Fluoride-Induced Oxidative Stress in Non-Skeletal Tissues: A Systematic Review and Meta-Analysis

Linet Musungu Angwa (lynangwa@gmail.com)
Kabarik University
https://orcid.org/0000-0003-1514-7729

David Masinde
Maseno University

Systematic Review

Keywords: Fluorosis, Oxidative stress, Non-skeletal tissues, Systematic review, Meta-analysis

Posted Date: March 14th, 2023

DOI: https://doi.org/10.21203/rs.3.rs-2681038/v1

License: This work is licensed under a Creative Commons Attribution 4.0 International License. Read Full License
Abstract

Several studies have investigated the oxidative stress parameters in non-skeletal tissues of animals exposed to fluoride, however, the findings from these studies are inconsistent. We conducted a systematic review and meta-analysis to evaluate the levels of oxidative stress biomarkers in experimental animals treated with fluoride compared with the control group. The PubMed, Cochrane Library, EBSCO, and JSTOR databases were searched for studies reporting oxidative stress biomarkers in non-skeletal tissues of animals exposed to fluoride. A random effects model with the 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 was assessed using the SYRCLE’s risk of bias tool and publication bias using Egger’s test. Compared to the control, the levels of ROS, LPO, and NO were significantly elevated and the levels of SOD, CAT, GSH-Px, and GSH significantly reduced in the studied tissues. The level of GST however showed no significant difference. The test for subgroup differences suggested that different animal species and tissues have varying susceptibilities and tolerance to fluoride. Furthermore, the extent of fluoride-induced oxidative stress damage can be modified by the intervention period. Meta-regression analysis indicated that the studies’ effect size for LPO was influenced by animal species. This meta-analysis’s findings demonstrated the presence of oxidative stress and depletion of antioxidants in the non-skeletal tissues of experimental animals exposed to fluoride.

1. Introduction

It is well established that fluorosis is a worldwide health concern and is endemic in some areas where fluoride content is high in drinking water.\textsuperscript{1,2} The global prevalence of dental and skeletal fluorosis is not clear. However, it is estimated that excessive fluoride concentration in drinking water has caused tens of millions of dental and skeletal fluorosis cases worldwide over a range of years.\textsuperscript{3} After absorption from the gastrointestinal tract, 99% of the retained fluoride is deposited in mineralized tissues with 1% being found in soft tissues.\textsuperscript{4,5} A steady state of distribution between the extracellular and intracellular compartments is established in these tissues where intracellular fluoride concentrations change simultaneously and in proportion to changes in plasma fluoride levels.\textsuperscript{4} The remaining proportion is excreted by the kidneys. Generally, the tissue-to-plasma concentration ratios fall between 1.0 and 4.0 except for the brain (< 0.1) and the kidney (> 4.0). The low value in the brain is attributed to the relative impermeability of the blood-brain barrier to fluoride. The kidney has the highest fluoride concentration compared to all other soft tissues due to its high concentration in the tubular fluid.\textsuperscript{4,6} Excessive fluoride ingestion over a prolonged period can adversely influence several organs and tissues characterized by a vast array of symptoms and pathological changes. Apart from dental and skeletal fluorosis, Fluoride is now known to cause renal toxicity,\textsuperscript{7} hepatotoxicity,\textsuperscript{8} neurotoxicity,\textsuperscript{9} cardiovascular system toxicity,\textsuperscript{10,11} reproductive toxicity,\textsuperscript{12,13} thyroid toxicity,\textsuperscript{14} and Haematotoxicity.\textsuperscript{15} Despite this, the molecular mechanisms of fluorosis are still unclear.
In recent decades, extensive research has reported a key role of oxidative stress in causing fluorosis in non-skeletal tissues.\textsuperscript{16} This is further strengthened by evidence of consistent protection of cells from the lipid peroxidation (LPO) caused by fluoride exposure by antioxidant treatment.\textsuperscript{17} Excessive accumulation of reactive oxygen species (ROS) or reactive nitrogen species (RNS), as a result of either increased ROS/RNS generation or impaired ROS/RNS clearance, leads to oxidative stress. To counteract the effects of ROS/RNS, living organisms possess a large number of antioxidants such as superoxide dismutase (SOD), glutathione peroxidase (GSH-Px/GPx), glutathione S-transferase (GST) and catalase (CAT), and non-enzymatic antioxidants such as vitamins A, E, and C, GSH, and β-Carotene.\textsuperscript{18–20} Fluoride is thought to inhibit these antioxidants and increase oxidative stress.\textsuperscript{21–23} However, previous studies addressing the oxidative stress status or detecting the biomarkers of oxidative stress in tissues of experimental animals with fluoride yielded controversial results.\textsuperscript{24–27}

In this study, we systematically reviewed the literature on oxidative stress induced by fluoride in the blood, liver, kidney, heart, and brain of experimental animals and conducted a meta-analysis on relevant studies. The primary objective was to quantitatively evaluate the levels of oxidative stress biomarkers (SOD, CAT, GSH-Px, GSH, GST, ROS, lipid peroxidation (LPO), and nitric oxide (NO)) in the aforementioned non-skeletal tissues of experimental animals exposed to fluoride compared to the controls. We hypothesized that there will be an increase in oxidative biomarkers and a decrease in antioxidative biomarkers in experimental animals with induced fluoride toxicity.

2. Materials And Methods

2.1 Inclusion criteria

Studies were included in our analyses if they met the following criteria: 1) experimental studies measuring oxidative stress biomarkers in blood, brain, kidney, heart, and liver of experimental animals; 2) studies published in English; 3) studies that provided animal numbers, means, and standard deviations. Through our search strategy, we decided to focus on some enzymatic antioxidants (SOD, CAT, GSH-Px, and GST), non-enzymatic antioxidants (GSH), reactive oxygen species (ROS), free radicals (NO), and oxidative damage products (malondialdehyde (MDA)/ thiobarbituric acid reactive substances (TBARs): LPO), but not other oxidative stress biomarkers because of limited studies.

2.2 Exclusion criteria

Studies were excluded from our analyses if they met the following criteria: 1) conference abstracts, reviews, editorials, and letters; 2) human, \textit{in vitro}, or other unrelated studies; 3) full-text not available in English; 4) studies with unavailable data/ unextractable data; 5) combined exposure with no fluoride only group; 6) studies not done in tissues of interest; 7) multigenerational studies; 8) studies on amelioration of fluoride toxicity; 9) studies that did not measure oxidative stress; 10) studies with incomplete or unclear results.
2.3 Literature search

We conducted searches in PubMed, Cochrane Library, EBSCO, and JSTOR to identify eligible articles on July 22nd, 2022, with the following keywords and Boolean operators: (“Fluoride OR Fluorosis”) AND (“Oxidative stress OR Oxidative damage”) AND (“Reactive oxygen species OR Reactive nitrogen species OR Lipid peroxidation OR Malondialdehyde OR Thiobarbituric acid reactive substance OR Nitric oxide OR Lipid hydroperoxide OR Superoxide dismutase OR Catalase OR Glutathione OR Vitamin C”) and articles were exported to Mendeley. An additional manual search of references and cited/related articles was done. Search terms were validated by ensuring the search retrieved a selection of articles, representative of relevant works. Searches were restricted to the English language and Animals with no restriction on the date of publication. Abstracts were screened independently by two reviewers (LA and DM). Full-text screens were conducted to confirm eligibility. Differences between the two reviewers were resolved through discussion and consensus.

2.4 Data extraction and quality assessment

Two reviewers (LA and DM) extracted eligible studies independently through the review of titles, abstracts, and full texts. In case of disagreement, a final decision was made by consensus. Information was extracted in a systematic way as follows: 1) author; 2) year of publication; 3) animal species/strain; 4) sex; 5) age; 6) weight; 7) tissue/organ studied; 8) the number of animals in experimental and control groups; 9) study period; and 10) oxidative stress biomarkers (Table 1). In studies with multiple intervention groups, only one pair was selected and others were excluded from the meta-analysis: only the high-dose group was included in studies with multiple fluoride groups, the fluoride-only group was included in co-exposure studies, and in studies with different duration of treatment, the longest duration was selected for the study. In some studies, multiple datasets were extracted if reported and the mean of means and standard deviations (SD) were used for the meta-analysis. WebPlotDigitizer was used to facilitate graphical data extraction. The SD for studies that reported standard error (SE) of mean was obtained by multiplying by the square root of the sample size (SD = SE×).

The quality of included studies was assessed independently by two reviewers (LA and DM) by using the SYRCLE’s risk of bias tool and disagreements were resolved through consensus-oriented discussion. The SYRCLE’s risk of bias tool contains 10 entries related to 6 types of bias: selection bias, performance bias, detection bias, attrition bias, reporting bias, and other biases. Signaling questions are used to assign a judgment of low, high or unclear risk of bias to each item mentioned in the tool.

2.5 Statistical Analysis

All statistical analysis was performed using the R project software version 4.2.2 (R package meta). We used Hedge's g standardized mean difference (SMD) as a measure of effect size because the measures used are not the same in all studies and corresponding 95% confidence intervals (95% CIs) were presented. Pooled effect sizes (ES) were calculated according to DerSimonian and Laird for the random effects model because of the diversity of methods, species, and intervention protocols. The statistical
heterogeneity was determined by the value of the $I^2$ index. A value of the $I^2$ index of around 25%, 50%, and 75% was considered as low-, moderate-, and high-heterogeneity, respectively. For rigor, Publication bias was estimated by inspecting the funnel plot asymmetry and Egger's regression test. 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. For additional insight, 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, heart, blood) to determine the factors associated with differences among study results in the outcome indicators. Statistical significance was defined at $p < 0.05$.

3. Results

3.1. Study Identification and Selection

We systematically identified a total of 619 articles using electronic databases (PubMed = 451, Cochrane Library = 2, EBSCO = 91, and JSTOR = 75) of which 614 were retrieved after deduplication. An additional 8 articles were identified through a manual search. Title and abstract screening excluded 531 articles. A full-text evaluation was conducted for the remaining 91 articles, and 29 articles were excluded for not fulfilling the inclusion criteria. Thus, 62 eligible studies were selected (Fig. 1). The studies presented a high prevalence of rodents as laboratory animals, (52 out of 62). 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 is categorized as high, low, or unclear. 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 general result of the risk of bias assessment of this systematic review is presented in Fig. 2. Concerning selection bias, 74.2% 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 90.3% of the studies, the groups were similar at baseline with 9.7% presenting insufficient information. Additionally, 95.1%, and 100%, of the included studies registered unclear risk of bias regarding performance bias items "random housing" and "blinding" with 85.5% and 100% categorized as having unclear risk of bias regarding detection bias “random outcome assessment” and “blinding” respectively. Regarding the attrition bias, 75.8% had all the animals included in the study while 24.2% had insufficient information. All studies were free of reporting bias and 25.8% registered a low risk of bias from other sources.

3.3. Meta-analysis of oxidative stress biomarkers

3.3.1. Meta-analysis of Superoxide Dismutase (SOD)
The overall effect size of SOD in the non-skeletal tissues was −4.2117 (95% CI: -5.4626; -2.9607, Z= -6.60, p< 0.0001) showing that the level of SOD was significantly lower in the treatment group compared to the controls. The statistical heterogeneity was notable (I² = 92%, p < 0.0001). More specifically, the effect sizes for SOD in blood, liver, kidney, heart, and brain were −1.4491 (95% CI: -3.2640; 0.3657, Z= -1.56, P= 0.1176; I² = 89%, P < 0.0001), -4.9682 (95% CI: -7.3196; -2.6169, Z= -4.14, P < 0.0001; I² = 94%, P < 0.0001), -3.9651 (95% CI: -7.2393; -0.6909, Z= -2.37, P= 0.0176; I² = 90%, P < 0.0001), -5.0754 (95% CI: -8.3959; -1.7549, Z= -3, P= 0.0027; I² = 90%, P < 0.0001), and −5.9072 (95% CI: -8.8593; -2.9551, Z= -3.92, P < 0.0001; I² = 88%, P < 0.0001) respectively (Fig. 3a-e). A visual inspection of the funnel plot showed asymmetry (Fig. 3f). Egger's regression analysis displayed evidence of publication bias for this marker (Intercept = −3.2373, t = -4.67, p-value < 0.0001). The exclusion of studies with a high risk of bias in any of the entries 23, 25, 34, 39, 40, 48 – 50, 55, 57, 63, 79, 82 yielded similar results and publication bias (SMD= -2.7346, 95%CI: -3.8546; -1.6146, Z = -4.79, P < 0.0001; I² = 91%, p < 0.0001; Egger's regression intercept = −3.5527, t = -3.50, p-value = 0.0014).

3.3.2. Meta-analysis of catalase (CAT)

The levels of CAT in non-skeletal tissues of experimental animals were remarkably lower than in the controls (SMD = -3.5470, 95% CI: -4.7155; -2.3786, Z= -5.95, p < 0.0001). The statistical heterogeneity was high (I² = 92%, P < 0.0001). The effect sizes of CAT in blood (SMD = -2.8730, 95% CI: -5.5312; -0.2148, Z= -2.12, p = 0.034; I² = 86%, P < 0.0001), liver (SMD = -5.5689, 95% CI: -8.9565; -2.1813, Z= -3.22, P < 0.0001; I² = 97%, p < 0.0001), heart (SMD = -4.0529, 95% CI: -5.3520; -2.7539, Z= -6.11, p < 0.0001; I² = 18%, p = 0.2972) and brain (SMD = -3.1932, 95% CI: -4.7950; -1.5915, Z= -3.91, P < 0.0001; I² = 85%, P < 0.0001) were also statistically significant (Fig. 4a-d). The pooled effect size for kidney CAT (SMD = -2.1930, 95% CI: -4.4329; 0.0469, Z= -1.92, P = 0.0550; I² = 18%, p = 0.2972) and brain (SMD = -3.1932, 95% CI: -4.7950; -1.5915, Z= -3.91, p < 0.0001; I² = 85%, P < 0.0001) were also statistically significant (Fig. 4a-d). The pooled effect size for kidney CAT (SMD = -2.1930, 95% CI: -4.4329; 0.0469, Z= -1.92, P = 0.0550; I² = 18%, p = 0.2972) and brain (SMD = -3.1932, 95% CI: -4.7950; -1.5915, Z= -3.91, p < 0.0001; I² = 85%, P < 0.0001) were also statistically significant (Fig. 4a-d). The pooled effect size for kidney CAT (SMD = -2.1930, 95% CI: -4.4329; 0.0469, Z= -1.92, P = 0.0550; I² = 18%, p = 0.2972) and brain (SMD = -3.1932, 95% CI: -4.7950; -1.5915, Z= -3.91, p < 0.0001; I² = 85%, P < 0.0001) were also statistically significant (Fig. 4a-d). The funnel plot was asymmetrical (Fig. 4f) and an Egger's test was performed to detect publication bias and the results indicated the presence of publication bias (Egger's regression intercept = -5.3276, t = -5.77, p-value < 0.0001). A sensitivity analysis with a random effect model was performed to calculate the pooled estimate of the effect after the exclusion of the studies with a high risk of bias 23, 36, 37, 48 – 50, 57, 63, 79, 82, 84, 85, which showed no substantial variation of the results (SMD = -2.8297, 95% CI: -4.0840; -1.5754, Z= -4.42, P < 0.0001; I² = 92%, P < 0.0001; Egger's regression intercept = -5.8654, t = -4.66, p-value = 0.0001).

3.3.3. Meta-analysis of glutathione peroxidase (GSH-Px)

The overall effect size for GSH-Px in non-skeletal tissues was −2.5319 (95% CI: -3.9412; -1.1226, Z = -3.52, p = 0.0004) indicating that the levels of GSH-Px were significantly lower in the treatment groups compared to the controls. High heterogeneity of the studies was observed (I² = 86%, p < 0.0001). Statistically significant results were also seen in the meta-analysis of the articles reporting GSH-Px levels in the blood (SMD = -3.3938, 95% CI: -6.5339; -0.2538, Z = -2.12, P = 0.0341; I² = 88%, p < 0.0001), liver (SMD = -2.6349, 95% CI: -3.9062; -1.3636, Z = 4.06, p < 0.0001; I² = 76%, p < 0.0001), kidney (SMD = -1.7080, 95% CI: -2.6243; -0.7917, Z = -3.65, p = 0.0003; I² = 75%, p = 0.0003), and heart (SMD = -5.6537,
95% CI: -10.7131; -0.5942, \( Z = -2.19, p = 0.0285; I^2 = 89\%, p = 0.0001 \). No significant change in GSH-Px was seen in the brain (SMD = -0.0574, 95% CI: -5.3120; 5.1972, \( Z = -0.02, p = 0.9829; I^2 = 91\%, p = 0.0001 \)) (Fig. 5a-e). The Egger's test for asymmetry of the funnel plot showed evidence of publication bias (Egger's regression intercept = -1.2193, \( t = -1.42, p = 0.1634 \)). The funnel plot is presented in Fig. 5f. The effect size was \(-2.9586 \) (95% CI: -4.6038; -1.3135, \( Z = -3.52, p = 0.0004; I^2 = 85\%, p < 0.0001 \)), and Egger's regression (intercept = -3.6063, \( t = -3.56, p = 0.0022 \)) after excluding articles with a high risk of bias.34,36,40–51,55,76,79,82,84

### 3.3.4. Meta-analysis of Glutathione (GSH)

The level of GSH was significantly lower in non-skeletal tissues of animals treated with fluoride compared to the controls (SMD = -3.2790, 95% CI: -3.9433; -2.6147, \( Z = -9.67, p < 0.0001 \)). The heterogeneity was found to be high (\( I^2 = 76\%, p < 0.0001 \)). The level of GSH in blood (SMD = -4.6776, 95% CI: -6.3518; -3.0034, \( Z = -5.48, p < 0.0001; I^2 = 73\%, p = 0.0003 \)), liver (SMD = -2.61, 95% CI: -3.7148; -1.5052, \( Z = -4.63, p < 0.0001; I^2 = 80\%, p = 0.0001 \)), kidney (SMD = -3.4747, 95% CI: -4.7208; -2.2285, \( Z = -5.46, p < 0.0001; I^2 = 80\%, p < 0.0001 \)), heart (SMD = -3.9344, 95% CI: -7.6808; -0.1881, \( Z = -2.06, p = 0.0396; I^2 = 80\%, p = 0.0253 \)), and brain (SMD = -1.6222, 95% CI: -2.7999; -0.4445, \( Z = -2.70, p = 0.0069; I^2 = 71\%, p = 0.0156 \)) of animals treated with fluoride compared to the controls was found to be significantly lower (Fig. 6a-e). The Egger's test for asymmetry of the funnel plot showed evidence of publication bias (Egger's regression intercept = -3.0807, \( t = -4.52, p < 0.0001 \)). The funnel plot is presented in Fig. 6f. A sensitivity analysis was performed by the exclusion of studies with a high risk of bias35–37,39,50,51,62,72,78,82,84 which showed similar results (SMD = -3.6340, 95% CI: -4.6631; -2.6050, \( Z = -6.92, p < 0.0001; I^2 = 82\%, p < 0.0001 \); Egger's regression intercept = -2.9614, \( t = -2.89, p = 0.0101 \)).

### 3.3.5. Meta-analysis of glutathione S-transferase (GST)

The difference in the levels of GST between the treatment groups and the controls was non-significant (SMD = -1.5579, 95% CI: -4.1415; 1.0257, \( Z = -1.18, p = 0.2373 \)). Significant heterogeneity was found (\( I^2 = 94\%, p < 0.0001 \)). Similarly, results from the analysis of the level of GST in individual tissues was found to be non-significant: liver (SMD = -2.8939, 95% CI: -7.0783; 1.2906, \( Z = -1.36, p = 0.1753; I^2 = 95\%, p < 0.0001 \)), kidney (SMD = -1.7370, 95% CI: -4.3792; 0.9052, \( Z = -1.29, p = 0.1976; I^2 = 88\%, p = 0.0047 \)) and brain (SMD = 0.6964, 95% CI: -3.8534; 5.2463, \( Z = 0.30, p = 0.7642; I^2 = 89\%, p < 0.0001 \)) (Fig. 7a-c). No studies measured GST in blood and heart. A visual inspection of the funnel plot showed asymmetry (Fig. 7d) with evidence of publication bias on Egger's regression test (Egger's regression intercept = -5.2524, \( t = -2.25, p = 0.0439 \)). The significance of the results did not change after a sensitivity analysis was done with the exclusion of the studies with a high risk of bias37,51,57,72 (SMD = -1.2318, 95% CI: -4.4643; 2.0008, \( Z = -0.75, p = 0.4552; I^2 = 95.2\%, p < 0.0001 \)). However, Egger's regression test showed no evidence of publication bias (Egger's regression intercept = -5.4283, \( t = -1.52, p = 0.1665 \)).
3.3.6. Meta-analysis of Lipid peroxidation (LPO)

The LPO levels in non-skeletal tissues of experimental animals were significantly higher compared to the controls (SMD = 3.4165, 95% CI: 2.8569; 3.9761, Z = 11.97, p < 0.0001). High statistical heterogeneity was found (I² = 85%, p < 0.0001). Meta-analysis of levels of LPO in individual tissues was also significant, blood (SMD = 3.0534, 95% CI: 1.6614; 4.4454, Z = 4.30, p < 0.0001; I² = 84%, p < 0.0001), liver (SMD = 3.4668, 95% CI: 2.4776; 4.4560, Z = 6.87, p < 0.0001; I² = 88%, p < 0.0001), kidney (SMD = 2.9527, 95% CI: 2.0353; 3.8700, Z = 6.31, p < 0.0001; I² = 81%, p < 0.0001), heart (SMD = 4.8766, 95% CI: 1.3816; 8.3715, Z = 2.73, p = 0.0062; I² = 87%, p = 0.0006), and brain (SMD = 4.0750, 95% CI: 2.5517; 5.5984, Z = 5.24, p < 0.0001; I² = 84%, p < 0.0001) (Fig. 8a-e). The funnel plot showed evidence of publication bias (Fig. 8f) which was confirmed by Egger's regression analysis (Egger's regression intercept 3.9938, t = 10.22, p < 0.0001). The pooled estimate of the effect did not vary substantially with the exclusion of the studies with a high risk of bias23,25,34–37,39,40,48–51,57,62,63,72,76,78,79,82,84,85 (SMD = 3.5875, 95% CI: 2.8567; 4.3183, Z = 9.62, p < 0.0001; I² = 89%, p < 0.0001; Egger's regression intercept 5.2682, t = 7.77, p < 0.0001).

3.3.7. Meta-analysis of Lipid peroxidation (ROS)

The results on ROS indicate a statistically significant increase in the levels of ROS in the non-skeletal tissues as compared to the controls (SMD = 4.5185 (95% CI: 3.1926; 5.8444, Z = 6.68, p < 0.0001). The heterogeneity of the included studies was high (I² = 86%, p < 0.0001). Only one study included in the meta-analysis reported the levels of ROS in the heart. A meta-analysis of the studies on ROS in the blood (SMD = 4.9560, 95% CI: 2.5505; 7.3616, Z = 4.04, p < 0.0001; I² = 78%, p = 0.0004), liver (SMD = 5.0346, 95% CI: 1.4152; 8.6540, Z = 2.73, p = 0.0064; I² = 92%, p < 0.0001), kidney (SMD = 6.2111, 95% CI: 3.8443; 8.5780, Z = 5.14, p < 0.0001; I² = 60%, p = 0.057), and brain (SMD = 2.3064, 95% CI: 0.0871; 4.1256, Z = 2.48, p = 0.0130; I² = 79%, p = 0.0028) was statistically significant (Fig. 9a-d). The funnel plot was asymmetrical (Fig. 9e) and the publication bias evaluated through Egger's test showed statistically significant evidence of publication bias (Egger's regression intercept 2.9315, t = 3.27, p = 0.004). The results were unchanged after the exclusion of two studies with a high risk of bias35,79 (SMD = 4.6235, 95% CI: 3.1309; 6.1162, Z = 6.07, p < 0.0001; I² = 87%, p < 0.0001; Egger's regression intercept 4.0824, t = 4.14, p = 0.0007).

3.3.8. Meta-analysis of Nitric Oxide (NO)

NO level was found to be significantly higher in non-skeletal tissues of experimental animals treated with fluoride compared to the controls (SMD = 3.1115, 95% CI: 0.0142; 6.2087, Z = 1.97, p = 0.049). High heterogeneity was found among the studies measuring NO (I² = 95%, p < 0.0001). The NO level in the blood (SMD = -0.5690, 95% CI: -5.0046; 3.8666, Z = -0.25, p = 0.8015; I² = 96%, p < 0.0001), kidney (SMD = 0.6776, 95% CI: -4.2842; 5.6394, Z = 0.27, p = 0.7890; I² = 97%, p < 0.0001), and heart (SMD = 4.8567, 95% CI: -13.3212; 23.0345, Z = 0.52, p = 0.6005; I² = 96%, p < 0.0001) of experimental animals treated with fluoride was non-significant compared to the controls. The NO level was however, significantly higher in the brain (SMD = 8.6347, 95% CI: 4.9441; 12.3253, Z = 4.59, p < 0.0001; I² = 53%, p = 0.1426) and liver
(SMD = 2.4717, 95% CI: 1.8812; 3.0622, Z = 8.20, p < 0.0001; I² = 0%, p = 0.5301) of experimental animals treated with fluoride compared to the controls (10a-e). The funnel plot was asymmetrical (Fig. 10b). However, a Egger’s regression test showed no evidence of publication bias (Egger’s regression intercept 1.1559, t = 0.5, p = 0.6311). Sensitivity analysis done by removing three studies39,62,79 was non-significant (SMD = 1.6737, 95% CI: -2.9603; 6.3078, Z = 0.71, p = 0.4790) but the heterogeneity remained unchanged (I² = 95%, p < 0.0001). As bias examination using a funnel plot is not recommended for the analysis with less than 10 studies,87 we did not examine the studies included in the sensitivity analysis for publication bias.

3.4. Subgroup Analysis

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) was conducted. The test for subgroup differences suggests that there is a statistically significant subgroup effect (intervention period for SOD (p = 0.0003), CAT (p = 0.03) and LPO (p = 0.007) (Fig. 11); animal species for LPO (p = 0.04; Fig. 12); and sample source for SOD (p = 0.04), GSH (p = 0.04), ROS (p = 0.048), and NO (p = 0.02) (Fig. 13)). No statistical difference was detected for other indicators.

3.5. Meta-Regression

A meta-regression analysis was performed with each indicator of oxidative stress 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, heart, blood) as factors. There was a significant influence of animal species (p = 0.02) for LPO. All the other moderators had no influence on the studies’ effect size (Table 2). The source of heterogeneity was found to be from all factors (intervention: LPO (4.1%); animal species: SOD (2.7%), GSH (3.7%), LPO (11%), and ROS (8.2%); sample source: SOD (6%), GSH-Px (3.5%), GSH (3.3%), ROS (3.8%) and NO (2.2%).

4. Discussion

In this study, we have undertaken the first meta-analysis to investigate the alterations of oxidative stress biomarkers in non-skeletal tissues of experimental animals exposed to fluoride compared with the controls. We included 62 studies measuring 8 oxidative stress biomarkers. Overall, in comparison to the controls, animals treated with fluoride showed a significant increase in the levels of ROS, LPO, and NO and a significant decrease in the antioxidant levels of SOD, CAT, GSH-Px, and GSH. The results on the levels of GST were, however, not significant. All biomarkers showed high levels of heterogeneity. Significant publication bias was found in all biomarkers except for NO. The sensitivity analysis showed significant differences for the oxidative stress biomarkers 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. However, the effect size for studies measuring NO was not significant and there was no publication bias for studies measuring GST after a sensitivity analysis.
We demonstrated an increase in oxidative stress in blood, liver, kidney, heart, and brain in line with the available evidence suggesting fluoride-induced oxidative stress as a mechanism involved in fluoride toxicity.\textsuperscript{16,88} The level of ROS and LPO were uniformly elevated in these tissues compared to the controls. However, the results on NO were only found to be significantly elevated in the brain and liver. NO is one of the reactive nitrogen species (RNS) produced by the catalytic action of nitric oxide synthase (NOS) during the generation of L-citrulline from L-arginine and oxygen.\textsuperscript{89} Fluoride can either induce\textsuperscript{90} or suppress the synthesis of NO.\textsuperscript{91} RNS initiates lipid peroxidation, reacts with thiols including glutathione (GSH), creating S-nitrosothiols which can inactivate proteins, leading to increased impaired cellular respiration, oxidative stress, or necrotic cell death. Further, excess NO combines with superoxide, producing peroxynitrite (ONOO\textsuperscript{-}) that is responsible for much of the cytotoxicity.\textsuperscript{89} Evidence suggests that oxidative stress also inhibits NO production by impairing endothelial NOS expression and activity. Excessive or deficient NO increases ROS/RNS production while lowering antioxidant levels.\textsuperscript{92} Of the 12 studies included in the meta-analysis, 2 recorded lower levels of NO in blood, heart, and kidney.\textsuperscript{19,44} The results of this meta-analysis should be interpreted with caution since only 2 or 3 studies were included in the analysis of each organ.

SOD forms the first line of defense against superoxide radicals by conversion to hydrogen peroxide ($H_2O_2$). $H_2O_2$ is either detoxified to $H_2O$ and $O_2$ by GSH-Px or diffuses into the cytosol and is detoxified by catalase (CAT) in peroxisomes. GSH protects against oxygen radicals and toxic compounds and acts as a coenzyme for enzymes.\textsuperscript{93} The role of GSH-Px is dependent upon the availability of GSH.\textsuperscript{94} Our study found a significant decrease in SOD, CAT, GSH-Px, and GSH in all the tissues studied except for the level of CAT in the kidney and GSH-Px in the brain which were not significant. A decrease in these antioxidants suggests an impaired ability of the antioxidant defense mechanism to inactivate ROS and scavenge free radicals. GST through their Se-independent glutathione peroxidase activity can reduce lipid hydroperoxides and detoxify lipid peroxidation end products such as 4-hydroxynonenal.\textsuperscript{95} No significant change was found in the level of GST in non-skeletal tissues of experimental animals compared with the control.

We observed a statistically significant subgroup effect suggesting that different animal species and tissues have varying susceptibility and tolerance to fluoride, and the intervention period can determine the level of oxidative damage in experimental animals. However, since there was substantial heterogeneity between the studies within each of these groups, the validity of the treatment effect estimate for each subgroup is uncertain. The absolute SMD for SOD and LPO was higher in the 30–90 days subgroup while that of CAT was higher in < 30 days subgroup. The severity of fluorosis is dependent on the dose and duration of fluoride exposure. In an in vivo study on the effect of sodium fluoride on sperm motility, sodium fluoride decreased sperm motility in a dose and time-dependent manner. The sperm abnormality was significantly increased at 10 and 100mg/ml of NaF at the 30 minutes time interval.\textsuperscript{96} In another study, the alterations found in the liver of rats at 60 days were less evident than those observed at 20 days, in both groups treated with 15mg/l and 50mg/l.\textsuperscript{97} Mukhopadhyay, et al.\textsuperscript{75} however, found that the effect of fluoride was different for the measured parameters e.g., Cytochrome P450 1 A (Cyp1A) mRNA
expression increased in a dose-dependent manner up to 30 mg NaF for 30 days treatment group but decreased in the 90 days treatment group while the downregulation of Kelch-like ECH-associated protein 1 (keap 1) was most prominent after 15 mg NaF treatment for 90 days. The differences observed in these studies are likely to be a result of different study designs, dosage, animal species, and duration of treatment. The significant animal species subgroup effect seen in LPO confirms the variation in genetic susceptibility found in different strains of animals. This difference is likely to be greater in animals from different species. There was also a significant sample source subgroup effect in SOD, GSH, ROS, and NO with the absolute SMD for SOD being lower in samples from brain and GSH in samples from blood. The SMD for ROS was higher in samples from the kidney and NO in samples from the brain. The available evidence suggests that the fluoride levels in the brain are generally low due to the relative impermeability of the blood-brain barrier. Conversely, the kidneys are exposed to high levels of fluoride and tend to have the highest fluoride concentration compared to all non-skeletal tissues. These results should, however, be interpreted with caution since the number of studies contributing data to different subgroups was unequal thus the analysis may not be able to detect subgroup differences.

5. Limitations

Our study had some limitations. First, the heterogeneity between the included studies was high. The dissimilarity seen in the studies analysed could be as 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. Second, due to the limited number of studies, some comparisons had to contain only two or three studies per item. Thirdly, a subgroup analysis based on fluoride dose could not be done since it was not possible to generate different dose ranges. Further meta-analysis with more studies included would be necessary to verify the results of this study.

6. Conclusion

In conclusion, our meta-analysis findings demonstrated the presence of oxidative stress and depletion of antioxidants in the non-skeletal tissues of experimental animals exposed to fluoride. This finding strengthens the evidence that fluoride toxicity is accompanied by increased oxidative stress response not only in skeletal but also in non-skeletal tissues, and manipulation of oxidative stress marker concentrations should be investigated for potential therapeutic strategies of the disease. Further, studies in humans are needed to explore the effect of fluoride toxicity in non-skeletal tissues.

Declarations

Funding

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.
Competing Interests

The authors declare no conflict of interest

Author contributions

Linet Musungu Angwa: Conceptualization, Methodology, Literature search, Data curation, Statistical analysis, Writing - original draft preparation. David Masinde: Validation, Writing – review and editing.

References


80. Inkielewicz-Stepniak I, Czarnowski W. Oxidative stress parameters in rats exposed to fluoride and caffeine. Food and Chemical Toxicology. 2010 Jun 1;48(6):1607-11. doi: 10.1016/j.fct.2010.03.033


Tables

Tables 1 to 2 are available in the Supplementary Files section

Figures
Figure 1
Flow chart of the literature search
Figure 2

Risk of bias, average per item
Figure 3

Forest plots for SOD meta-analysis: (a) Blood; (b) Liver; (c) Kidney; (d) Heart; (e) Brain

f: SOD Funnel plot
Figure 4

Forest plots for CAT meta-analysis: (a) Blood; (b) Liver; (c) Heart; (d) Brain; (e) Kidney

f: CAT Funnel plot
Forest plots for GSH-Px meta-analysis: (a) Blood; (b) Liver; (c) Kidney; (d) Heart; (e) Brain

f: GSH-Px Funnel plot
Figure 6

Forest plots for GSH meta-analysis: (a) Blood; (b) Liver; (c) Kidney; (d) Heart; (e) Brain

f: GSH Funnel plot
Figure 7

Forest plots for GST meta-analysis: (a) Liver; (b) Kidney; (c) Brain

d: GST Funnel plot
Figure 8

Forest plots for LPO meta-analysis: (a) Blood; (b) Liver; (c) Kidney; (d) Heart; (e) Brain

f: LPO Funnel plot
Figure 9

Forest plots for ROS meta-analysis: (a) Blood; (b) Liver; (c) Kidney; (d) Brain

(e) ROS Funnel plot
Figure 10

Forest plots for NO meta-analysis: (a) Blood; (b) Kidney; (c) Heart; (d) Brain; (e) Liver f: NO Funnel plot
Subgroup analysis to determine the effect of intervention period on oxidative stress induced by fluoride. There is a statistically significant subgroup effect for SOD ($p = 0.0003$), CAT ($p = 0.03$) and LPO ($p = 0.007$). No statistical difference was detected for other indicators.
Figure 12

Subgroup analysis to determine the effect of animal species on oxidative stress induced by fluoride. There is a statistically significant subgroup effect for LPO (p = 0.04). No statistical difference was detected for other indicators.
Figure 13

Subgroup analysis to determine the effect of sample source on oxidative stress induced by fluoride. There is a statistically significant subgroup effect for SOD (p = 0.04), GSH (p = 0.04), ROS (p =0.048) and NO (p = 0.02). No statistical difference was detected for other indicators.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- Tables.docx