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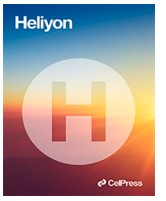

Review article

Fluoride-induced apoptosis in non-skeletal tissues of experimental animals: A systematic review and meta-analysis

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ABSTRACT

Different studies have suggested that fluoride can induce apoptosis in non-skeletal tissues, however, evidence from these experimental studies is still controversial. This meta-analysis aims to clarify the mechanism of fluoride-induced apoptosis in non-skeletal tissues of experimental animals. Primary studies which measured apoptosis were identified through exhaustive database searching in PubMed, Embase, Web of Science Core Collection, Scopus, and references of included studies. A random effects model with standardized mean difference (SMD) was used for meta-analyses. The heterogeneity of the studies was evaluated using Higgin's I^2 statistics. The risk of bias and publication bias were assessed using the SYRCLE's risk of bias tool and Egger's test, respectively. There was an increase in total apoptotic cells, and the expression of Bax, Bax/Bcl-2 ratio, caspase-3, caspase-8, caspase-9, Cyt *c*, and p53, and a decrease in the expression of Bcl-2 in the fluoride-treated groups as compared to the control groups. However, there was no evidence of a difference in the expression of APAF-1 in the two groups. The subgroup analysis highlighted the role of the intervention period in modification of the apoptotic effect of fluoride and that the susceptibility and tolerance of different animal species and tissues vary. Meta-regression analysis indicated that the studies' effect size for total apoptotic cells was influenced by animal species and that of Bax by the sample source. The results of this meta-analysis revealed that fluoride causes apoptosis by up-regulating caspase-3, -8, and -9, Cyt *c*, p53, Bax, and down-regulating Bcl-2 with a concomitant up-regulation of the Bax/Bcl-2 ratio.

1. Introduction

Fluorine, the 13th most abundant element in the earth's crust is ubiquitously present in the soil, water, plants, and air [1]. The recommended optimal level of fluoride in drinking water is between 0.6 and 1.1 mg/L [2]. Fluoride is considered beneficial for the integrity of bone and teeth at optimal levels [3]. Fluoride concentrations of less than 0.5 mg/L in drinking water could cause dental caries, lack of formation of dental enamel, and reduction of bone mineralization. On the other hand, fluoride consumption at higher

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Table 1
Characteristics of included studies.

Author	Year	Species/ Strain	Animal sex	Age	Weight	Tissue/ Organ	Animal Number: Control/ Intervention	Intervention period	Indexes
Agalakova & Gusev [24]	2013	Rats	Male	6 weeks	N/A	Other	10/10	>90 days	% apoptosis
Bai et al. [17]	2010	Broilers	N/A	1 day	N/A	Kidney	5/5	30–90 days	% apoptosis
Bontemps et al. [25]	2019	Mice	Male	8 weeks	25 ± 5 g	Kidney	5/6	<30 days	Caspase-3
Campos-Pereira et al. [18]	2017	Rats	Male	Adult	180–200 g	Liver	10/10	30–90 days	% apoptosis
Cao et al. [26]	2014	Carp	N/A	Juvenile	55.8 ± 0.24 g	Other	18/18	90 days	Caspase-3
Chen et al. [27]	2009	Broilers	N/A	1 day	N/A	Other	5/5	30–90 days	% apoptosis
Chen et al. [28]	2015	Carp	N/A	Juvenile	15.8 ± 0.24 g	Kidney	18/18	30–90 days	% apoptosis, caspase-3, -8, -9
Chouhan & Flora [19]	2008	Rats	Male	Adult	100–120 g	Brain	6/6	30–90 days	Caspase-3
Deng et al. [29]	2017	Mice	N/A	N/A	N/A	Other	8/8	30–90 days	Number of apoptotic cells, Bax, Bcl, caspase-3, Bax/Bcl-2 ratio
Duan et al. [30]	2017	Rats	Male	N/A	180–200 g	Kidney	10/10	30–90 days	Caspase-3
Geng et al. [31]	2014	Rats	Female	8–10 weeks	160–250 g	Other	10/10	>90 days	Bcl-2, Bax, caspase-3
Gutiérrez-Salinas et al. [22]	2010	Rats	Male	N/A	250 ± 5 g	Other	5/5	30–90 days	Bcl-2, caspase-3, p53
He & Chen [32]	2006	Rats	Male	N/A	80–120 g	Liver	5/5	<30 days	% apoptosis
Li et al. [33]	2020	Zebrafish	Female	3 months	N/A	Other	8/8	30–90 days	Bax, Bcl-2, caspase-3, -8, -9, Bax/Bcl-2 ratio, Cyt *c*
Li et al. [34]	2021	Ducks	N/A	7 days	N/A	Kidney	7/7	<30 days	Bax, Bcl-2, caspase-3, Cyt *c*, p53, APAF-1
Liu et al. [35]	2011	Rats	Male/ Female	N/A	90–120 g	Brain	12/12	>90 days	% apoptosis
Liu et al. [36]	2013	Broilers		1 day	N/A	Other	5/5	30–90 days	% apoptosis, Bax, Bcl-2, caspase-3
Liu et al. [37]	2016	Rats	Male/ Female	1 month	110–130 g	Other	10/10	>90 days	% apoptosis
Lou et al. [38]	2014	Rats	Male/ Female	N/A	90–120 g	Brain	20/20	>90 days	Apoptotic cells, Bax, Bcl-2, Bax/ Bcl-2 ratio
Lu et al. [39]	2017	Mice	N/A	N/A	N/A	Liver	8/8	30–90 days	% apoptosis, caspase-3, -8
Mohammed et al. [20]	2017	Rats	Female	12 weeks	40–60 g	Other	8/8	30–90 days	Bcl-2, caspase-3
Mondal et al. [40]	2021	Zebrafish	Female	N/A	0.25–0.30 g	Brain	10/10	30–90 days	Bax, Bcl-2, p53
Niu et al. [41]	2018	Rats	Female	N/A	180–220 g	Brain	10/10	30–90 days	Caspase 3
Ouyang et al. [42]	2021	Ducks	N/A	1 day	N/A	Liver	7/7	<30 days	Bax, Bcl-2, caspase-3, Cyt *c*, p53, APAF-1
Panneerselvama et al. [21]	2015	Rats	Male	3 months 2 weeks	130–150 g	Other	3/3	<30 days	Bax/Bcl-2 ratio, caspase-3, Cyt *c*
Qing-Feng et al. [43]	2019	Rats	N/A	1 month	N/A	Other	30/30	>90 days	% apoptosis, p53
Quadri et al. [44]	2018	Rats	Male	75 day old	N/A	Other	18/18	30–90 days	% apoptosis, Bcl-2, caspase-3
Singh et al. [45]	2017	*Clarias gariepinus*	N/A	N/A	50–60 g	Other	9/9	30–90 days	Caspase-3
Song et al. [46]	2013	Rats	Male/ Female	N/A	120 ± 5 g	Kidney	12/12	30–90 days	% apoptosis
Song et al. [47]	2014	Rats	Male	N/A	90 ± 10 g	Kidney	6/6	>90 days	% apoptosis, caspase-3, -8, -9, Cyt *c*
Song et al. [48]	2015	Rats	Male	N/A	90 ± 10 g	Liver	6/6	>90 days	% apoptosis, caspase-3, -9

(*continued on next page*)

Table 1 (*continued*)

Author	Year	Species/ Strain	Animal sex	Age	Weight	Tissue/ Organ	Animal Number: Control/ Intervention	Intervention period	Indexes
Wang et al. [23]	2011	Mice	Male	8 weeks	20 g	Other	8/8 (Apoptosis) 5/5 (Cyt *c*, caspase-3)	30–90 days	% apoptosis, Cyt *c*, caspase-3
Wang et al. [49]	2017	Mice	N/A	30 day old	20 ± 1.5 g	Others	10/10	90 days	Cyt *c*, caspase-3, -9, Bax, Bcl-2
Wei et al. [50]	2018	Mice	Male/ Female	4 weeks	N/A	Kidney	8/8	30–90 days	% apoptosis, Bax, Bcl-2, Bax/Bcl-2 ratio, caspase-3, 9, p53
Wei et al. [51]	2018	Rats	Male/ Female	1 month	N/A	Brain	15/15	>90 days	% apoptosis
Wei et al. [52]	2019	Rats	Male	N/A	120–140 g	Other	7/7	30–90 days	% apoptosis
Yan et al. [53]	2016	Rats	Male/ Female	5 weeks	N/A	Brain	10/10 (Apoptosis) 8/8 (Bax, Bcl-2)	30–90 days	Apoptotic cells, Bax, Bcl-2, Bax/ Bcl-2 ratio
Zhan et al. [54]	2006	Pig (Barrows)	Male	N/A	17 kg	Liver	8/8	30–90 days	Caspase-3, -9
Zhang & Zhang [55]	2013	Rats	Male	N/A	60–80 g	Brain	6/6	>90 days	Bax, Bcl-2, Bax/ Bcl-2 ratio
Zhang et al. [56]	2016	Rats	Male	Pups	N/A	Other	4/4	30–90 days	Cyt *c*, caspase-3
Zhao et al. [57]	2018	Mice	Male	8 weeks	N/A	Liver	12/12	>90 days	Bcl-2, p53, Cyt *c*, caspase-3, APAF-1
Zhou et al. [58]	2020	Mice	Female	21 days	N/A	Liver	6/6	30–90 days	% apoptosis, caspase-3, -9, Cyt *c*
Kumar et al. [59]	2021	Golden Hamster	Male	90–100 days	125 ± 5 g	Testes	5/5	>90 days	Bax, Bcl-2
Cheng et al. [60]	2013	Rats	N/A	30 days	56 ± 7.1 g	Myocardium	3/3	90 days	Caspase-9, apoptotic cells
Khan et al. [61]	2021	Wistar Rats	Female	3 months	140 ± 20 g	Liver	5/5	>90 days	% apoptosis, Bax, Bcl-2, Bax/Bcl-2 ratio
Shao et al. [62]	2020	Mice	Male	N/A	15–20 g	Kidney	10/10	>90 days	Bax, Bcl-2, caspase-3, -9, p53, Cyt *c*, APAF-1
Bhowmik et al. [63]	2020	Mice	Male	1 month	20–23 g	Brain, Liver	8/10	>90 days	Bax, Bcl-2

APAF-1: Apoptotic protease-activating factor; Bax: Bcl-2-associated X protein: Bcl-2: B-cell lymphoma/leukemia-2; Cyt *c*: Cytochrome *c*.
N.B: Total number of study animals was used in studies that did not report the number of animals for each experiment.

levels may lead to a range of detrimental effects [4,5]. High fluoride exposure can induce acute toxicity, occasionally reported, and the more common chronic toxicity, which is dependent on the dose and duration of exposure among other factors [6]. Endemic fluorosis is global in scope, affecting millions of people and occurring on all continents [7]. Fluorosis can present as milder phenotypes such as dental mottling as well as relatively more severe skeletal manifestations such as severe crippling, osteosclerosis, osteoporosis, and osteomalacia or osteopenia [8]. Additionally, fluoride is now known to affect soft tissues leading to structural changes and disorders in their function [9]. Although numerous studies in recent years have focused on the molecular mechanisms associated with fluoride toxicity, the underlying mechanisms of chronic fluorosis are still not well understood. The results of previous studies indicated that fluoride can induce oxidative stress, endoplasmic reticulum stress, mitochondrial damage, cell cycle arrest, alteration of gene expression, and apoptosis [10,11].

Apoptosis is a highly regulated and programmed cell death characterized by specific biochemical and morphological features that culminate in cellular shrinkage to apoptotic bodies that are engulfed by neighboring macrophages [12]. This occurs without an accompanying inflammatory response. Characterized morphological changes accompanying apoptosis include cleavage of cytoskeletal filament fibers, cell cytoplasm, and nucleus condense, DNA fragmentation by the action of endonucleases, fragmentation of cell organelles (Golgi apparatus, endoplasmic reticulum, and mitochondria), and blistering of the cell plasma membrane. The cells become round and separate from neighboring cells [13]. Apoptosis plays a key role in the elimination of unnecessary or damaged cells and a variety of normal biological processes such as cell proliferation and differentiation, aging, and tissue homeostasis [12,14].

A growing body of literature has shown that apoptosis induced by oxidative stress plays a key role in the pathogenesis of fluorosis. Oxidative stress can be triggered by promoting reactive oxygen species (ROS) production and reducing antioxidant function [15]. Excess cellular levels of ROS cause damage to proteins, nucleic acids, lipids, membranes, and organelles, which can lead to the activation of cell death processes such as apoptosis [15]. Other molecular mechanisms underlying fluoride-induced apoptosis include

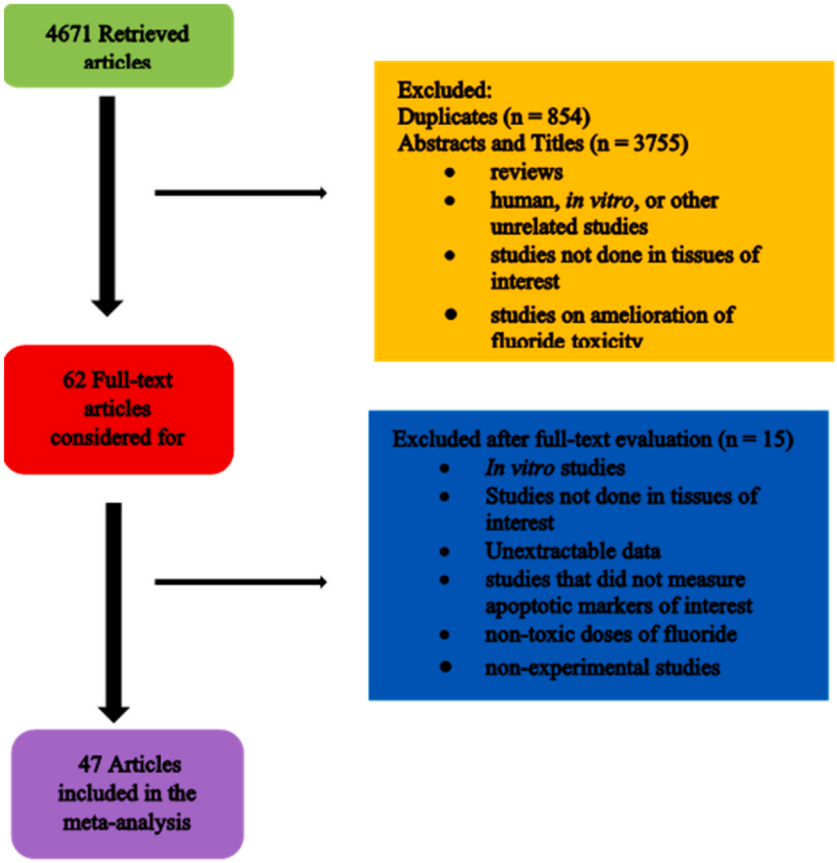


Fig. 1. Flow chart of identification and screening procedure.

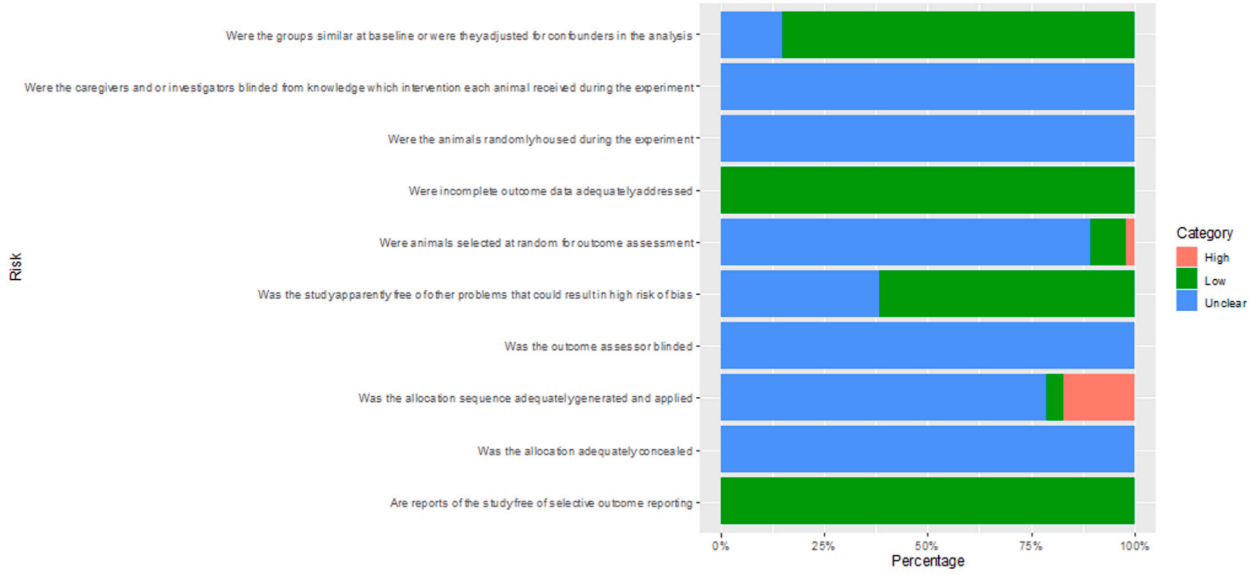


Fig. 2. Risk of bias, average per item.

disruption of mitochondria outer membrane and release of cytochrome *c* into the cytosol, which activates caspases-9 and -3 (intrinsic) apoptotic pathway, activation of the cell surface death receptors (extrinsic Fas/FasL-caspase-8 and -3 pathway), alterations in the ratio of anti-apoptotic-apoptotic Bcl-2 proteins, upregulation of p53 expression, expression of apoptosis-related genes, endoplasmic

Table 2
Summary of meta-analysis and sensitivity analysis results.

Apoptosis biomarkers	Main meta-analysis				Sensitivity analysis			
	Number of studies	Effect estimate (95% CI)	I^2	Egger' test *p* value	No. of excluded studies	Effect estimate (95% CI)	I^2	Egger' test *p* value
Total apoptotic cells	26	3.78 (2.72; 4.83)	90%	0.02	5	3.59 (2.58; 4.61)	88%	0.13
APAF-1	4	1.94 (−0.66; 4.54)	92%	N/A	N/A	N/A	N/A	N/A
Bax	15	3.04 (1.78; 4.29)	89%	0.04	4	3.34 (1.99; 4.70)	87%	0.01
Bcl-2	19	−1.99 (−2.91;-1.06)	89%	0.001	4	−1.37 (−2.31; −0.44)	89%	0.02
Bax/Bcl-2 Ratio	9	3.95 (1.38; 6.51)	93%	N/A	2	5.08 (1.23; 8.93)	96%	N/A
Caspase-3	28	4.32 (3.14; 5.51)	90%	<0.0001	3	4.60 (3.28; 5.93)	90%	<0.0001
Caspase-8	4	3.06 (0.98; 5.14)	86%	N/A	N/A	N/A	N/A	N/A
Caspase-9	10	4.81 (3.01; 6.60)	88%	N/A	N/A	N/A	N/A	N/A
Cytochrome *c*	11	3.36 (2.25; 4.47)	69%	0.005	N/A	N/A	N/A	N/A
p53	8	4.89 (2.21; 7.57)	95%	N/A	1	5.63 (2.14; 9.12)	96%	N/A

reticulum stress and disturbances in protein synthesis [16]. Even though this subject has been thoroughly and extensively evaluated, studies employed different protocols and methods, different test groups and sizes, and different types of experimental animals which, among other factors, yielded conflicting findings. Bai et al. [17] for example, reported an increase in the percentage of apoptotic renal cells in chicken whereas Campos-Pereira et al. [18] reported no evident increase in positive terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) induced by fluoride in rat hepatocytes indicating the absence of apoptosis. In another study, fluoride exposure did not produce any signs of apoptosis evidenced by the lack of an increase in caspase-3 [19]. In contrast, several studies reported an increase in caspase activity after exposure to fluoride [20–23]. It is therefore important to provide a systematic evaluation and meta-analysis of these studies to clarify the mechanism of fluoride-induced apoptosis in experimental animals.

2. Materials and methods

2.1. Inclusion criteria

The following criteria were used to include studies in our analyses: 1) experimental studies evaluating apoptosis biomarkers in experimental animals; 2) studies published in English; 3) studies that provided animal numbers, means, and standard deviations. Because of limited studies, we focused on studies that reported % apoptosis/number of apoptotic cells, Bax (Bcl-2-associated X protein), Bcl-2 (B-cell lymphoma/leukemia-2), caspase-3, -8, -9, Bax/Bcl-2 ratio, p53, Cyt *c* (cytochrome *c*), and APAF-1 (apoptotic protease-activating factor). A tissue-specific analysis was not done because of limited studies evaluating apoptosis markers in individual tissues.

2.2. Exclusion criteria

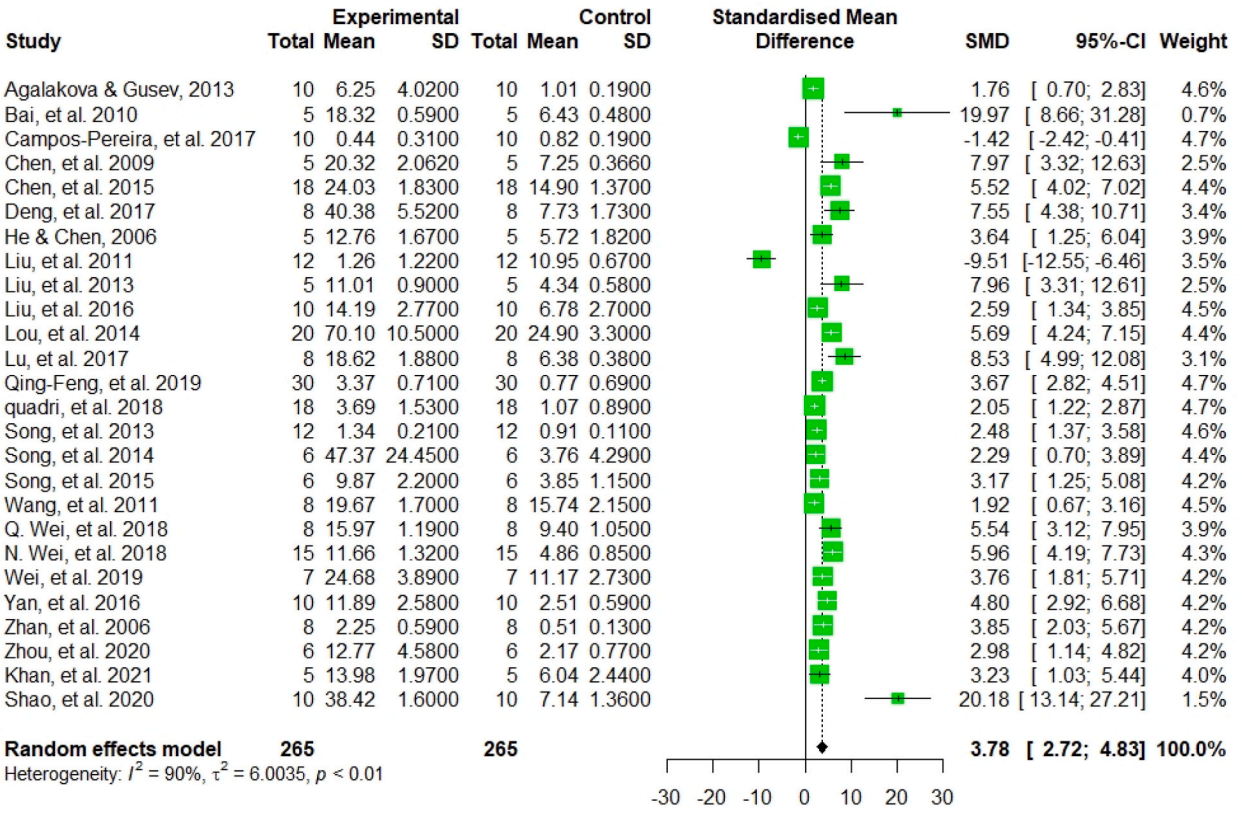
The following criteria were used to exclude studies in our analyses: 1) conference abstracts, reviews, editorials, and letters; 2) human, *in vitro,* or other unrelated studies; 3) full-text not available in English; 4) studies with unavailable data/unextractable data; 5) studies that used non-toxic doses of fluoride; 6) co-exposure with no fluoride only group; 7) multigenerational studies; 9) studies on amelioration of fluoride toxicity.

2.3. Literature search

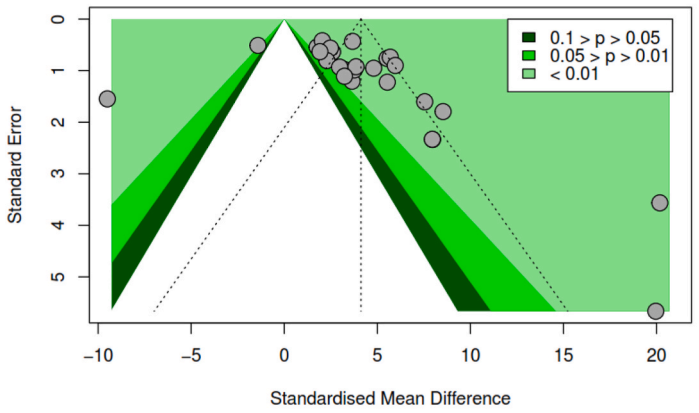
Structured and electronic database searches were done in PubMed, Embase, Web of Science Core Collection, and Scopus to identify eligible articles on June 14th, 2022, with the following keywords and Boolean operators: ("Fluoride OR Fluorosis") AND ("Apoptosis") AND ("Caspase OR Bcl-2 OR BAX OR Cytochrome C OR PARP OR p53 OR APAF-1") and articles were exported to Mendeley. Further, a manual search of references of the eligible studies was done. Search terms were validated by ensuring the search retrieved a selection of articles, representative of relevant works. Searches were restricted to animals and the English language with no restriction on the date of publication. Titles and abstracts were screened independently by two investigators (LA and AK). Full-text screens were conducted to confirm eligibility. The divergence of opinion between the two investigators was settled through concurrence and discussion with a third investigator (SN, TA, and GP).

2.4. Data extraction and quality assessment

Two investigators (LA and AK) extracted data from eligible studies independently through the review of titles, abstracts, and full texts. In case of a difference in opinion, the ultimate decision was made by concurrence and discussion with a third investigator (SN, TA, and GP). The following information was extracted: author, year of publication, animal species/strain, sex, age, weight, tissue/



a *Forest plot for total apoptotic cells meta-analysis*

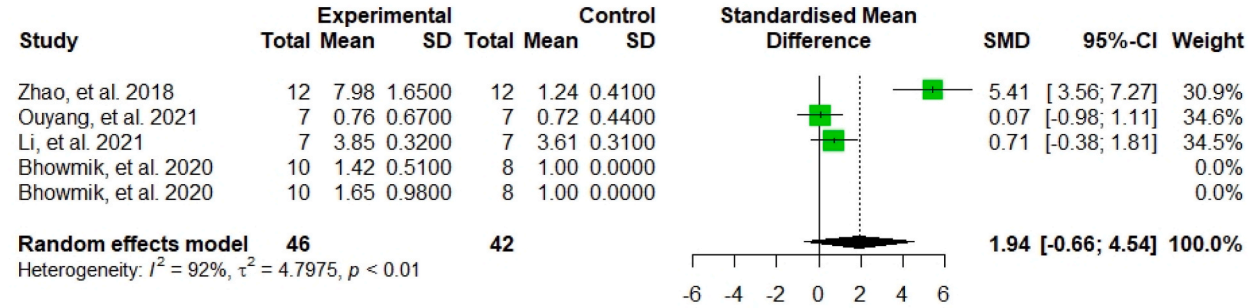


b *Total apoptotic cells contour-enhanced funnel plot*

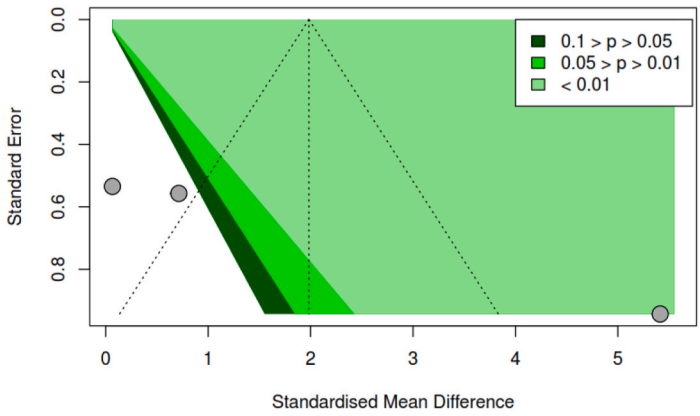
Fig. 3a. Forest plot for total apoptotic cells meta-analysis. Total apoptotic cells contour-enhanced funnel plot.

organ studied, number of animals in experimental and control groups, study period, and apoptosis biomarkers (Table 1). In studies with numerous intervention groups, a single pair was selected and others were excluded from the meta-analysis: the high dose group was included in studies with numerous fluoride groups; the fluoride-only groups were included in co-exposure studies; and in studies with varied treatment period, the longest duration was selected for the study. In some studies, multiple datasets were extracted if reported that is, in studies that measured apoptosis biomarkers in different organs/tissues. WebPlotDigitizer was used to facilitate graphical data extraction. The standard deviation (SD) for studies that recorded standard error (SE) of mean was achieved by multiplying the SE by the square root of the sample size ($SD = SE \times \sqrt{n}$).

The quality of included studies was evaluated independently by two investigators (LA and TA) through the SYRCLE's risk of bias



a *Forest plot for APAF-1 meta-analysis*



b *APAF-1 contour-enhanced funnel plot*

Fig. 4a. Forest plot for APAF-1 meta-analysis. APAF-1 contour-enhanced funnel plot.

tool and divergence was determined through consensus-oriented discussion. The SYRCLE's risk of bias tool contains 10 entries involving 6 types of bias: selection bias, performance bias, detection bias, attrition bias, reporting bias, and other biases. Signaling questions are utilized to designate a verdict of low, high, or unclear risk of bias to each item mentioned in the tool [64].

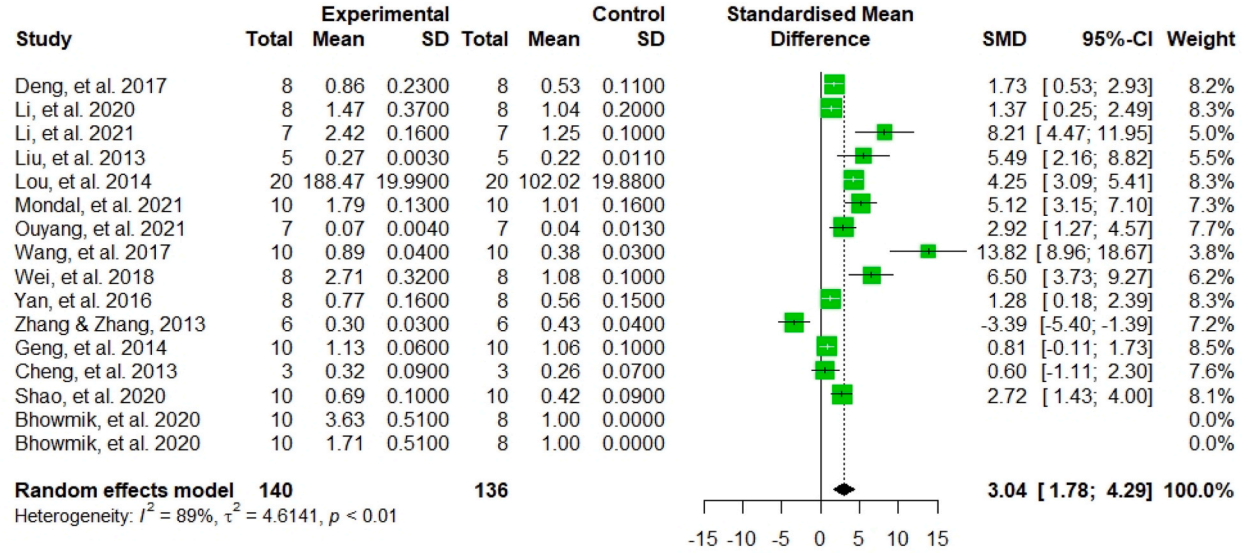
2.5. Statistical analysis

All statistical analysis was performed using the R project software version 4.1.1(R package meta). Hedge's g standardized mean difference (SMD) was used as a measure of effect size since the measures used were not the same in all studies. Corresponding 95% confidence intervals (95% CIs) were presented. Because of the diversity of methods, species, and intervention protocols, the pooled effect sizes (ES) were calculated according to DerSimonian and Laird for the random effects model. The I^2 index was used to determine the statistical heterogeneity. An I^2 index value of around 25%, 50%, and 75% was considered low-, moderate-, and high-heterogeneity, respectively [65]. Egger's regression test was used to assess publication bias via the funnel plot asymmetry. The leave-one-out sensitivity analysis was performed by excluding the studies identified as having a high risk of bias using the SYRCLE's risk of bias tool one at each analysis. Subgroup analyses were performed based on the intervention period (<30, 30–90, >90 days); species of animals (mice, rats, others), and sample source (liver, kidney, brain, other tissue samples) to determine the factors associated with differences among study results in the outcome indicators.

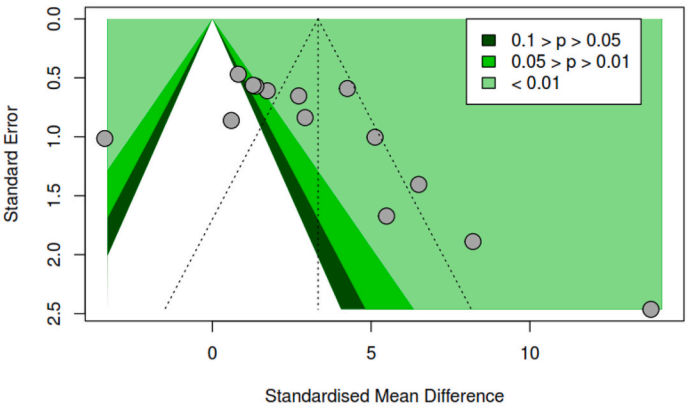
3. Results

3.1. Study identification and selection

We systematically identified a total of 4665 articles using electronic databases (PubMed = 215, Embase = 1180, Web of Science = 367, and Scopus = 2903) and an additional 6 articles through a manual search of references of the eligible studies. After deduplication, 3817 articles were screened for title and abstract and 3755 articles were excluded according to the eligibility criteria. A full-text evaluation was conducted for the remaining 62 articles, and 15 articles were excluded for not fulfilling inclusion criteria. Finally, 47 eligible studies were selected. The flow chart of the selection and identification process of our analysis is shown in Fig. 1. All study



a *Forest plot for Bax meta-analysis*



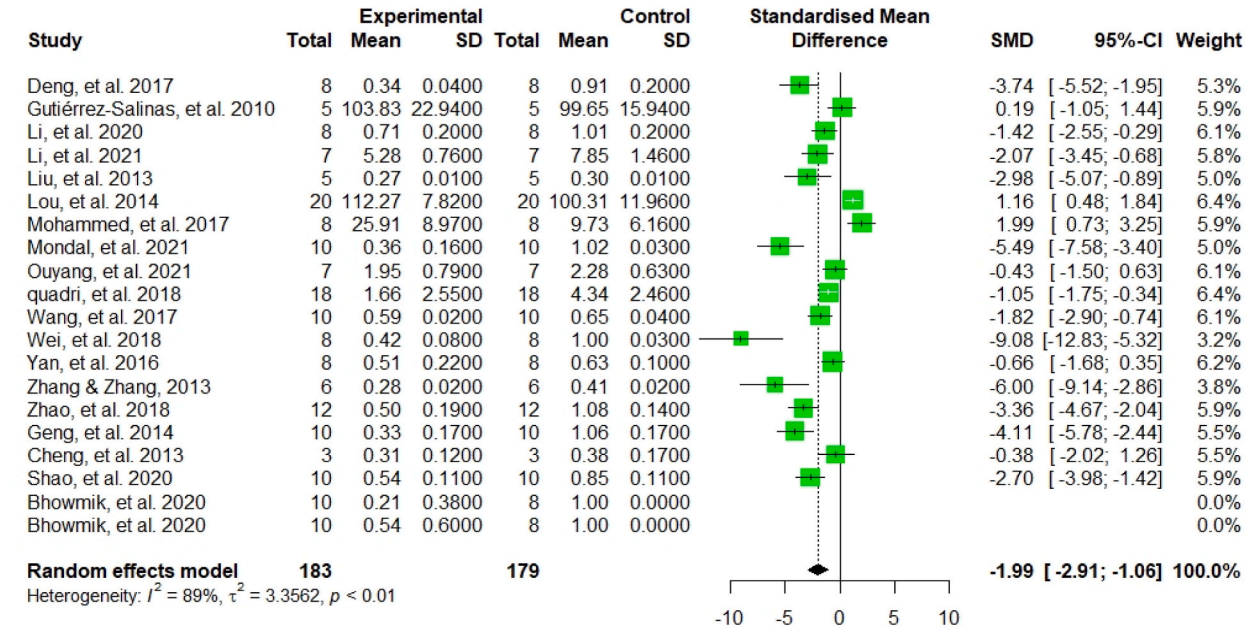
b *Bax contour-enhanced funnel plot*

Fig. 5a. Forest plot for Bax meta-analysis. Bax contour-enhanced funnel plot.

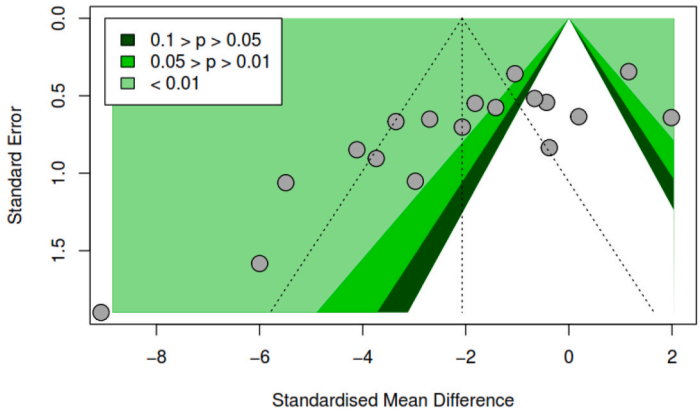
characteristics are presented in Table 1. The studies presented a high prevalence of rodents as laboratory animals (36 out of 47), although the characteristics of the animals and study design differed substantially among the studies.

3.2. Risk of bias and quality of included studies

The risk of bias was categorized as high, low, or unclear. In view of selection bias, 78.7% of the included studies reported randomization of the experimental units but the information provided was insufficient to assess whether the allocation sequence was adequately generated or adequately concealed. In 85.1% of the studies, the control and intervention groups were identical at the beginning of the experiment with 14.9% presenting insufficient information. Furthermore, all of the included studies registered unclear risk of bias regarding performance bias items "random housing" and "blinding" with 89.4% and 100% categorized as having unclear risk of bias regarding detection bias "random outcome assessment" and "blinding", respectively. Regarding the attrition bias, 100% had all the animals included in the study. All the studies were free of reporting bias and 61.9% registered a low risk of bias from other sources. The majority of the studies presented a high number of unclear scores, indicating incomplete information related to the study design, resulting in difficulty accessing the actual risk of bias and not fully reproducible experimental protocols. The overall result of the risk of bias assessment of the included studies is presented in Fig. 2.



a *Forest plot for Bcl-2 meta-analysis*



b *Bcl-2 contour-enhanced funnel plot*

Fig. 6a. Forest plot for Bcl-2 meta-analysis. Bcl-2 contour-enhanced funnel plot.

3.3. Meta-analysis of apoptosis biomarkers

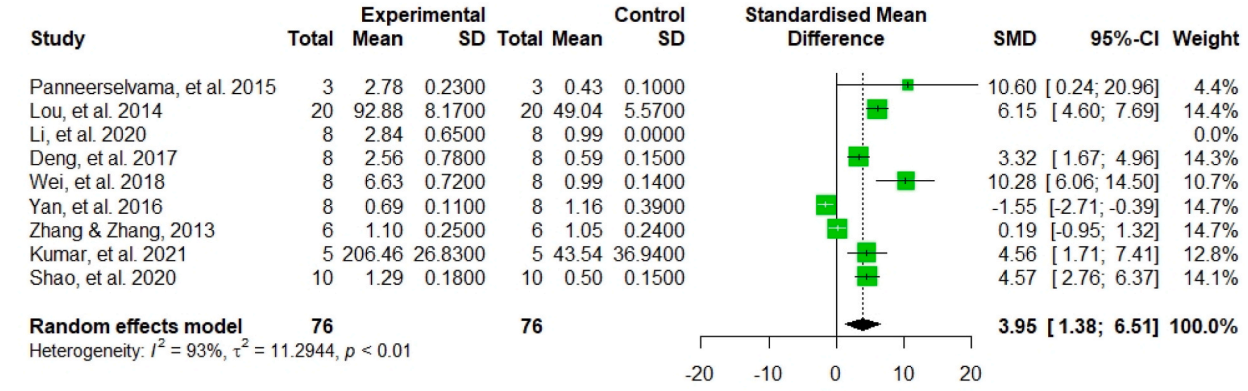
A summary of the meta-analysis and sensitivity analysis results of all biomarkers is presented in Table 2.

3.3.1. Meta-analysis of total apoptotic cells

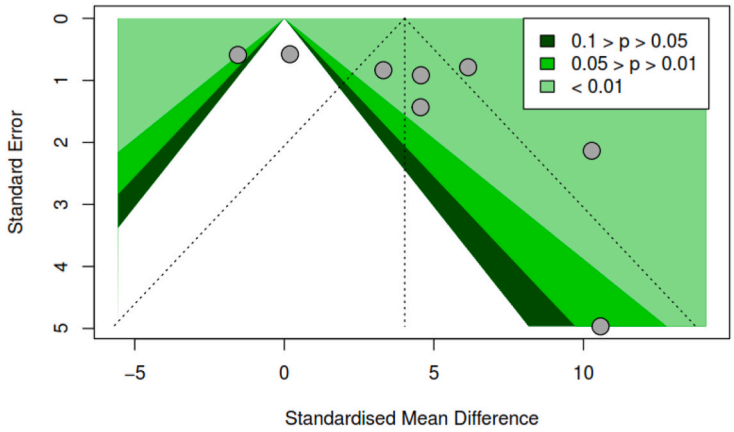
Results from 26 studies were pooled in the meta-analysis of total apoptotic cells. The total apoptotic cells in tissues of fluoride-treated animals was higher than in the controls (SMD = 3.78, 95% CI: 2.72; 4.83, $Z = 6.99$, $p < 0.0001$). The statistical heterogeneity was high ($I^2 = 90\%$, $p < 0.0001$) (Fig. 3a). The funnel plot was asymmetrical (Fig. 3b), however, an Egger's test performed to detect publication bias showed evidence of publication bias (Egger's regression intercept 3.01, t = 2.41, $p = 0.02$). A sensitivity analysis done by exclusion of five studies with high risk of bias [18,27,29,32,36] showed similar results with the Egger's test showing no evidence of publication bias (SMD = 3.59, 95% CI: 2.58; 4.61, $Z = 6.93$, $p < 0.0001$; $I^2 = 88\%$, $p < 0.0001$; Egger's regression intercept 2.25, t = 1.59, $p = 0.13$).

3.3.2. Meta-analysis of APAF-1

A total of 4 studies were included in the meta-analysis of APAF-1. No evidence of a difference in the expression of APAF-1 between the fluoride-treated groups and the control (SMD = 1.94, 95% CI: −0.66; 4.54, $Z = 1.46$, $p = 0.14$) was found. There was high



a *Forest plot for Bax/Bcl-2 meta-analysis*



b *Bax/Bcl-2 contour-enhanced Funnel plot*

Fig. 7a. Forest plot for Bax/Bcl-2 meta-analysis. Bax/Bcl-2 contour-enhanced Funnel plot.

heterogeneity (I^2 = 92%, $p < 0.0001$) (Fig. 4a). The funnel plot was asymmetrical (Fig. 4b), however, owing to the small number of studies, we did not perform a publication bias examination for the studies evaluating the expression levels of APAF-1 [66]. No study was excluded for sensitivity analysis as none of the included studies had a high risk of bias.

3.3.3. Meta-analysis of Bax

Results from 15 studies measuring Bax were pooled. The expression of Bax was increased in the fluoride-treated groups as compared to the controls (SMD = 3.04, 95% CI: 1.78; 4.29, $Z = 4.74$, $p < 0.0001$). Heterogeneity of the studies included was high (I^2 = 89%, $p < 0.0001$) (Fig. 5a). The funnel plot was asymmetrical (Fig. 5b) and the publication bias evaluated through the Egger's test showed evidence of publication bias (Egger's regression intercept 3.92, t = 2.24, $p = 0.04$). The sensitivity analysis was performed by removing four studies with high risk of bias [29,36,40,55] and the results remained unchanged (SMD = 3.34, 95% CI: 1.99; 4.70, Z = 4.83, $p < 0.0001$ I^2 = 87%, $p < 0.0001$; Egger's regression intercept 5.15, t = 3.38, $p = 0.01$).

3.3.4. Meta-analysis of Bcl-2

A total of 19 studies measuring Bcl-2 were include in the meta-analysis of Bcl-2. Compared to the control groups, the expression of Bcl-2 was decreased in the fluoride treatment groups (SMD = −1.99, 95% CI: −2.91; −1.06, $Z = -4.20$, $p < 0.0001$) and there was high heterogeneity among the studies included (I^2 = 89%, $p < 0.0001$) (Fig. 6a). A visual inspection of the funnel plot showed asymmetry (Fig. 6b). Publication bias was present (Egger's regression intercept −5.58, t = −3.98, $p = 0.001$). The results did not change after a sensitivity analysis was done with exclusion of the four studies with high risk of bias [29,36,40,55] (SMD = -1.37, 95% CI: −2.31; −0.44, $Z = -2.88$, $p = 0.004$; I^2 = 89%, $p < 0.0001$; Egger's regression intercept −5.27, t = −2.57, $p = 0.02$).

3.3.5. Meta-analysis of Bax/Bcl-2 ratio

Results from 9 studies measuring Bax/Bcl-2 ratio were pooled. The results of Fig. 7a show higher levels of the Bax/Bcl-2 ratio in the fluoride-treated groups as compared to the controls (SMD = 3.95, 95% CI: 1.38; 6.51, $Z = 3.02$, $p = 0.003$). High heterogeneity of the

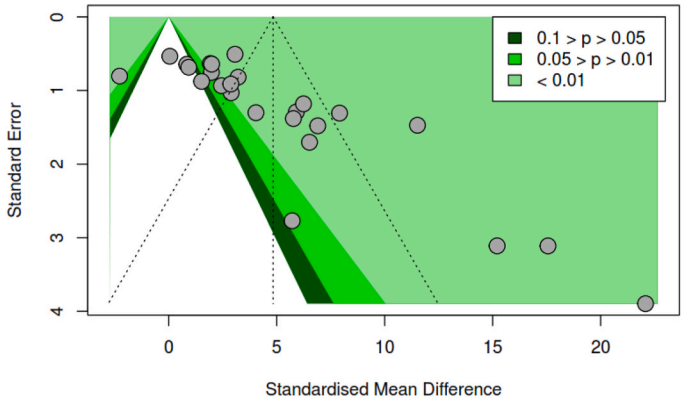
Study	Experimental Total	Mean	SD	Control Total	Mean	SD	SMD	95%-CI	Weight
Bontemps, et al. 2019	6	124.00	32.7000	5	100.00	14.8300	0.83	[-0.43; 2.10]	4.7%
Cao, et al. 2014	18	2.60	0.1200	18	1.00	0.1500	11.52	[8.62; 14.41]	3.9%
Chen, et al. 2015	18	33.92	1.2100	18	30.11	1.2200	3.07	[2.07; 4.06]	4.8%
Chouhan & Flora, 2008	6	15.97	1.4000	6	27.86	6.6600	-2.28	[-3.87; -0.69]	4.6%
Deng, et al. 2017	8	0.76	0.0500	8	0.38	0.0700	5.91	[3.35; 8.46]	4.1%
Duan, et al. 2017	10	2.98	0.4200	10	0.98	0.1100	6.24	[3.91; 8.57]	4.2%
Gutiérrez-Salinas, et al. 2010	5	148.89	15.1800	5	100.36	15.1800	2.89	[0.84; 4.93]	4.4%
Li, et al. 2020	8	1.36	0.0800	8	1.01	0.2300	1.92	[0.68; 3.17]	4.8%
Li, et al. 2021	7	1.25	0.0600	7	0.88	0.0600	5.77	[3.04; 8.50]	4.0%
Liu, et al. 2013	5	0.31	0.0260	5	0.26	0.0030	2.44	[0.59; 4.29]	4.5%
Lu, et al. 2017	8	0.89	0.0500	8	0.31	0.0100	15.21	[9.06; 21.36]	2.1%
Mohammed, et al. 2017	8	38.13	7.7800	8	16.00	4.9500	3.21	[1.60; 4.82]	4.6%
Niu, et al. 2018	10	2.04	0.2700	10	0.99	0.0000			0.0%
Ouyang, et al. 2021	7	0.80	0.2700	7	0.79	0.0600	0.05	[-1.00; 1.10]	4.8%
Panneerselvama, et al. 2015	3	2.67	0.4000	3	0.54	0.1300	5.73	[-0.03; 11.49]	2.3%
Singh, et al. 2017	9	2.35	1.4700	9	1.00	0.0000			0.0%
Song, etal. 2014	6	1.97	0.4400	6	0.99	0.0700	2.87	[1.07; 4.67]	4.5%
Song, et al. 2015	6	0.34	0.0700	6	0.23	0.0200	1.97	[0.49; 3.46]	4.6%
Wang, et al. 2011	5	157.04	12.6100	5	100.70	12.6100	4.04	[1.45; 6.62]	4.0%
Wang, et al. 2017	10	0.61	0.0300	10	0.20	0.0100	17.56	[11.42; 23.70]	2.1%
Wei, et al. 2018	8	2.49	0.2900	8	0.99	0.0200	6.90	[3.98; 9.82]	3.8%
Zhan, et al. 2006	8	8.06	0.8900	8	6.05	1.0200	1.99	[0.73; 3.25]	4.7%
Zhang, et al. 2016	4	1.46	0.2900	4	1.01	0.2200	1.52	[-0.22; 3.26]	4.5%
Zhao, et al. 2018	12	2.50	0.1400	12	0.99	0.2200	7.91	[5.33; 10.48]	4.1%
Zhou, et al. 2020	6	2.29	0.2400	6	1.01	0.0900	6.52	[3.14; 9.90]	3.6%
Geng, et al. 2014	10	7.32	0.3300	10	1.03	0.2000	22.08	[14.39; 29.76]	1.6%
Kumar, et al. 2021	5	66.28	23.5900	5	43.11	21.6500	0.92	[-0.42; 2.27]	4.7%
Bhowmik, et al. 2020	10	5.00	1.3300	8	1.00	0.0000			0.0%
Bhowmik, et al. 2020	10	1.71	0.9200	8	1.00	0.0000			0.0%
Random effects model	**236**			**231**			**4.32**	**[3.14; 5.51]**	**100.0%**

Heterogeneity: $I^2 = 90\%$, $\tau^2 = 7.2500$, $p < 0.01$

Standardised Mean Difference

-20 -10 0 10 20

a *Forest plot for caspase-3 meta-analysis*



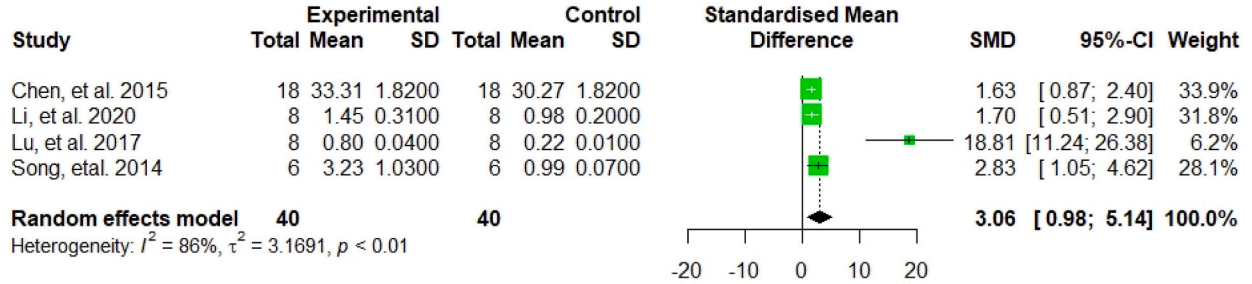
b *Caspase-3 contour-enhanced funnel plot*

Fig. 8a. Forest plot for caspase-3 meta-analysis. Caspase-3 contour-enhanced funnel plot.

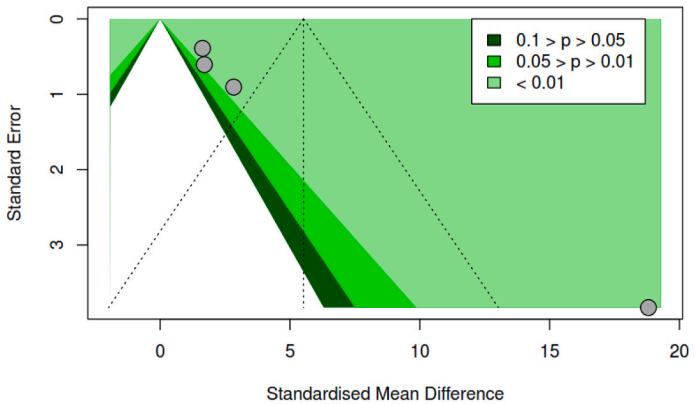
studies was observed ($I^2 = 93\%$, $p < 0.0001$). The funnel plot was asymmetrical (Fig. 7b) indicating publication bias. However, a test for publication bias was not performed due to the small number of studies measuring the Bax/Bcl-2 ratio [66]. Sensitivity analysis done by removing two studies with high risk of bias [29,55] showed similar results (SMD = 5.08, 95% CI: 1.23; 8.93, $Z = 2.59$, $p = 0.01$; $I^2 = 96\%$, $p < 0.0001$).

3.3.6. Meta-analysis of caspase-3

Results from 28 studies measuring caspase-3 were pooled. The expression of caspase-3 was found to be higher in the fluoride-treated groups as compared to the controls (SMD = 4.32, 95% CI: 3.14; 5.51, $Z = 7.17$, $p < 0.0001$). High heterogeneity was found among the studies measuring the expression of caspase-3 ($I^2 = 90\%$, $p < 0.0001$) (Fig. 8a). The funnel plot was asymmetrical (Fig. 8b) and the publication bias evaluated through the Egger's test showed evidence of publication bias (Egger's regression intercept 5.41, t = 5.71, $p < 0.0001$). The results were unchanged after exclusion of three studies with high risk of bias [25,29,36](SMD = 4.60, 95% CI:



a *Forest plot for caspase-8 meta-analysis*



b *Caspase-8 contour-enhanced funnel plot*

Fig. 9a. Forest plot for caspase-8 meta-analysis. Caspase-8 contour-enhanced funnel plot.

3.28; 5.93, $Z = 6.78$, $p < 0.0001$; $I^2 = 90\%$, $p < 0.0001$; Egger's regression intercept 5.32, t = 5.16, $p < 0.0001$).

3.3.7. Meta-analysis of caspase-8

The results on caspase-8 indicated an increase in the expression of caspase-8 in the fluoride-treated groups as compared to the controls (SMD = 3.06, 95% CI: 0.98; 5.14, $Z = 2.89$, $p = 0.004$). The heterogeneity of the included studies was high ($I^2 = 86\%$, $p = 0.0001$) (Fig. 9a) and the funnel plot was asymmetrical (Fig. 9b). As bias examination using a funnel plot is not recommended for the analysis with less than 10 studies [66], we did not examine the studies evaluating the expression level of caspase-8. No study was excluded for sensitivity as none of the included studies had a high risk of bias. A total of 4 studies were include in this meta-analysis.

3.3.8. Meta-analysis of caspase-9

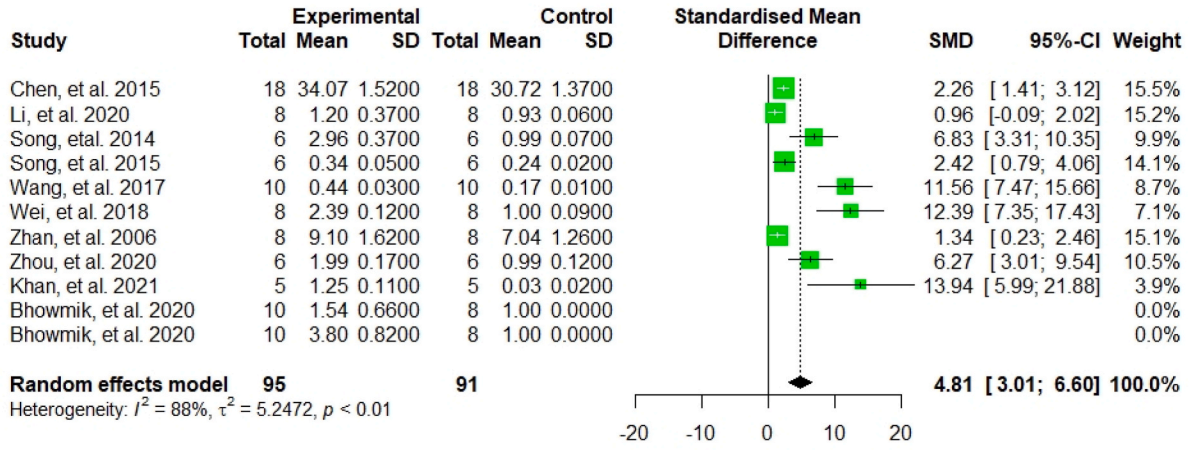
The expression of caspase-9 was found to be higher in the fluoride-treated groups as compared to the controls (SMD = 4.81, 95% CI: 3.01; 6.60, $Z = 5.24$, $p < 0.0001$). The statistical heterogeneity was notable ($I^2 = 88\%$, $p < 0.0001$) (Fig. 10a). A visual inspection of the funnel plot showed asymmetry (Fig. 10b). A test for publication bias was not performed due to the small number of studies measuring caspase-9 [66]. No study was excluded for sensitivity analysis as none of the included studies had a high risk of bias. Results from 10 studies were pooled in this meta-analysis.

3.3.9. Meta-analysis of cytochrome c

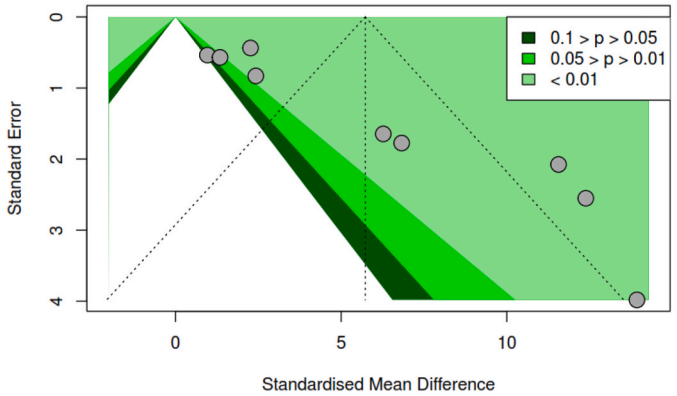
The expression level of Cyt *c* was higher in the fluoride-treated groups as compared to the controls (SMD = 3.36, 95% CI: 2.25; 4.47, $Z = 5.92$, $p < 0.0001$). The statistical heterogeneity was moderate ($I^2 = 69\%$, $p = 0.0006$) (Fig. 11a). The funnel plot was asymmetrical (Fig. 11b) and an Egger's test indicated the presence of publication bias (Egger's regression intercept 3.20, t = 3.82, $p = 0.005$). None of the included studies had a high risk of bias. Results from 11 studies were pooled in this meta-analysis.

3.3.10. Meta-analysis for p53

Results from 8 studies measuring p53 were pooled. Compared to the controls, the expression of p53 was higher in the fluoride treated groups (SMD = 4.89, 95% CI: 2.21; 7.57, $Z = 3.57$, $p = 0.0004$). There was high heterogeneity among the studies included ($I^2 = 95\%$, $p < 0.0001$) (Fig. 12a) and the funnel plot showed asymmetry (Fig. 12b). We did not perform a test for publication bias because of the small number of studies evaluating the expression levels of p53 [66]. The results remained unchanged after exclusion of one study with high risk of bias [40] (SMD = 5.63, 95% CI: 2.14; 9.12, $Z = 3.16$, $p = 0.0016$; $I^2 = 96\%$, $p < 0.0001$).



a *Forest plot for caspase-9 meta-analysis*



b *Caspase-9 contour-enhanced funnel plot*

Fig. 10a. Forest plot for caspase-9 meta-analysis. Caspase-9 contour-enhanced funnel plot.

3.4. Subgroup analysis

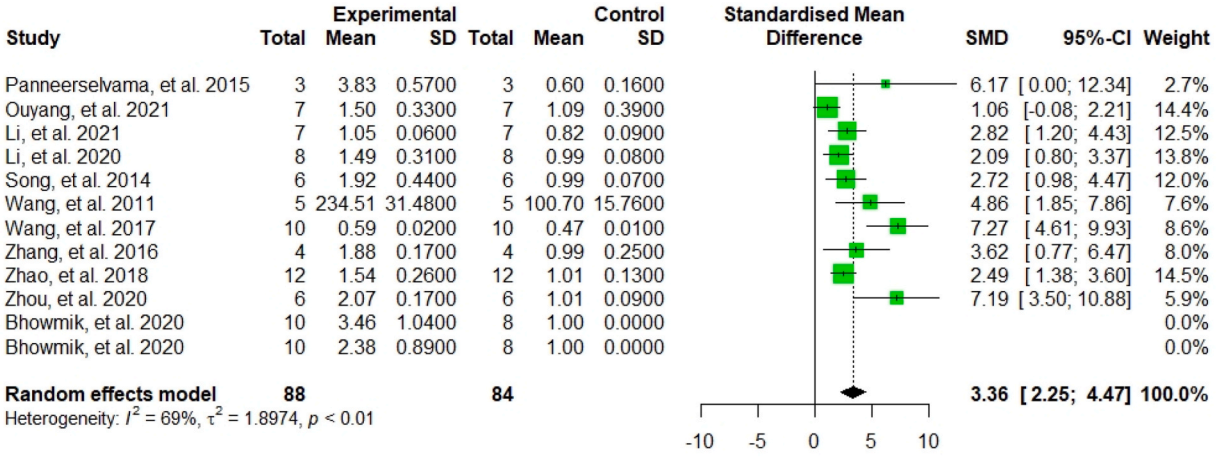
We conducted a subgroup analysis assessing the intervention period (<30, 30–90, >90 days); species of animals (mice, rats, others), and sample source (liver, kidney, brain, other tissue). The test for subgroup differences suggested that there is a subgroup effect; intervention period for Bax ($p = 0.01$) (Fig. 13), animal species for total apoptotic cells ($p = 0.002$), Bax ($p = 0.02$), Cyt *c* ($p = 0.05$), and Bcl-2 ($p = 0.02$) (Fig. 14). No difference was detected for other indicators (Figs. 13–15). The subgroup analysis for APAF-1, Bax/Bcl-2 ratio, caspase-8, -9, and p53 was not done because according to Schwarzer et al. [67] subgroup analyses are more appropriate when the meta-analysis contains at least 10 studies.

3.5. Meta-regression analysis

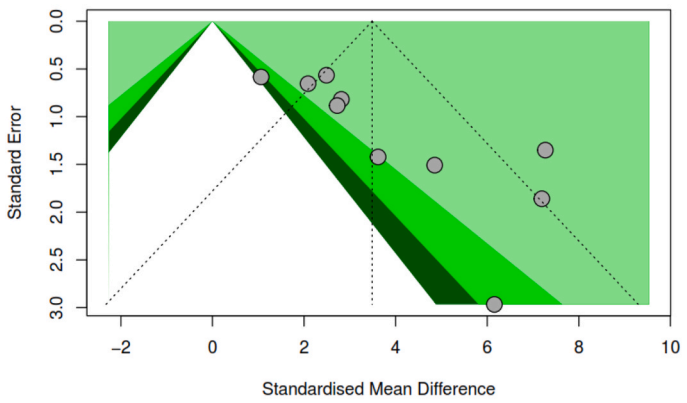
A meta-regression analysis was performed with each indicator of apoptosis as the outcome and with the intervention period (<30, 30–90, >90 days); animal species (rats, mice, others); and source of samples (kidney, liver, brain, other tissues) as factors. Based on the meta-regression analysis (Table 3), the animal species influenced the studies' effect size for total apoptotic cells ($p = 0.01$) and the sample source influenced the studies' effect size for Bax ($p = 0.045$). There was no evidence of association of all other predictors with the effect size. The source of heterogeneity was probably from animal species (apoptotic cells ($R^2 = 18.67\%$), Bax ($R^2 = 19.89\%$), Bcl-2 ($R^2 = 20.21\%$), caspase-3($R^2 = 7.60\%$), Cyt *c* ($R^2 = 27.66\%$)), and intervention period (Bax ($R^2 = 24.39\%$), Cyt *c* ($R^2 = 9.73\%$)).

4. Discussion

In this study, we presented the results of the first systematic review and meta-analysis aimed at elucidating whether fluoride causes apoptosis in non-skeletal tissues of experimental animals. The systematic review is based on 47 studies measuring 10 biomarkers of



a *Forest plot for Cyt c meta-analysis*

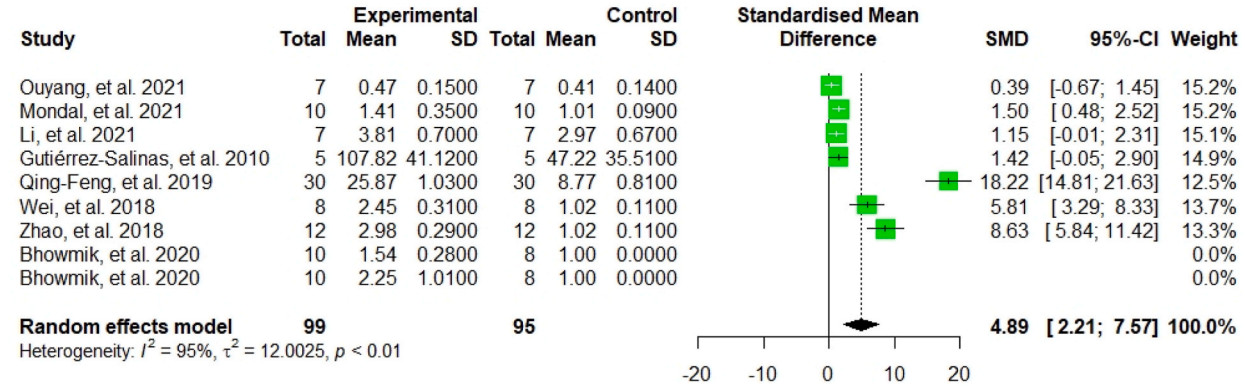


b *Cyt c contour-enhanced funnel plot*

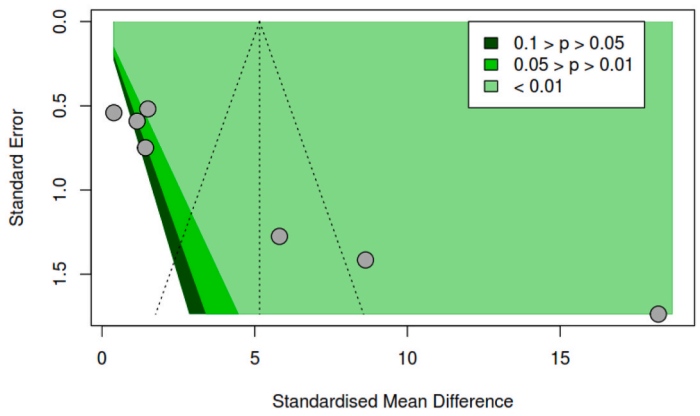
Fig. 11a. Forest plot for Cyt *c* meta-analysis.Cyt *c* contour-enhanced funnel plot.

apoptosis identified systematically from four major scientific databases. Results from the meta-analysis reported here have shown that fluoride increased the total apoptotic cells, the level of Bax/Bcl-2 ratio, and the expression of Bax, caspase-3, -8, -9, Cyt *c*, and p53 and decrease the expression of Bcl-2. There was, however, no evidence of a difference in the expression of APAF-1 between the fluoride and control groups. All biomarkers showed high levels of heterogeneity except for Cyt *c* which had moderate heterogeneity. There was evidence of publication bias in studies measuring apoptotic cells, Bax, Bcl-2, caspase-3, and Cyt *c*. The sensitivity analysis for studies measuring total apoptotic cells and the expression of Bax, Bcl-2, Bax/Bcl-2 ratio, caspase-3, and p53 showed that differences in the biomarkers of apoptosis between animals treated with fluoride and the controls were not influenced by any single study, suggesting the robustness of the outcome of the meta-analysis. Our study also showed that the apoptotic effect of fluoride might be dependent on the intervention period. In addition, different animal species have different sensitivity and tolerance to fluoride. Our meta-analysis, therefore, provides a theoretical basis for the molecular mechanism of fluoride-induced toxicity in non-skeletal tissues of experimental animals.

The known molecular mechanisms underlying fluoride-induced apoptosis are different and varied. They include amongst others, disruption of outer mitochondria membrane, activation of caspases, alterations in the ratio of anti-apoptotic-apoptotic Bcl-2 proteins, upregulation of p53 expression, expression of apoptosis-related genes, and disturbances in protein synthesis [10,16]. Caspases are divided into initiator (−2, −8, −9, −10) and effector (−3, −6, −7) caspases and function by a cascade, depending on whether the lethal stimuli are generated at the cell membrane, activating the extrinsic pathway (death receptor-mediated pathway) or intracellularly, activating the intrinsic pathway (mitochondria-mediated pathway) [12,16]. Most of the morphological changes associated with apoptosis can be attributed to the cleavage of initiator caspases at specific target sites which activate a set of effector caspases [68]. The disruption of the outer mitochondrial membrane by apoptotic stimuli initiates the mitochondrial pathway with the subsequent release of Cyt *c* from mitochondria to the cytosol. The release of Cyt *c* triggers apoptosome assembly from APAF-1, dATP (deoxyadenosine triphosphate), and procaspase-9, which in turn activates caspase-3 and -7, leading to oligonucleosomal DNA fragmentation [16,69]. The extrinsic pathway originates from the plasma membrane through the association of transmembrane death receptors TNF-α or Fas



a *Forest plot for p53 meta-analysis*



b *p53 contour-enhanced funnel plot*

Fig. 12a. Forest plot for p53 meta-analysis.p53 contour-enhanced funnel plot.

and their ligands inducing oligomerization of the receptor. This leads to receptor aggregation that binds adaptor molecules such as Fas-associated death domain (FADD) which then bind to initiator caspases prodomain e. g caspase-8 or -10 to promote their activation with subsequent stimulation of caspase-3. Caspase-3 cleaves one of its substrates, poly(ADP-ribose) polymerase (PARP), resulting in the degradation of nuclear DNA. Thus, intrinsic and extrinsic pathways converge at the activation of caspase-3 [16,68].

The ability of fluoride to trigger an intrinsic pathway has been demonstrated both *in vitro* and *in vivo* in many cell types. Song et al. [48] demonstrated increased caspase-3 and -9 protein and mRNA relative expression in the liver of rats chronically exposed to fluoride. In other studies, an increase in cytosolic Cyt *c* [70] and activation of caspase-3 was reported in HL-60 (human leukemia cell line) cells [71]. The role of the death receptor-dependent pathway in fluoride-induced apoptosis has also been documented. Fluoride exposure resulted in up-regulation of the Fas-ligand (Fas-L), a ligand of death receptor in human gingival fibroblasts. This was associated with an increased level of Cyt *c* and enhanced activities of the caspase-3, -8, and -9 [72]. The participation of the Fas-L was also demonstrated in SH-SY5Y (human neuroblastoma) cells [73]. Our results are consistent with these findings. We found that Cyt *c*, APAF-1, caspase-3, -8, and -9 in non-skeletal tissues of the fluoride intervention groups had an increasing trend compared to the control groups which indicated the involvement of the caspase pathway.

Another key component of the apoptotic signaling is the regulation of pro-apoptotic (e.g. Bcl-2 and Bcl-xL (Bcl-extra long)) and anti-apoptotic (e.g. Bax, Bad (Bcl-2 antagonist of cell death), and Bid (Bcl-2 interacting domain death agonist)) genes [74]. Bax and Bcl-2 are major members of the Bcl-2 family [75]. The release of Cyt *c*, dATP, and other critical molecules from the mitochondrial intermembrane space to the cytosol is controlled by proteins of the Bcl-2 family by regulation of the mitochondrial permeability transition pore [16]. Evidence of the involvement of the Bcl-2 family of proteins in fluoride-induced apoptosis is well documented. The up-regulation of Bax and Bak (Bcl-2 antagonistic killer) protein expression and corresponding suppression of Bcl-2 and Bcl-xL protein expression have been shown in cultured splenic lymphocytes treated with fluoride [76]. A similar picture was observed in primary cultured rat chondrocytes, where apoptosis induced by fluoride was also associated with downregulation of the Bcl-2 protein and upregulation of Bax protein [77]. Our study showed an increase in the expression of Bax and a decrease in the expression of Bcl-2 in the fluoride intervention groups as compared to the controls. The Bax/Bcl-2 ratio was also found to be higher in the fluoride groups. The Bax/Bcl-2 ratio is considered to be a crucial determinant in the occurrence of apoptosis. A higher expression of the Bax/Bcl-2 ratio

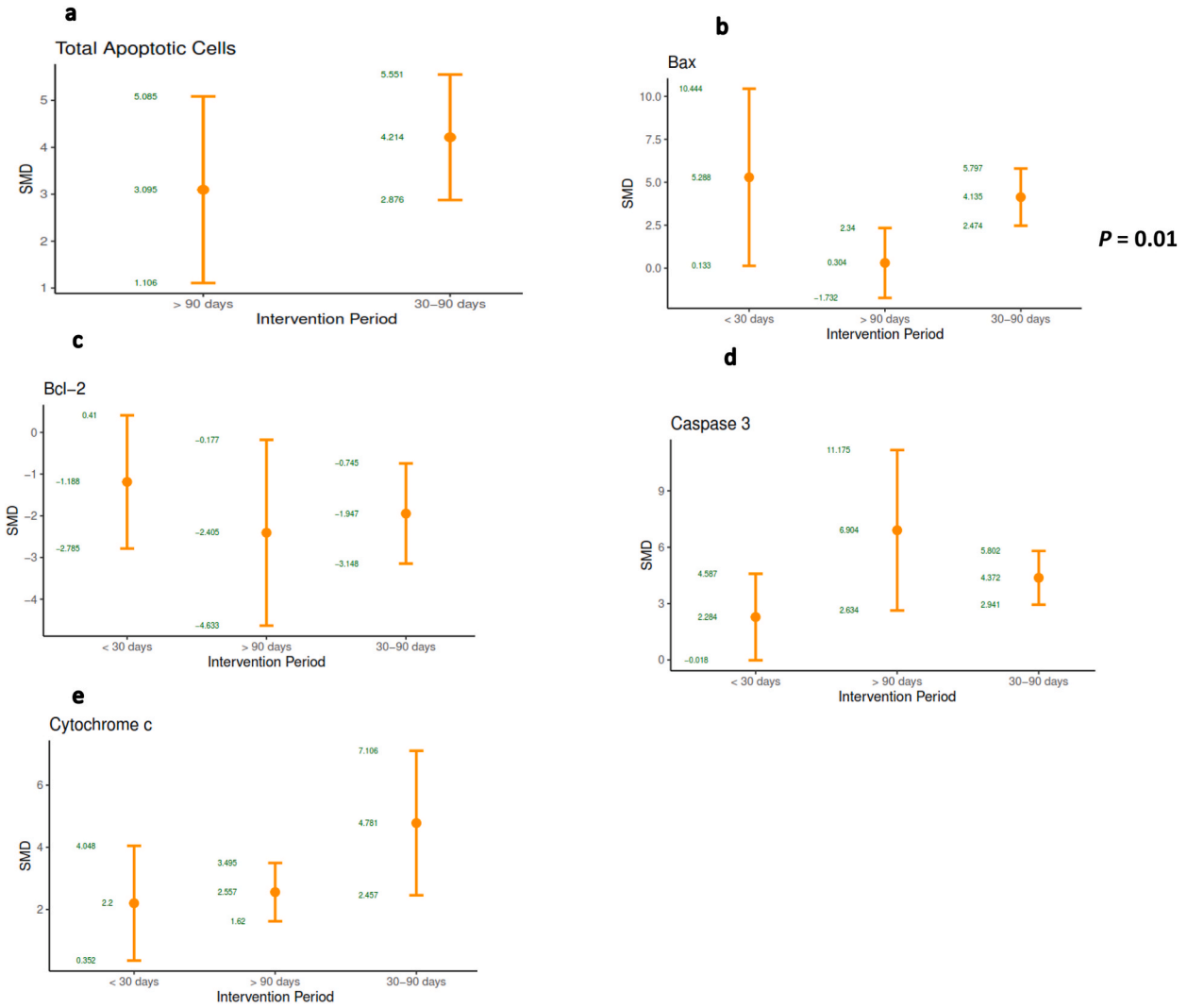


Fig. 13. Subgroup analysis to determine the effect of intervention period on apoptosis induced by fluoride. A significant subgroup effect was observed in (b) intervention period for Bax ($p = 0.01$). The subgroup effect for (a) total apoptotic cells, (c) Bcl-2, (d) caspase-3, and (e) Cyt *c* was, however, not significant.

increases cell susceptibility to apoptosis [75,78].

The expression level of p53 was found to be higher in fluoride intervention groups in our study. The p53 tumor suppressor protein has been described as an inductor of cell cycle arrest and apoptosis. p53 is also able to mediate the expression of apoptotic-related proteins like Fas, Bax, and Bcl-2 and regulate normal metabolic homeostasis, senescence, fertility, and differentiation [10,79–81]. The involvement of p53 in fluoride-induced apoptosis had previously been reported in lymphocytes of aluminum smelter workers [82], SH-SY5Y (human neuroblastoma) cells [83], and L-02 (human embryo hepatocyte) cells [84].

Our meta-analysis found a subgroup effect, suggesting that the apoptotic effect of fluoride could be dependent on the intervention period, and the susceptibility and tolerance of different animal species to fluoride. We observed variation in different animal species, with the absolute SMD for total apoptotic cells, Bax, and Cyt *c* being higher and that of Bcl-2 being lower in mice than in rats and other species. Few studies have explored the genetic basis underlying fluoride susceptibility. In their study on bone responses to fluoride, Kobayashi et al. [85] found that the A/J mouse strain was more sensitive to developing dental fluorosis and to alterations in the quality of bone, while the 129P3/J strain was less affected. The mineral apposition rate was higher in the 50 ppm 129P3/J mice than in the 50 ppm A/J mice showing that fluoride increased bone formation in a strain-specific manner. Fluoride exposure was associated with dose-specific and strain-specific alterations in the expression of proteins involved in osteogenesis and osteoclastogenesis [85]. Similar studies have also shown genetic differences in response to fluoride [86–94]. We postulated that these differences could be greater between different animal species. The number of studies contributing data to different subgroups was, however, not equal thus the analysis may not be able to detect subgroup differences.

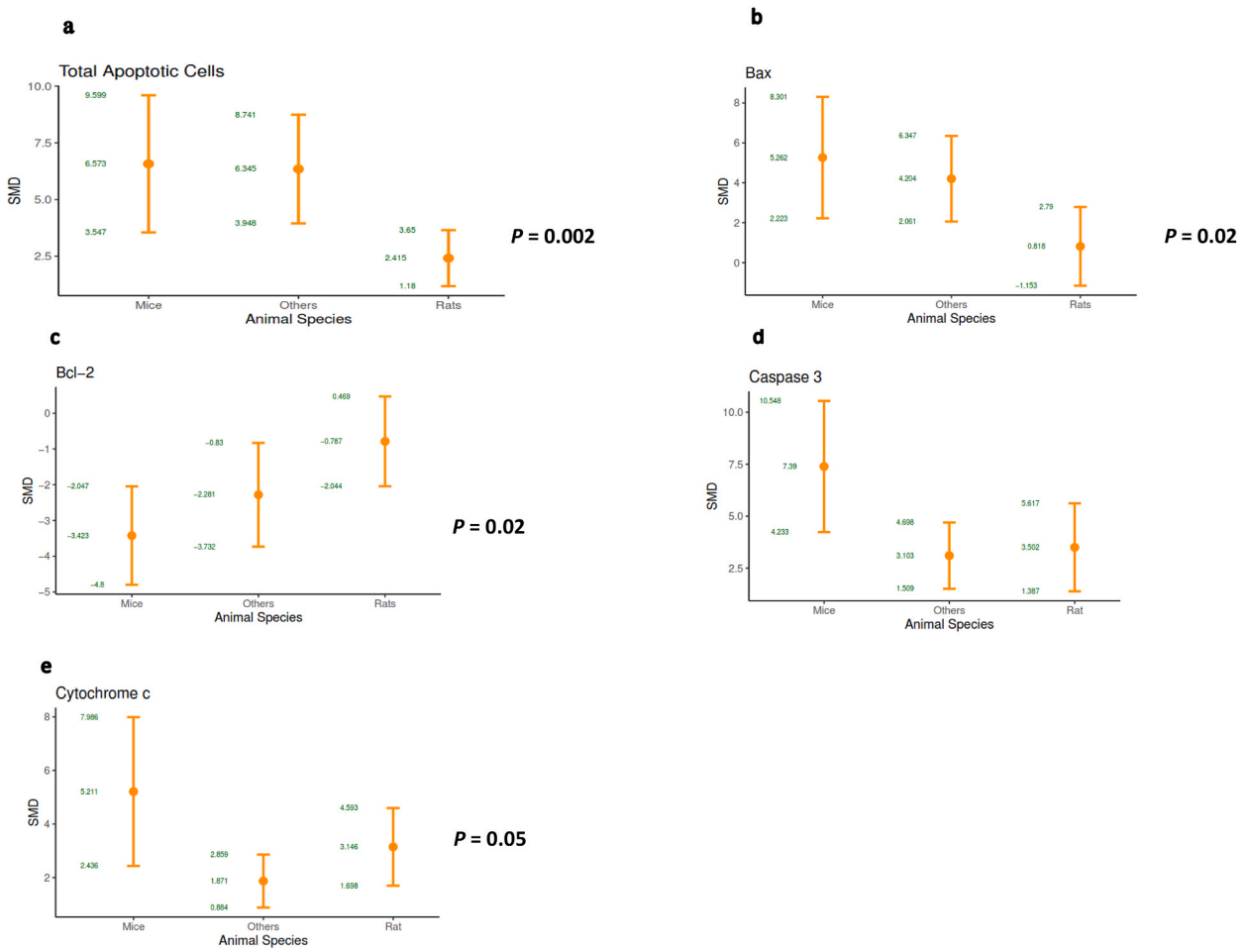


Fig. 14. Subgroup analysis to determine the effect of animal species on fluoride-induced apoptosis. A significant subgroup effect was observed in animal species for (a) total apoptotic cells (p = 0.002), (b) Bax (p = 0.02), (c) Bcl-2 (p = 0.02), and (e) Cyt *c* (p = 0.05). The subgroup effect for (d) caspase-3 was, however, not significant.

5. Strengths and limitations

To the best of our knowledge, this is the first systematic review and meta-analysis that comprehensively assessed, evaluated, and provided evidence of fluoride-induced apoptosis in non-skeletal tissues of experimental animals. We used extensive searches from four different databases to ensure we found all relevant studies. Standard guidelines were followed during the review process.

The limitations of this study include obvious heterogeneity of the studies, publication bias, and few published articles measuring apoptosis. The dissimilarity seen in the studies analyzed could be a result of differences in ages of experimental animals, animal species, kind of tissue examined, dose and mode of fluoride exposure, time of exposure, and methods for biochemical assay. Due to the small number of included literature, subgroup analyses for APAF-1, Bax/Bcl-2 ratio, caspase-8, -9, and p53 were not carried out in this study. Non-English studies were not included in the search which might have contributed to insufficient literature.

6. Conclusion

The results of this systematic review and meta-analysis support the fact that apoptosis is one of the mechanisms by which fluoride causes toxicity in the non-skeletal tissues of experimental animals. We also found that the extent of fluoride-induced apoptotic damage might be associated with the intervention period. Moreover, different animal species have different susceptibilities and tolerance to fluoride. Because of the limitations of publication bias, study heterogeneity, and fewer studies in some analyses, these results should be interpreted with caution. Additional studies in the non-skeletal tissues of humans are needed to validate these results.

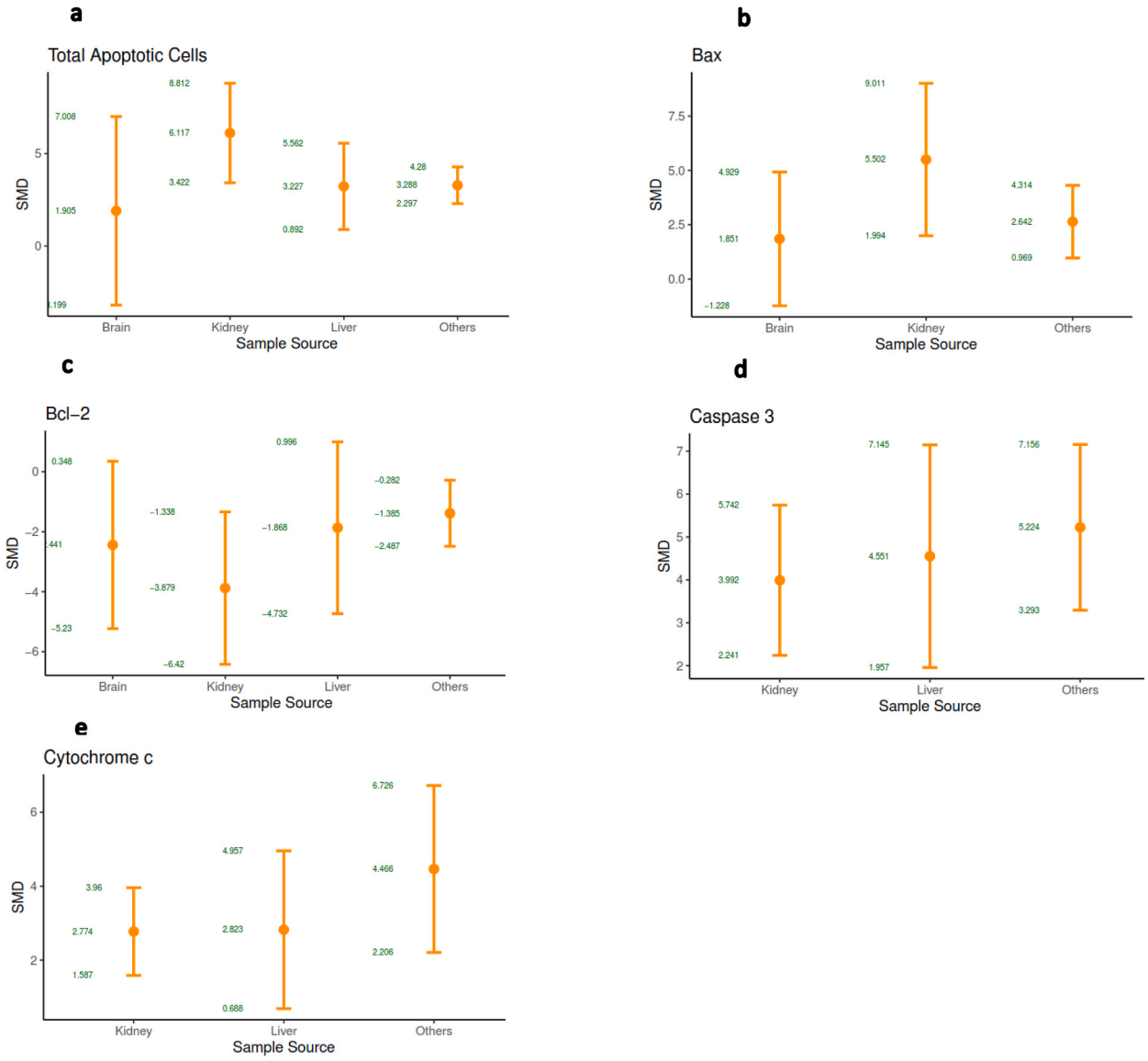


Fig. 15. Subgroup analysis to determine the effect of source of sample on fluoride induced-apoptosis. The subgroup effect of sample source for (a) total apoptotic cells, (b)Bax, (c) Bcl-2, (d) caspase-3, and (e) Cyt *c* was not significant.

Author contribution statement

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Data availability statement

Data will be made available on request.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to

Table 3
Results of the meta-regression analysis.

	Coefficient	SE	Z value	*P* value	95% CI
Apoptotic cells					
-Intervention period (Reference >90 days)					
✓30–90 days	1.302	1.820	0.716	0.474	−2.264–4.869
-Animal species (Reference mice)					
✓Rats	−4.282	1.858	−2.305	0.021	−7.924–−0.641
✓Others	0.518	2.472	0.209	0.834	−4.328–5.363
-Sample source (Reference brain)					
✓Kidney	5.084	2.886	1.761	0.078	−0.573–10.741
✓Liver	1.338	2.707	0.494	0.621	−3.968–6.644
✓Others	2.142	2.603	0.823	0.411	−2.960–7.243
Bax					
Intervention period (Reference <30 days)					
✓30–90 days	−0.800	2.578	−0.310	0.756	−5.853–4.253
✓> 90 days	−4.971	2.778	−1.789	0.074	−10.416–0.475
-Animal species(Reference mice)					
✓Rats	−4.598	2.179	−2.110	0.035	−8.868–−0.328
✓Others	−0.984	2.226	−0.442	0.658	−5.346–3.379
-Sample source (Reference brain)					
✓Kidney	3.785	2.993	1.264	0.206	−2.082–9.651
✓Others	1.588	2.499	0.635	0.525	−3.311–6.486
Bcl-2					
-Intervention period (Reference <30 days)					
✓30–90 days	−0.865	1.941	−0.446	0.656	−4.668–2.939
✓> 90 days	−1.144	2.050	−0.558	0.577	−5.163–2.875
-Animal species(Reference mice)					
✓Rats	2.812	1.244	2.261	0.024	0.374–5.250
✓Others	1.309	1.380	0.948	0.343	−1.396–4.013
-Sample source (Reference brain)					
✓Kidney	−1.666	1.988	−0.838	0.402	−5.563–2.231
✓Liver	0.498	2.160	0.231	0.818	−3.736–4.732
✓Others	0.940	1.528	0.615	0.538	−2.054–3.934
Caspase-3					
-Intervention period (Reference <30 days)					
✓30–90 days	1.968	2.665	0.739	0.460	−3.255–7.192
✓> 90 days	4.344	3.436	1.264	0.206	−2.391–11.078
-Animal species(Reference mice)					
✓Rats	3.444	2.213	−1.556	0.120	−7.782–0.895
✓Others	−4.054	2.230	−1.818	0.069	−8.424–0.316
-Sample source (Reference Kidney)					
✓Liver	0.799	2.692	0.297	0.767	−4.477–6.075
✓Others	1.541	2.334	0.660	0.509	−3.034–6.116
Cytochrome *c*					
-Intervention period (Reference <30 days)					
✓30–90 days	2.272	1.481	1.534	0.125	−0.630–5.174
✓> 90 days	0.248	1.687	0.147	0.883	−3.058–3.555
-Animal species(Reference mice)					
✓Rats	−1.417	1.537	−0.922	0.357	−4.430–1.596
✓Others	−2.931	1.315	−2.229	0.026	−5.508–−0.354
-Sample source (Reference kidney)					
✓Liver	0.147	1.866	0.079	0.937	−3.511–3.805
✓Others	1.641	1.776	0.924	0.355	−1.840–5.122

influence the work reported in this paper.

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