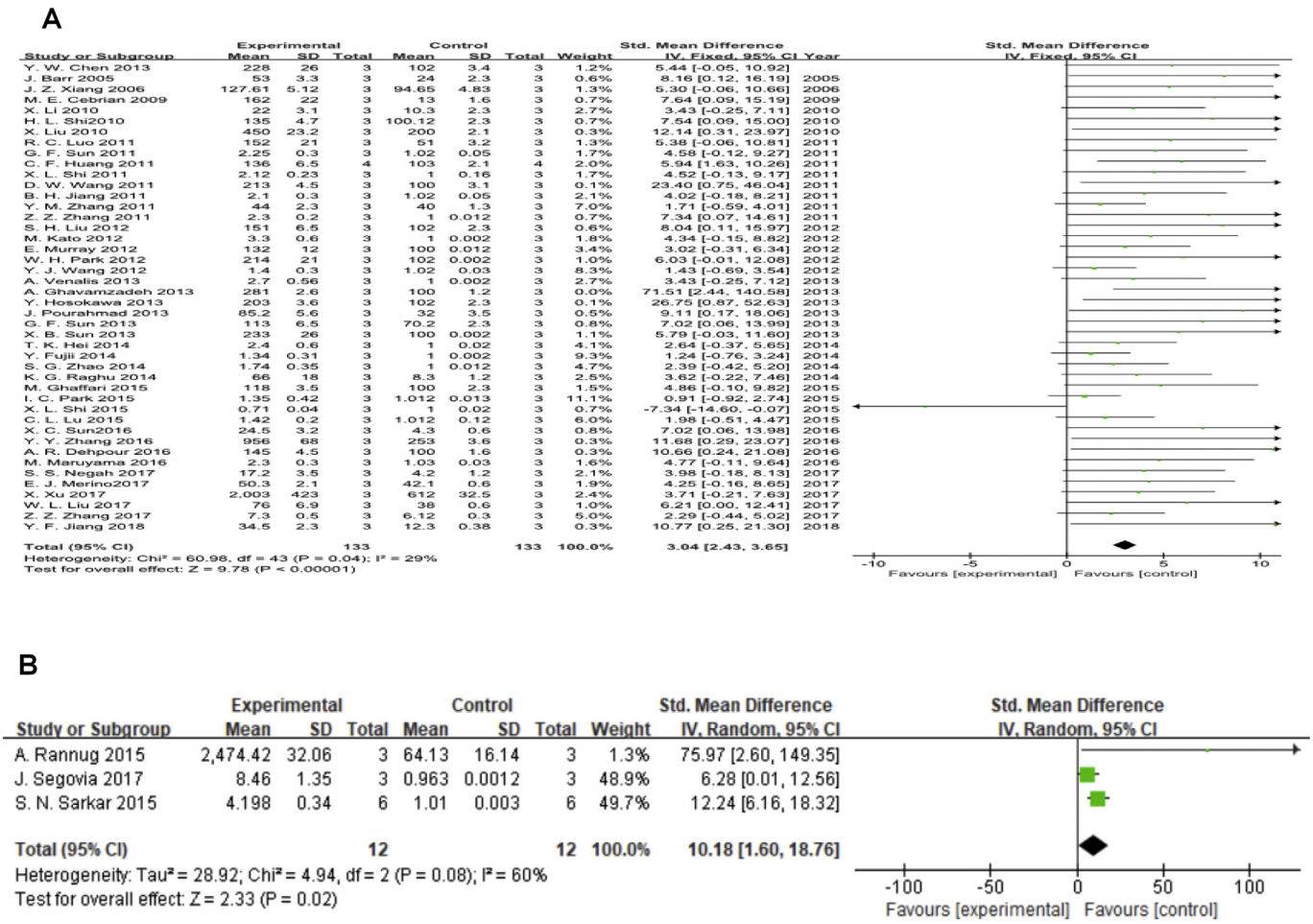


Fig. 3. In vitro studies. (A) The effects of arsenic on ROS were evaluated using forest plot results. (B) Effect of arsenic on NADPH oxidase (NOX4). Show it as a forest map. Abbreviations: SMD, standardized mean difference; IV, independent variable; 95% CI, 95% confidence interval.

principles [20]. Participants (P): Cells, animals or people; Intervention (I): The experimental group treated with arsenic or arsenic compounds, the highest dose or longest duration of arsenic was used in the study; Comparison (C): The control group was a blank control group without any intervention; Outcome (O): NADPH oxidase subunits (NOX2, NOX4), ROS, TGF-β1, p-Smad2, p-Smad3 and fibrosis-related indicators (α-SMA, Collagen1, Collagen3, FN, PAI -1, Collagen content). (2) Type of research: Experimental research published either in Chinese or English.

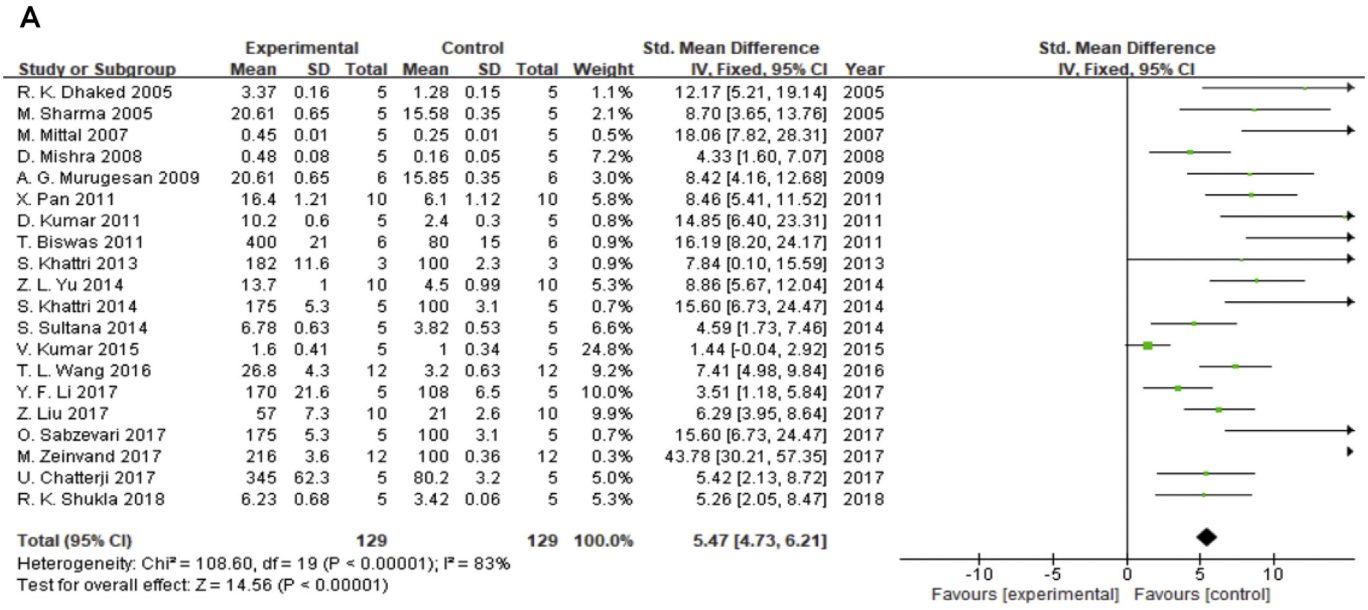
Exclusion criteria: (1) Not a Chinese or English literature, title is not relevant, abstract does not contain arsenic and TGF-β. (2) Repeated articles (the same article is published in both Chinese and English magazines). (3) The data information in the article is incomplete and cannot be extracted. (4) The experimental group is not simply using arsenic intervention. (5) Non-experimental research.

2.3. Search results

This meta-analysis complied with Preferred Reporting Items for Systematic Reviews and Meta-analyses (PRISMA) guideline [21]. A total of 256 articles were identified using the search strategy and 47 articles were eventually retained for meta-analysis according to the inclusion and exclusion criteria. Included in our search strategy was two researchers using the same search terms. Following the PICO principle, 256 articles were first included based on the article title and summary information, then, according to the exclusion criteria, 95 duplicate articles and 46 review articles were excluded, leaving 115 in total. Next, it was found that there were 24 and 36 articles which, in their titles and abstracts respectively, did not contain arsenic and TGF-β, leading to their exclusion and thus 55 articles remain. After reading the full text of the articles, data on arsenic and TGF-β in eight of the articles could not be extracted efficiently and therefore, were excluded. Finally, 47 articles were included in the study. The publication time of the literature was taken until July 14, 2018. The search results are shown in Fig. 1.

2.4. Data analysis

The mean ± standard deviation was used to describe the expression of each index in the experimental and control groups. The standardized mean difference (SMD) was used to compare and analyze the numerical data with different units or mean difference. This method was used in the research of M. C. Xu et al. [22] The calculation formula of SMD was $d_i = \frac{\overline{x_{1i}} - \overline{x_{2i}}}{s_d}$, $i = 1,2,3\ldots k$. I^2 is used to reflect the heterogeneity of the included literature, according to the Cochrane Handbook [23,24]. Heterogeneity is divided into three categories: 0% to 25%, indicating mild heterogeneity; 25% to 50% signifying moderate heterogeneity; 50% to 75% with high heterogeneity. The random effect model was used when $P < 0.05$ and $I^2 > 50\%$. The fixed effect model was employed when $P > 0.05$ and $I^2 \leq 50\%$. Further, a subgroup analysis was utilized to investigate the source of heterogeneity. Based on the dose-response relationship study of Orsini et al. [25], we used spline models and subgroup analyses to analyze the bidirectional regulation of arsenic on TGF-β1. The meta subgroup analysis was based on treatment time (< 48 h vs. ≥ 48 h), arsenic concentration (≤ 5 μmol/L vs. > 5 μmol/L), cell type (normal vs. cancer cells), mice species (mice



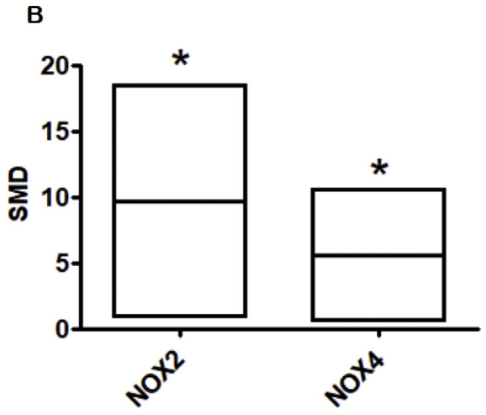


Fig. 4. In vivo studies. (A) Effects of arsenic on ROS. (B) Effect of arsenic on NADPH oxidase (NOX2, NOX4). Abbreviations: SMD, standardized mean difference; IV, independent variable; 95% CI, 95% confidence. *$P < 0.05$ versus control group.

vs. rat vs. guinea pigs), intervention (drinking vs. gavage), and arsenic species ($NaAsO_2$ vs. AS_2O_3 vs. DMA) in determining the relationship between grouping factors and outcome measures. The results of the combination of the experimental and the control group were expressed by SMD and 95% confidence interval (95% CI). Bilateral tests were used for statistical analysis of all indicators and when $P \leq 0.05$ was considered statistically significant.

Data analysis was performed using Review Manager Version 5.3 (The Nordic Cochrane Centre, The Cochrane Collaboration 2012, Portland, OR, USA) and Stata 12.0 [26] (Stata Corp LP, College Station, TX, USA) software. Graphpad Prism 6 software is also used to make images. The funnel plot was applied to reflect literature publication bias, and the hypothesis test used chi-square values. When $P < 0.05$, we considered it a publication bias. Stabilities of synthetic results were evaluated with sensitivity analyses.

3. Results

3.1. Basic characteristics of included studies

3.1.1. Related to in vitro reports, this study included basic features of 24 kinds of literature

The experimental group treated with different kinds of arsenic including $NaAsO_2$ and AS_2O_3 and the control group was taken as blank without arsenic exposure. In the subgroup analysis, we divided the articles according to the arsenic exposure dose, < 5 μmol/L (n = 18) and > 5 μmol/L (n = 6); according to the arsenic intervention time, < 48 h (n = 12) and ≥ 48 h (n = 12); and according to the cell type, normal (n = 12) and cancer cells (n = 12). The study outcome variables were NADPH oxidase (NOX2, NOX4), ROS, TGF-β1, p-Smad2, p-Smad3, and pro-fibrotic outcome markers (α-SMA, FN, Collagen1, Collagen3, PAI-1). M. Nanjundan [27], B. F. Yang [28] and W. F. Chu [29] provided the most valuable information and their three articles were analyzed with at least four indicators (Table 1).

3.1.2. Pertaining to in vivo studies, we included the basic features of 23 kinds of literature

The experimental groups treated with different kinds of arsenic including $NaAsO_2$, AS_2O_3 and DMA, and the control blank group without arsenic exposure. In the subgroup analysis, we divided the articles according to the mice, mice (n = 12), rats (n = 10) and guinea pigs (n = 1); according to the different ways of arsenic intake, by drinking water (n = 12) and gavage (n = 11); and according to the dose of arsenic, (≤ 50 mg/L (n = 5) vs. > 50 mg/L (n = 7)) and (< 5 mg/kg (n = 6) vs. ≥ 5 mg/kg (n = 5)). The study outcome variables were NADPH subunit (NOX2, NOX4), ROS, TGF-β1, p-Smad2, p-Smad3, and

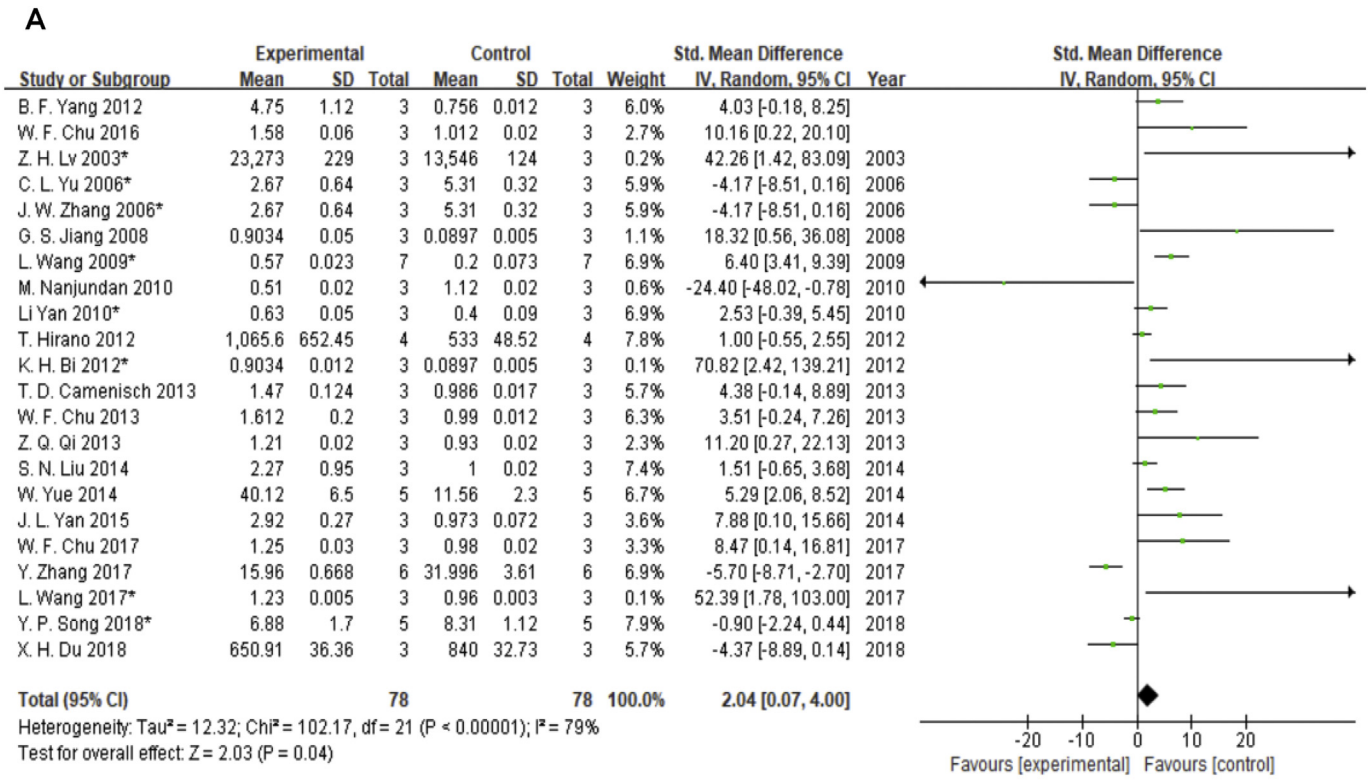


Fig. 5. In vitro studies. (A) The forest plot reflects the effects of arsenic on TGF-β1. (B) Effect of arsenic on p-Smad3 and p-Smad2 proteins. P-Smad represents the activated Smad protein. Abbreviations: SMD, standardized mean difference; IV, independent variable; 95% CI, 95% confidence interval.

profibrotic markers (α-SMA, FN, Collagen1, Collagen3, PAI-1). Q. Yang [18], X. Pan [7], J. A. Lasky [8] and G. F. Sun [23] provided the most valuable information and these four articles were analyzed with at least four indicators (Table 2).

3.2. Relationship between arsenic and fibrosis index

3.2.1. Relationship between arsenic and fibrosis markers in the in vitro studies

Arsenic exposure can up-regulate the expression of fibrosis markers such as α-SMA, Collagen1 and FN, thus induces liver fibrosis [7]. In the in vitro studies, the expression level of Collagen content in the arsenic group was increased by 5.15 times (95% CI, 2.40–7.90; Z = 3.67; $P = 0.0002$) compared with the control group; compared with the control group, the expression level of Collagen1 in the arsenic exposure group was down-regulated by 27.64 times; there was no significant difference in the expression level of PAI-1 ($P > 0.05$) (Fig. 2A). The results showed that arsenic exposure could up-regulate the expression of Collagen content and induce tissue fibrosis.

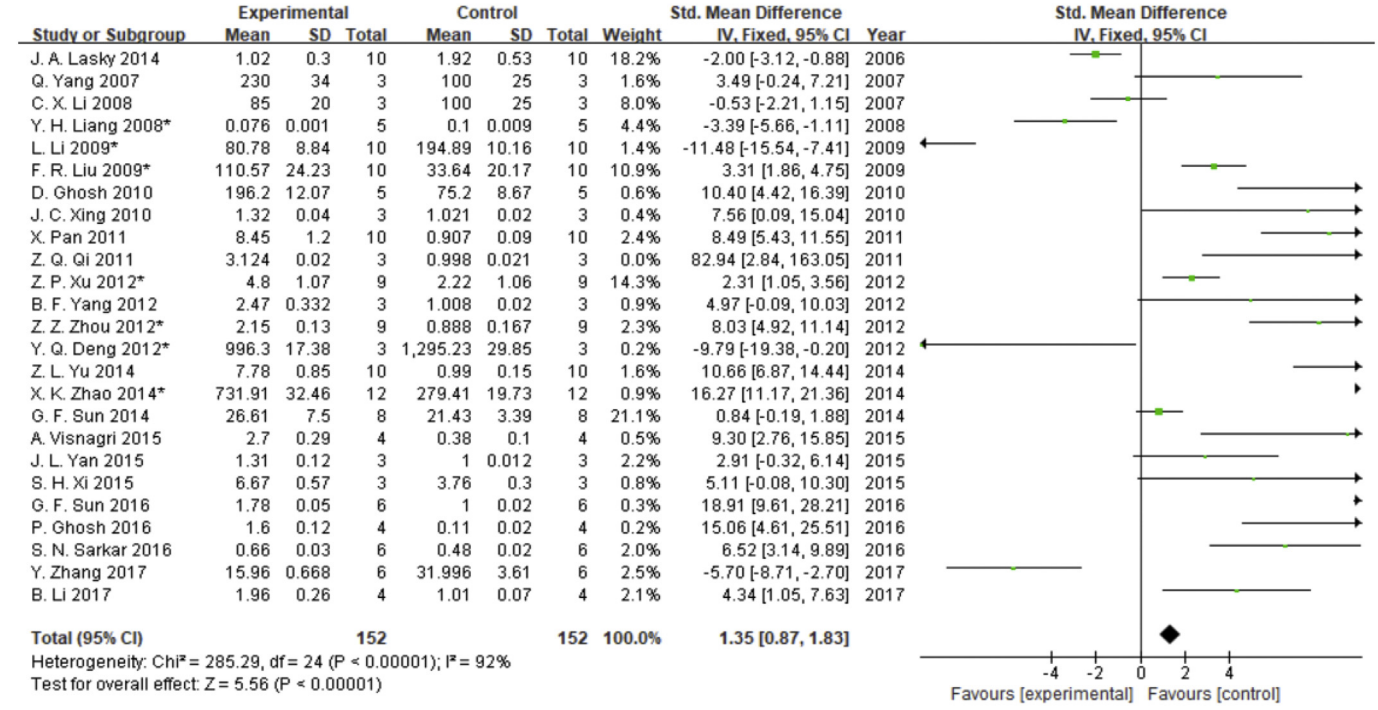
3.2.2. Relationship between arsenic and fibrosis markers in the in vivo studies

In the in vivo studies (Fig. 2B), the expression levels of Collagen1 and Collagen3 in the arsenic-stained group were 6.14 times (95% CI, 3.28–9.00) and 7.78 times (95% CI, 5.95–9.61) higher than those in the control group; The expression level of α-SMA in the arsenic group was 6.67 times that of the control group (95% CI, 0.92–12.41); The expression level of Collagen content was 4.58 times that of the control group (95% CI, 3.33–5.83); The expression level of FN was 13.95 times that of the control group (95% CI, 2.05–20.80); The expression level of PAI-1 was not statistically significant compared with the control group. The results showed that arsenic could up-regulate the expression of Collagen1/3, α-SMA, FN and other fibrosis indexes and induces body fibrosis.

A

Study or Subgroup	Experimental Mean	SD	Total	Control Mean	SD	Total	Weight	Std. Mean Difference IV, Fixed, 95% CI	Year
J. A. Lasky 2014	1.02	0.3	10	1.92	0.53	10	18.2%	-2.00 [-3.12, -0.88]	2006
Q. Yang 2007	230	34	3	100	25	3	1.6%	3.49 [-0.24, 7.21]	2007
C. X. Li 2008	85	20	3	100	25	3	8.0%	-0.53 [-2.21, 1.15]	2007
Y. H. Liang 2008*	0.076	0.001	5	0.1	0.009	5	4.4%	-3.39 [-5.66, -1.11]	2008
L. Li 2009*	80.78	8.84	10	194.89	10.16	10	1.4%	-11.48 [-15.54, -7.41]	2009
F. R. Liu 2009*	110.57	24.23	10	33.64	20.17	10	10.9%	3.31 [1.86, 4.75]	2009
D. Ghosh 2010	196.2	12.07	5	75.2	8.67	5	0.6%	10.40 [4.42, 16.39]	2010
J. C. Xing 2010	1.32	0.04	3	1.021	0.02	3	0.4%	7.56 [0.09, 15.04]	2010
X. Pan 2011	8.45	1.2	10	0.907	0.09	10	2.4%	8.49 [5.43, 11.55]	2011
Z. Q. Qi 2011	3.124	0.02	3	0.998	0.021	3	0.0%	82.94 [2.84, 163.05]	2011
Z. P. Xu 2012*	4.8	1.07	9	2.22	1.06	9	14.3%	2.31 [1.05, 3.56]	2012
B. F. Yang 2012	2.47	0.332	3	1.008	0.02	3	0.9%	4.97 [-0.09, 10.03]	2012
Z. Z. Zhou 2012*	2.15	0.13	9	0.888	0.167	9	2.3%	8.03 [4.92, 11.14]	2012
Y. Q. Deng 2012*	996.3	17.38	3	1,295.23	29.85	3	0.2%	-9.79 [-19.38, -0.20]	2012
Z. L. Yu 2014	7.78	0.85	10	0.99	0.15	10	1.6%	10.66 [6.87, 14.44]	2014
X. K. Zhao 2014*	731.91	32.46	12	279.41	19.73	12	0.9%	16.27 [11.17, 21.36]	2014
G. F. Sun 2014	26.61	7.5	8	21.43	3.39	8	21.1%	0.84 [-0.19, 1.88]	2014
A. Visnagri 2015	2.7	0.29	4	0.38	0.1	4	0.5%	9.30 [2.76, 15.85]	2015
J. L. Yan 2015	1.31	0.12	3	1	0.012	3	2.2%	2.91 [-0.32, 6.14]	2015
S. H. Xi 2015	6.67	0.57	3	3.76	0.3	3	0.8%	5.11 [-0.08, 10.30]	2015
G. F. Sun 2016	1.78	0.05	6	1	0.02	6	0.3%	18.91 [9.61, 28.21]	2016
P. Ghosh 2016	1.6	0.12	4	0.11	0.02	4	0.2%	15.06 [4.61, 25.51]	2016
S. N. Sarkar 2016	0.66	0.03	6	0.48	0.02	6	2.0%	6.52 [3.14, 9.89]	2016
Y. Zhang 2017	15.96	0.668	6	31.996	3.61	6	2.5%	-5.70 [-8.71, -2.70]	2017
B. Li 2017	1.96	0.26	4	1.01	0.07	4	2.1%	4.34 [1.05, 7.63]	2017
Total (95% CI)			**152**			**152**	**100.0%**	**1.35 [0.87, 1.83]**	

Heterogeneity: Chi² = 285.29, df = 24 (P < 0.00001); I² = 92%
Test for overall effect: Z = 5.56 (P < 0.00001)



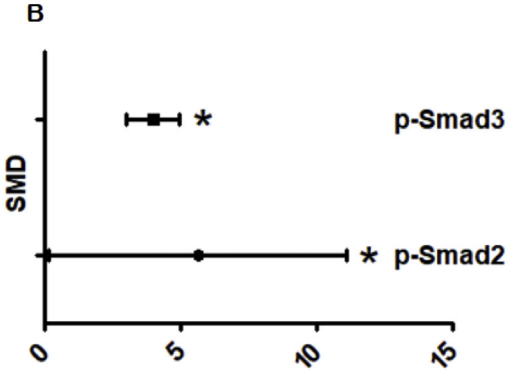


Fig. 6. In vivo studies. (A) The forest plot reflects the effects of arsenic on TGF-β1. (B) Effect of arsenic on p-Smad3 and p-Smad2 proteins. Abbreviation: SMD, standardized mean difference; IV, independent variable; 95% CI, 95% confidence interval. *$P < 0.05$ versus control group.

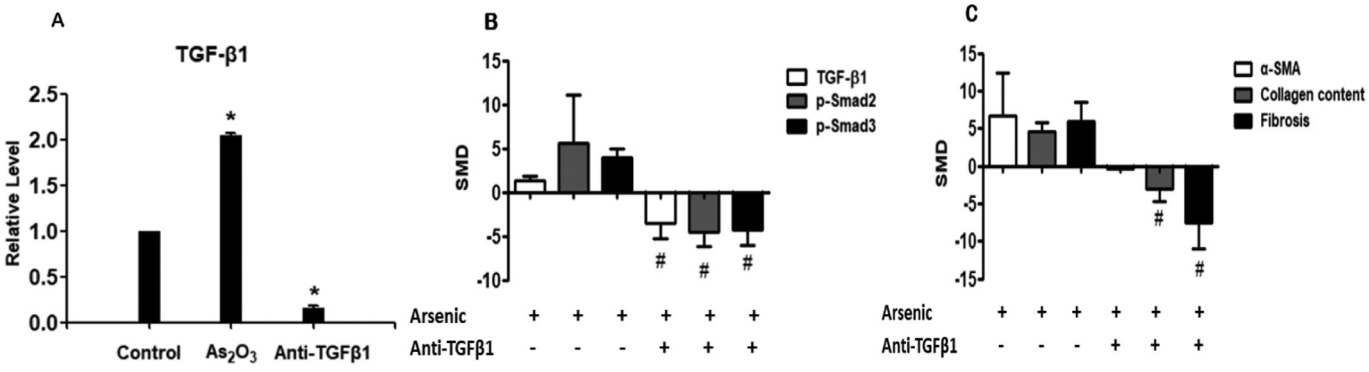


Fig. 7. (A) The effects of TGF-β1 inhibitor were administered alone. (B) The effects of arsenic on TGF-β1, p-Smad2 and p-Smad3. (C) The effects of arsenic on α-SMA, Collagen content and Fibrosis. Cells were treated with 0.5 μM–5 μM As_2O_3 for 2, 4, 12 and 24 h. The inhibitor of TGF-β1 was LY364947 or LY3200882. Abbreviation: Anti-TGFβ1, TGF-β1 inhibitor; TGF-β1, transforming growth factor-β1; α-SMA, α-Smooth Muscle Actin; SMD, standardized mean difference. *$P < 0.05$ versus control group, #$P < 0.05$ versus arsenic group.

Table 3
In vitro studies, seven articles comprehensive dose-response data of TGF-β1 with different doses of arsenic.

ID	Author	Year	Dose (μmol/L) (Mean (SD))				
			0	1	2	5	10
1	W. F. Chu [12]	2013	0.99 (0.012)	1.32 (0.02)	1.612 (0.2)	2.15 (0.03)	–
2	M. Nanjundan [27]	2010	1.12 (0.02)	1.01 (0.02)	9.62 (0.03)	7.31 (0.012)	7.25 (0.016)
3	B. F. Yang [28]	2012	0.756 (0.012)	2.45 (0.8)	4.75 (1.12)	3.26 (0.56)	–
4	T. F. Huang [14]	2013	0.986 (0.017)	1.38 (0.06)	1.47 (0.124)	1.56 (0.05)	–
5	S. N. Liu [40]	2014	1.03 (0.001)	1.68 (0.1)	2.34 (0.2)	2.52 (0.51)	1.78 (0.3)
6	J. L. Yan [61]	2014	1.012 (0.001)	2.86 (0.53)	–	3.56 (0.35)	2.55 (0.36)
7	L. Wang [42]	2017	0.96 (0.073)	–	1.35 (0.02)	2.35 (0.3)	1.79 (0.3)

A dose-response relationship between arsenic and TGF-β1 was explored using seven in vitro studies. Abbreviation: SD, standard deviation.

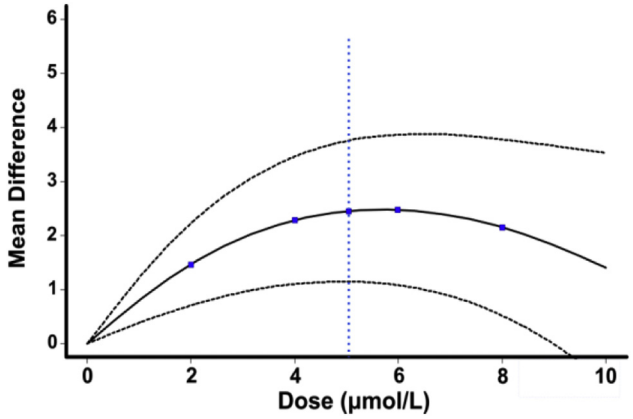


Fig. 8. The relationship between arsenic and TGF-β1 was analyzed using a random effects model. The dashed line indicates the 95% confidence interval for the spline model. The solid line indicates mean of the spline model.

3.3. The effect of arsenic on ROS and NADPH oxidase

3.3.1. Effect of arsenic on ROS and NADPH oxidase in in-vitro studies

Studies have reported that the production of ROS is an important factor in arsenic-induced fibrosis [5]. The results of in vitro studies showed that the ROS expression level in the arsenic-treated group was 2.29 times that of the control group (95% CI, 2.37–3.60; Z = 9.46; $P < 0.00001$), indicating that arsenic exposure leads to ROS accumulation in cell experiments. It prompts the body to release a series of fibrotic cytokines (Fig. 3A). NADPH oxidase (NOX2, NOX4) is the main source of ROS [6]. Compared to control group in in-vivo studies, the expression level of NOX4 in the arsenic-exposed group increased by 10.18 times (95% CI, 1.60–18.76; Z = 0.20; $P < 0.05$). The results indicated that arsenic significantly increased the expression level of NADPH oxidase (NOX4) and induced ROS production in cell experiments (Fig. 3B).

3.3.2. Effect of arsenic on ROS and NADPH oxidase in in-vivo studies

The same results were obtained in in-vivo studies. The expression level of ROS in the arsenic exposure group was 5.41 times that of the blank control group (95% CI, 4.67–6.14; Z = 14.35; $P < 0.00001$). The results showed arsenic exposure in in-vivo studies, induces ROS production, leading to an increase in the expression of fibrosis markers (Fig. 4A). The expression level of NOX2 in the arsenic exposure group was 5.56 times higher than that in the control group (95% CI, 0.52–10.61), the expression level of NOX4 was 9.56 times higher than that of the control group (95% CI, 0.68–18.50). Our results showed that arsenic up-regulates the expression levels of NADPH oxidase (NOX2, NOX4), causing ROS accumulation to induce fibrosis (Fig. 4B).

3.4. Relationship between arsenic and TGF-β/Smad

3.4.1. Relationship between arsenic and TGF-β/Smad pathway in in-vitro studies

TGF-β/Smad is an important pathway in the process of fibrosis [10]. In vitro, our results showed that the expression level of TGF-β1 was increased by 2.04 times in the arsenic-stained group (95% CI, 0.07–4.00; Z = 2.03; $P < 0.05$) compared to control group (Fig. 5A). There was no significant difference in the expression levels of p-Smad3 and p-Smad2 between the arsenic exposure groups ($P > 0.05$). It is indicated that arsenic up-regulates the expression of TGF-β1 to induce expression of fibrosis markers (Fig. 5B). Here, the regulation of arsenic on the TGF-β/Smad pathway is still controversial.

3.4.2. Relationship between arsenic and TGF-β/Smad pathway in in-vivo studies

In vivo studies, we found out that the expression level of TGF-β1 in the arsenic-treated group was 1.35 times that of the control group (95% CI, 0.87–1.83; Z = 5.56; $P < 0.00001$) (Fig. 6A). The p-Smad3 and p-Smad2 proteins were up-regulated by 3.98-fold (95% CI, 2.99–4.97) and 5.63-fold (95% CI, 0.16–11.11; Z = 2.02), respectively (Fig. 6B). The results showed that arsenic exposure resulted in elevated levels of TGF-β1 and phosphorylated Smad2/3 in in-vivo studies. We exhibited that exposure to arsenic activates the TGF-β/Smad pathway and induces increased expression of fibrosis markers.

3.5. Relationship between arsenic, TGF-β1 and fibrosis indicators

Arsenic increased the expression of TGF-β1 [43]. Studies indicated that the expression of TGF-β1 in the arsenic exposure group was 2.04 times higher than that in the control group, and the TGF-β1 inhibitor group alone significantly reduced the level of TGF-β1 compared with the control group. (Fig. 7A).

To further clarify the regulation of arsenic on the TGF-β1 signaling pathway, we used arsenic in combination with TGF-β1 inhibitor as the experimental group and arsenic alone as the control group. Compared with the arsenic group, the expression of TGF-β1 itself was down-regulated 3.58 times (95% CI, −5.28-, −1.88) after adding TGF-β1 inhibitor; the p-Smad2 and p-Smad3 value was down-regulated by 4.82 and 4.52 times, respectively (Fig. 7B). α-SMA was down-regulated by 0.28-fold; Collagen content was down-regulated by 3.05-fold (95% CI, −4.63-, −1.47); Fibrosis was down-regulated by 7.48 times (95% CI, −11.03, −3.93) (Fig. 7C). The results showed that arsenic combined with TGF-β1 inhibitor group significantly inhibited the expression of TGF-β/Smad pathway and down-regulated the expression levels of fibrosis markers such as α-SMA, Collagen content and Fibrosis. It further explained that arsenic induces expression of fibrosis index through the TGF-β/Smad pathway.

3.6. The dose-response relationship between arsenic and TGF-β1

To study the dose-response relationship between arsenic and TGF-β1, we used a spline model to quantify the standardized mean difference in multiple studies. In the literatures, we found 7 articles that reported continuous 4 or more doses of arsenic and their induced expression of TGF-β1. Based on the 7 papers, which we analyzed the dose-effect relationship to elucidate the relationship between the arsenic and TGF-β1. The basic information is shown in Table 3. Our results showed that when the concentration of arsenic is ≤5 μmol/L, the dose-response relationship between arsenic and TGF-β1 is positive; When the

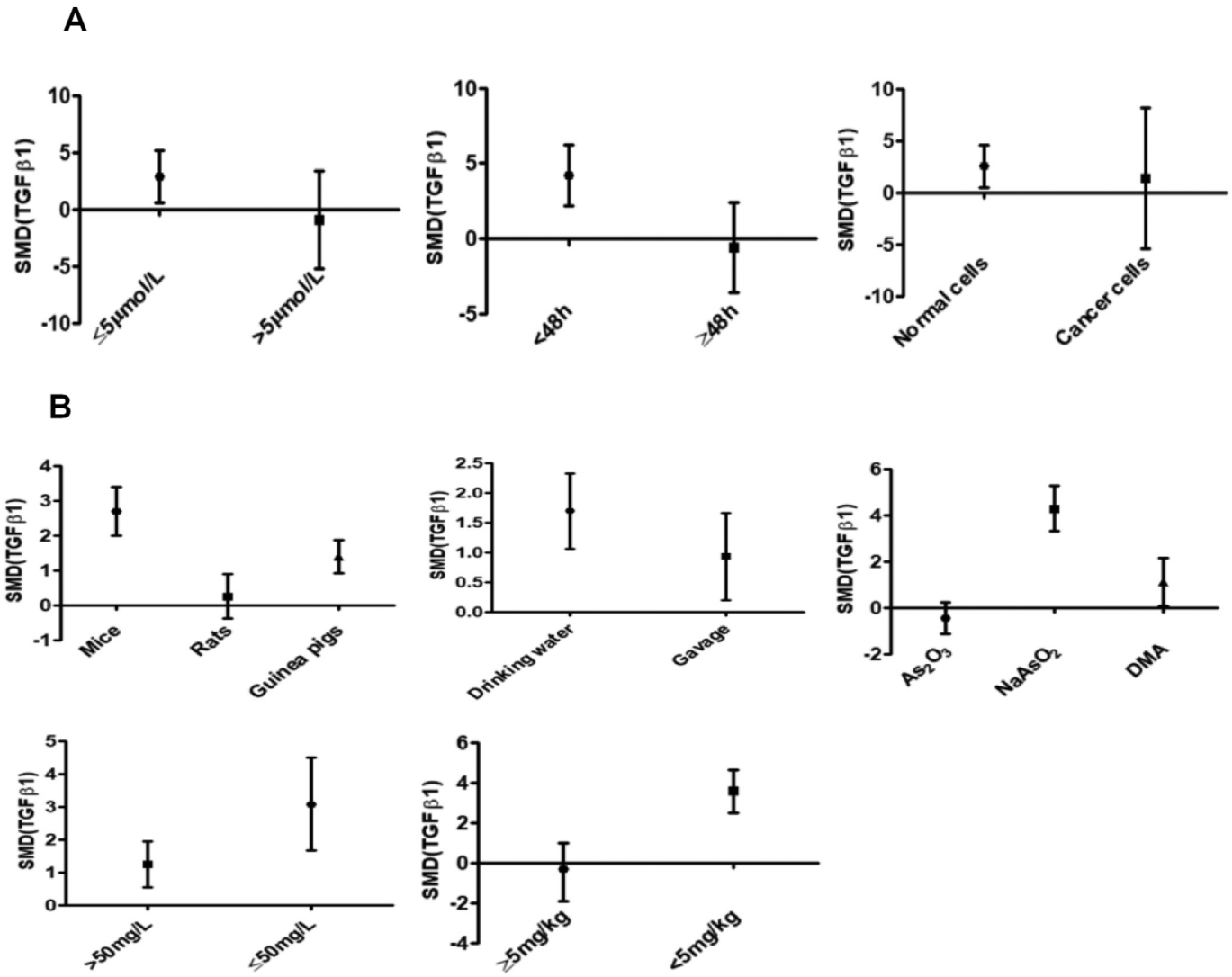


Fig. 9. (A) Subgroup analysis of the effect of arsenic on TGF-β1 in vitro. As was shown in the figure, arsenic treatment concentration(≤5 μmol/L, > 5 μmol/L), exposure time(< 48 h, ≥48 h) and cell types (Normal cells, Cancer cells) was used as an influencing factors respectively to analyze sources of heterogeneity. (B) Subgroup analysis of the effect of arsenic on TGF-β1 in vivo. Based on the types of mice (Mice, Rats, Guinea pigs), routes of administration of arsenic (Drinking water, Gavage), the species of arsenic (As_2O_3, $NaAsO_2$, DMA) and the concentration of arsenic(> 50 mg/L, ≤50 mg/L ; ≥5 mg/kg, < 5 mg/kg) respectively, a subgroup analysis was conducted. Abbreviation: SMD, standardized mean difference; TGF-β1, transforming growth factor-β1.

concentration of arsenic is > 5 μmol/L, the expression level of TGF-β1 decreases (Fig. 8). This result clearly shows that the dose of arsenic has an effect on the regulation of TGF-β1 and the low dose arsenic (≤5 μmol/L) can increase the expression of TGF-β1, whereas the high dose arsenic (> 5 μmol/L) has a tendency to inhibit the expression of TGF-β1.

3.7. Arsenic and TGF-β1 subgroup analysis results

3.7.1. Arsenic and TGF-β1 subgroup analysis results in in-vitro studies

We performed a subgroup analysis on in vitro studies for arsenic exposure time (< 48 h vs. 48 h), arsenic doses (≤5 μmol/L vs. > 5 μmol/L) and cell types (normal cells vs. cancer cells). It was discovered that short-term (< 48 h) and low-dose (≤5 μmol/L) arsenic exposure up-regulated the expression of TGF-β1 (SMD = 4.20, 95% CI: 2.21, 6.20; I^2 = 45.0%), (SMD = 2.87, 95% CI: 0.54, 5.20; I^2 = 79.0%); And increased the expression level of TGF-β1 in normal cells (SMD = 2.53 95% CI: 0.47, 4.60; I^2 = 82.0%); Long-term (≥48 h), high dose (> 5 μmol/L) arsenic exposure in cancer cells had no statistical significance for TGF-β1 expression (P > 0.05). These results indicated that low dose (≤5 μmol/L) and short-term (< 48 h) exposure to arsenic can up-regulate TGF-β1 expression, in normal cells compared to cancer cells (Fig. 9A).

3.7.2. Arsenic and TGF-β1 subgroup analysis results in in-vivo studies

Analyzing the in vivo studies, we performed a subgroup analysis for mice species (mice vs. rats vs. guinea pigs), arsenic intake (drinking vs. gavage), arsenic species (As_2O_3 vs. $NaAsO_2$ vs. DMA) and arsenic concentration (≤50 mg/L vs. > 50 mg/L or ≥5 mg/kg vs. < 5 mg/kg). It was realized that arsenic exposure in mice and guinea pigs had up-regulated the expression of TGF-β1 (SMD = 2.69, 95% CI: 1.99, 3.40), (SMD = 1.40, 95% CI: 0.92, 1.87). There was no statistically significant effect of arsenic on TGF-β1 expression in the study of rats (P > 0.05). Regardless of the mode of drinking (SMD = 1.69, 95% CI: 1.06, 2.32; I^2 = 93.0%) or the method of gavage (SMD = 0.93, 95% CI: 0.20, 1.66; I^2 = 90.0%), arsenic exposure up-regulated expression of TGF-β1 in both. Compared with As_2O_3 and DMA, $NaAsO_2$ up-regulated TGF-β1 expression more significantly (SMD = 4.28, 95% CI: 3.30, 5.26; I^2 = 90.0%); Low dose of arsenic (≤50 mg/L or < 5 mg/kg) can up-regulate the expression of TGF-β1 (SMD = 0.93, 95% CI: 0.20, 1.66;

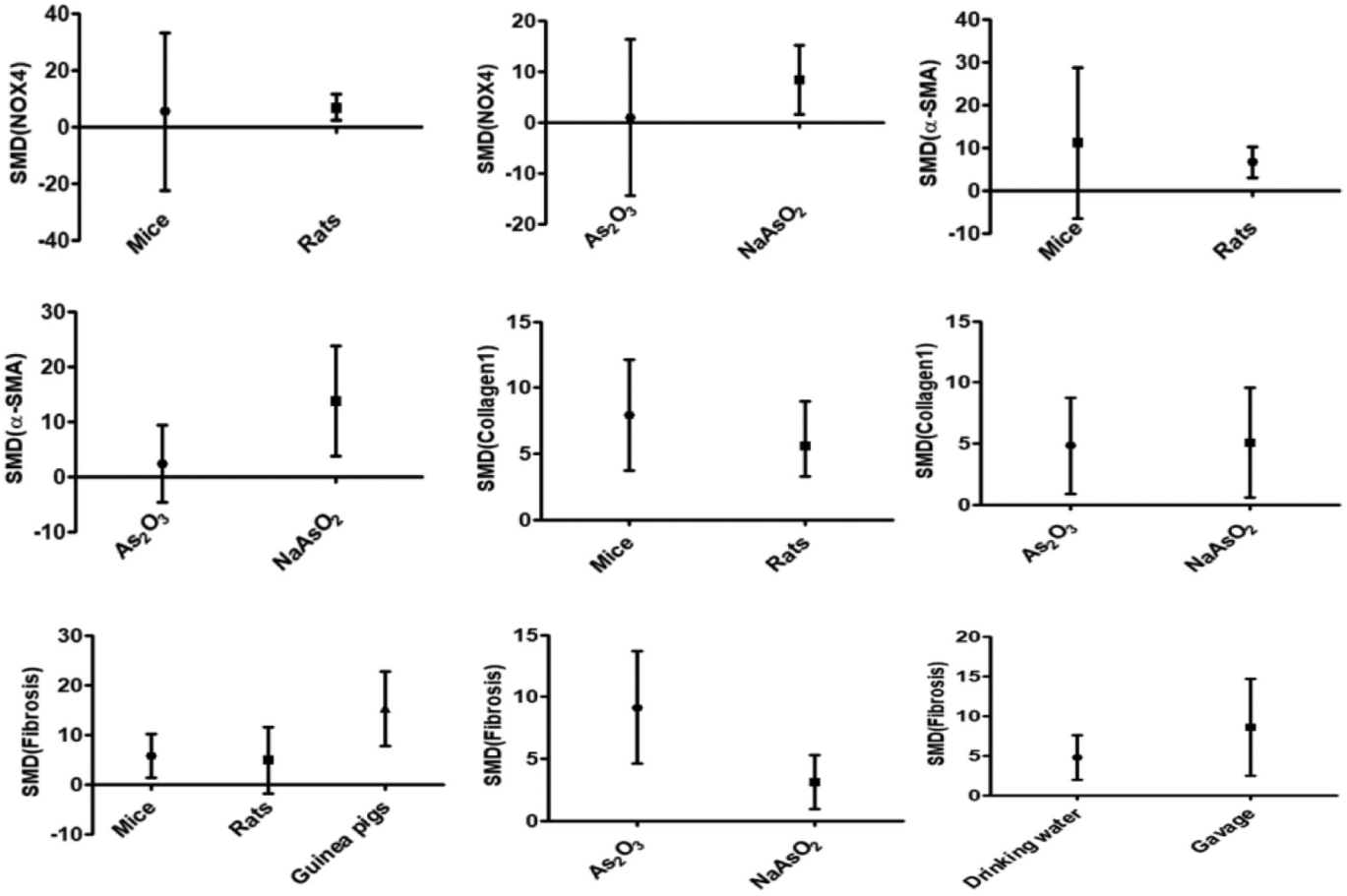


Fig. 10. Subgroup analysis of the effect of arsenic on NOX4, α-SMA, Collagen1 and Fibrosis in vivo. According to the types of mice (Mice, Rats) and the species of arsenic (As_2O_3, NaA_SO_2), an subgroup analysis of the effect of arsenic on NOX4, α-SMA, Collagen1 and Fibrosis was performed respectively to analyze the sources of heterogeneity. Routes of administration of arsenic (Drinking water, Gavage) was also used as an influencing factor. Abbreviation: SMD, standardized mean difference; NOX4, NADPH oxidase subunit; α-SMA, α-smooth muscle actin; Collagen1, Type-1 collagen.

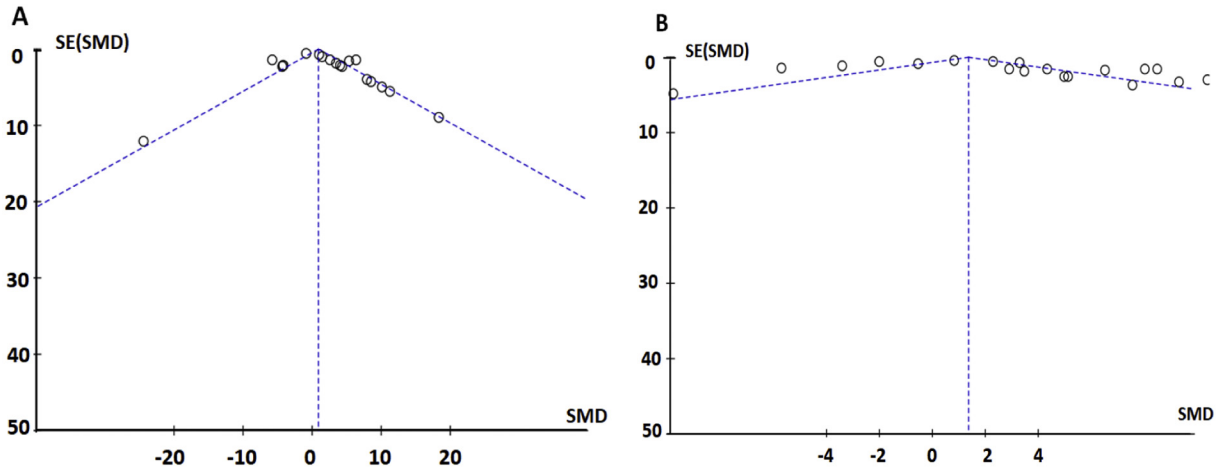


Fig. 11. (A) The funnel plot of TGF-β1 in vitro. (B) The funnel plot of TGF-β1 in vivo. The blue-dotted line shows the overall estimated standard mean difference. Evidence for publication bias was not found. Abbreviation: SMD, standard mean difference; SE, standard error; TGF-β1, transforming growth factor -beta 1. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

I^2 = 90.0%), (SMD = 3.57, 95% CI: 2.50, 4.65; I^2 = 78.0%). However, high-dose (> 50 mg/L or ≥5 mg/kg) arsenic had no statistical significance for the expression of TGF-β1 (P > 0.05) (Fig. 9B).

3.8. In vivo subgroup analysis of arsenic and NADPH oxidase (NOX4), α-SMA, Collagen1, and fibrosis

In vivo studies have shown that arsenic exposure in rats is more effective in up-regulating NOX4 and α-SMA than in mice (SMD = 6.86, 95% CI: 2.18, 11.54; I^2 = 84%), (SMD = 6.61, 95% CI: 2.94, 10.28;

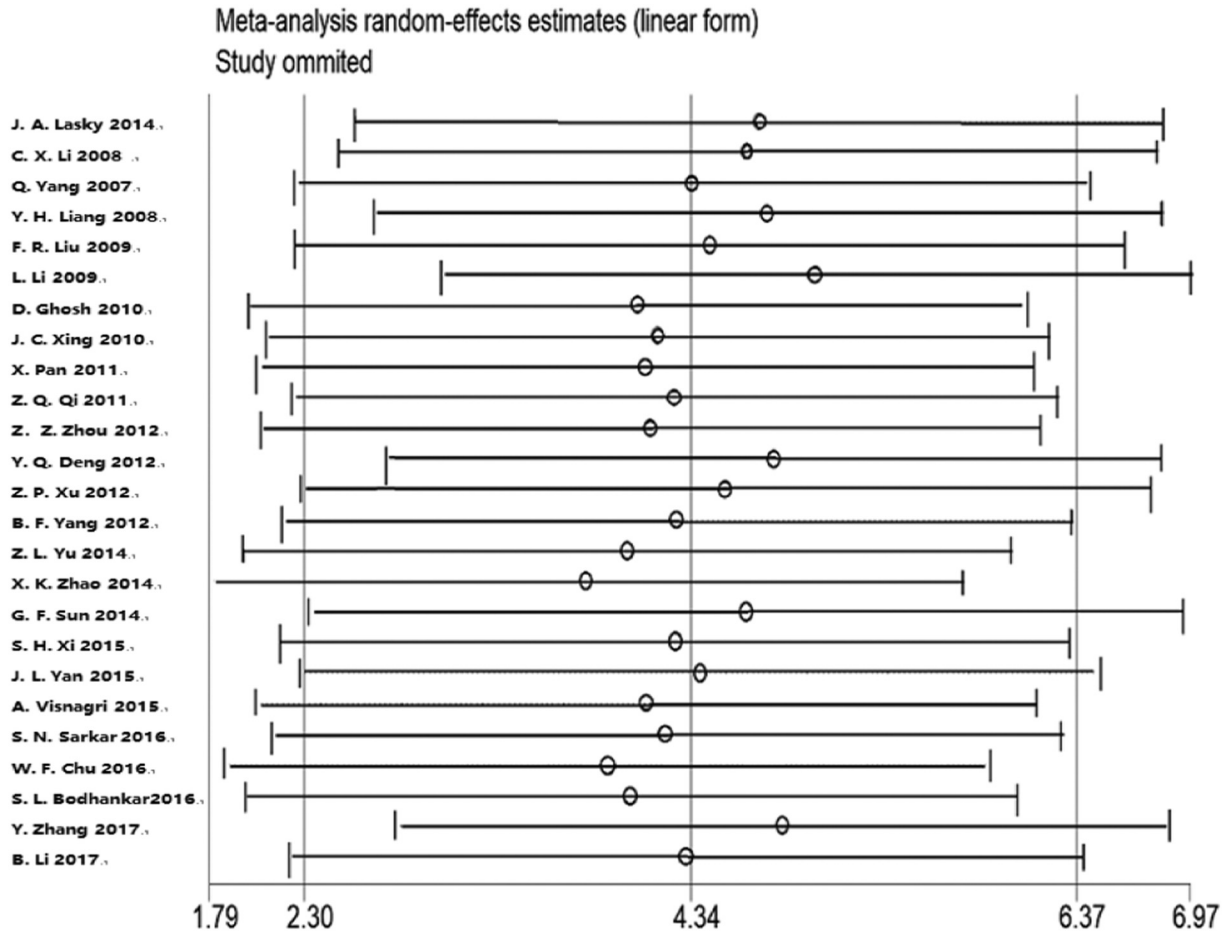


Fig. 12. Sensitivity analysis for TGF-β1. Abbreviation: CI, confidence interval; TGF-β1, transforming growth factor-beta 1; * Published in Chinese.

I^2 = 50.0%) and the type of mice had no significant effect on the up-regulation of Collagen1 protein and fibrosis. Compared with As_2O_3, $NaAsO_2$ exposure up-regulated the expression levels of NOX4 and α-SMA (SMD = 8.39, 95% CI: 1.58, 15.21; I^2 = 82%), (SMD = 13.72, 95% CI: 3.74, 23.70; I^2 = 70.0%). As_2O_3 has a more pronounced effect on Fibrosis (SMD = 9.15, 95% CI: 4.63, 13.67; I^2 = 71%). The type of arsenic has no effect on the up-regulation of Collagen1. In both drinking and gavage methods, the expression level of fibrosis was up-regulated. The results indicated that rats exposed to $NaAsO_2$ were able to up-regulate the expression of NADPH oxidase (NOX4) and α-SMA; arsenic exposure up-regulated Collagen1 protein expression, independent of mice and arsenic species; the up-regulation of fibrosis values were not affected by the type of mice and the mode of ingestion. As_2O_3 was able to up-regulate fibrosis expression compared to $NaAsO_2$ (Fig. 10).

3.9. Publication of bias and sensitivity analysis

The funnel plot shows that all the results of the study are symmetrically distributed, which indicates no publication bias (Fig. 11). Taking the sensitivity analysis of arsenic and TGF-β1 as an example. The results indicated that no individual results affect the overall outcomes and the results contained in the literature are relatively stable (Fig. 12).

4. Discussion

Inorganic arsenic is a potent human carcinogen [62], widely distributed in water, air and soil. Exposure to arsenic leads to fibrosis of multiple organs [3,4]. Based on many research results in vitro and vivo, we found that TGF-β1 was an important transcription factor for pro-fibrosis. However, there have been a few controversial issues about fibrosis induced by arsenic and the TGF-β/Smad signaling pathway [6,8,9]. This study showed that arsenic exposure could activate NADPH oxidase to induce ROS production, thereby activating the TGF-β/Smad pathway, increasing the expression of fibrosis markers such as α-SMA and collagen 1/3.

TGF-β1 is a cytokine closely related to the occurrence of organ fibrosis [63]. Smad protein family is an intracellular transduction molecule of TGF-beta family signal from receptor to nucleus, which participates in the regulation of cell proliferation and apoptosis [64]. This study clearly demonstrated that arsenic-induced fibrosis was related to ROS accumulation and the TGF-β/Smad signaling pathway, which not only provided a theoretical basis for exploring the mechanism of arsenic-induced fibrosis but also offered reference for prevention and treatment of arsenic poisoning.

Oxidative damage is one of the important mechanisms of arsenic-induced fibrosis [5]. Arsenic exposure induces ROS production, which is mainly caused by NADPH oxidase (NOX4, NOX2) [6]. On one hand, arsenic up-regulates AT_1R protein expression to increase the expression of NOX and NO, and induces ROS accumulation, while on the other, iNOS mediates NO production, accompanied by NOX and ROS production, which activates ERK1/2 and VEGF, and induces the release of pro-inflammatory and pro-fibrogenic factors [13]. In both in vitro and in vivo studies, arsenic up-regulated the expression of NOX2 and NOX4, resulting in a significant increase in ROS expression (Fig. 13①). Subgroup analysis showed that the up-regulation of NADPH oxidase (NOX4) was more pronounced in $NaAsO_2$ exposure in rats. These results confirm that arsenic exposure induces ROS production by NADPH

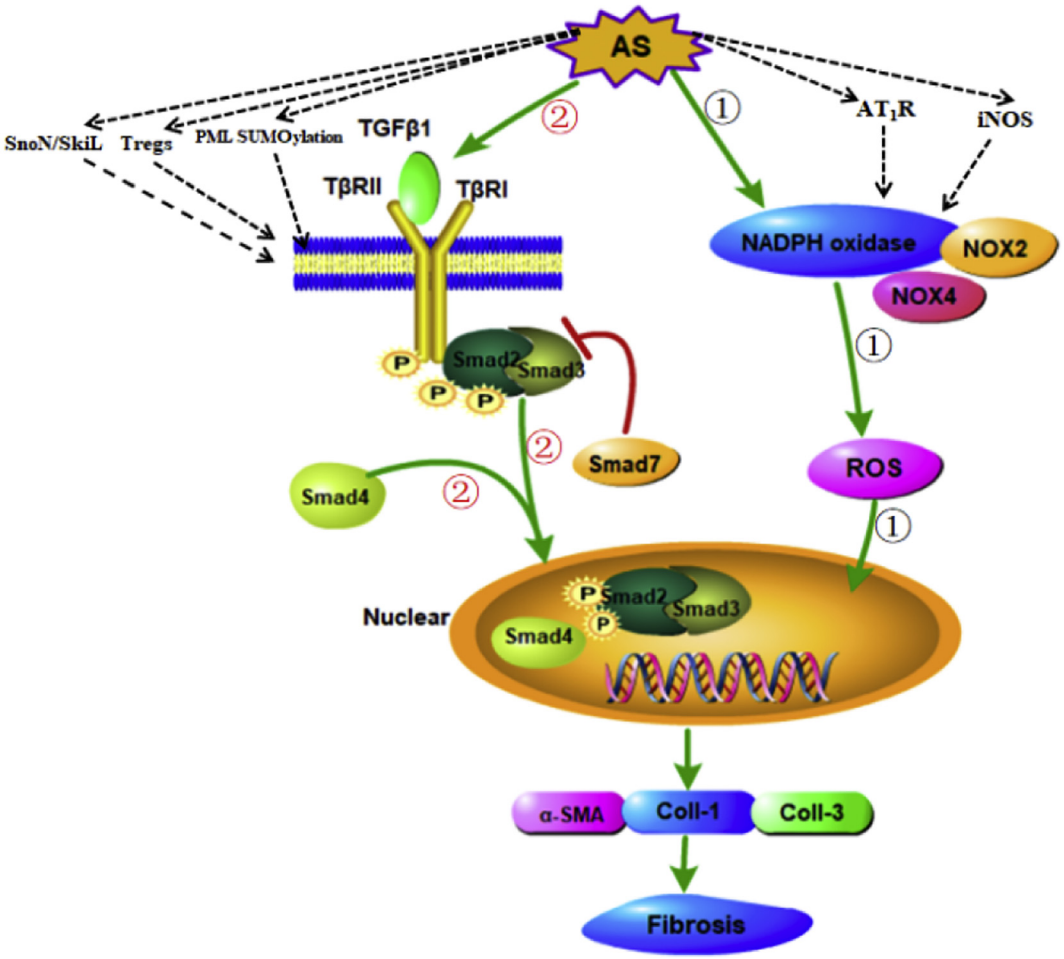


Fig. 13. Arsenic-induced fibrosis through the TGF-β/Smad pathway. ① indicates the pathway of NADPH oxidase-induced fibrosis; ② indicates the pathway of TGF-β/Smad pathway-induced fibrosis. The green arrow represents the promotion; the red arrow represents the inhibition. The dashed head represents the inclusion in the literature. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

oxidase, which is an important factor in oxidative damage.

The results also indicated that arsenic can activate the TGF-β/Smad pathway. It was reported that arsenic exposure could up-grade the miRNA-21 through inhibiting the expression of its target gene Smad7 [55], PDCD4, PTEN, and Spry1 [65] contributed to cell malignant proliferation. AS_2O_3 induces PML SUMOylation, which forms PML-NBs, which in turn up-regulates the expression of TGF-β1 and p-Smad2/3, and induces an increase in the expression of fibrogenic factors [29]. In addition, AS_2O_3 also down-regulates Tregs, up-regulates TGF-β1, and inhibits the expression of inflammatory factors such as IL-2, IFN-γ and Foxp3 [50]. These results showed that arsenic regulates the expression of the fibrosis index by the TGF-β/Smad pathway (Fig. 13②).

The relationship between arsenic and TGF-β1 inhibitors was further observed. The results showed that arsenic combined with TGF-β1 inhibitors significantly down-regulated the expression of fibrosis factors, indicating that arsenic induces expression of fibrosis markers through TGF-β/Smad pathway. The dose-response relationship between arsenic and TGF-β1 indicated that low dose (≤5 μmol/L) significantly up-regulated the expression of TGF-β1, and high doses of arsenic (> 5 μmol/L) have a tendency to down-regulate the expression of TGF-β1. Subgroup analysis indicated that arsenic exposure in normal cells for a short time (< 48 h) with a low dose (≤5 μmol/L) was more effective in up-regulating TGF-β1. Low dose (< 5 mg/kg or ≤ 50 mg/L) of $NaAsO_2$ in mice can up-regulate the expression level of TGF-β1, while the method of arsenic intake has no significant effect on the up-regulation of TGF-β1. These results suggested that high dose, long-term or arsenic exposure in cancer cells have a tendency to down-regulate TGF-β1 expression, providing a theoretical basis for further study of arsenic-induced fibrosis via TGF-β/Smad pathway.

Exposure to arsenic induces an increase in the expression level of TGF-β1, which in turn causes ECM to secrete large amounts of collagen to form fibrosis [66]. On the one hand, we found that long-term $NaAsO_2$ exposure in drinking water led to an increase in ECM components, thus up-graded the expression of TIMP-1 and Collagen3 genes in mouse liver tissue and decreased levels of MMP8 gene, which contributed to liver fibrosis [7]. In addition, AS_2O_3 activated the AKT/GSK-3β/Snail pathway, enhancing the expression of Collagen1/3, MMP2, MMP9, α-SMA and CD31 to induce cardiac fibrosis [41]. On the other hand, arsenic trioxide as an anticancer drug, played an vital role in down-regulating Collagen1. According to the report [67], arsenic trioxide could significantly inhibit the occurrence and development of multiple solid tumors such as breast cancer and liver cancer, through suppressing cell proliferation and inducing apoptosis, in addition to having significant effects on blood system diseases. C. H. Rhee et al. [68] pointed that arsenic trioxide significantly reduced the levels of Collagen1 and Collagen3 mRNA and restrained As_2O_3-induced invasion and migration of HT1080 cells. This study reveals that arsenic exposure increased the expression of fibrosis markers such as α-SMA, Collagen1/3, and FN. Subgroup analysis showed that the up-regulation effect of α-SMA on $NaAsO_2$ was more obvious in rats. The exposure of arsenic on mouse species and arsenic had no significant effect on the up-regulation of collagen1 and fibrosis.

5. Conclusion

In vitro and in vivo studies have shown that arsenic exposure activates the TGF-β/Smad pathway, up-regulates the expression levels of fibrosis markers such as α-SMA, Collagen1/3 and Fibronectin, and accelerates the process of tissue fibrosis, which further complements the molecular mechanism theory of arsenic-induced fibrosis.

6. Limitations and perspectives

Although this study reveals the regulation of arsenic on the TGF-β/Smad pathway, there is still no experimental study for further validation, and the specific molecular mechanisms seem insufficient such as the effect of PML SUMOylation on the TGF-β/Smad pathway. Moreover, it has also become an important direction for future research.

Conflict of interests

The authors declare no conflicts of interest. The authors are solely responsible for the writing and content of the paper. The author's affiliation is as shown on the cover page.

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