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Effects of Subacute Carvacrol Administration on Behavioral and Oxidative Stress Parameters During NaF Exposure

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ABSTRACT

Purpose: Fluoride is an element found in the earth's crust that leaches into drinking water from sources where the rocks contain fluoride-rich minerals. Excessive fluoride exposure can lead to complications in various tissues and metabolism, particularly affecting dental and skeletal tissues alongside others. This study aimed to examine the impact of carvacrol (CAR), one of the main active compounds in thyme, on behavior and oxidative stress in sodium fluoride (NaF) exposure. In the current study, the Open Field Test (OFT), Elevated Plus Maze (EPM), and Barnes Maze tests were used to examine anxiety-like behaviors and spatial memory.

Methods: Herein, 32 male Balb/C mice were divided into four groups: the Control group, CAR group: 20 mg/kg CAR intraperitoneally (i.p.), NaF group: 50 ppm NaF (in tap water), and NaF+CAR group: 50 ppm NaF (in tap water) + CAR 20 mg/kg i.p. The treatments continued for 14 days. At the end of the protocol, the OFT, EPM test, and Barnes Maze test were conducted. Oxidative stress parameters [superoxide dismutase (SOD), catalase (CAT), malondialdehyde (MDA)] were examined in the brain, kidney, and liver tissues.

Results: The oxidative stress data indicated significant changes caused by the NaF and CAR. In the liver and kidneys, the enzyme activity values of SOD and CAT decreased significantly in the NaF group compared to the control and CAR groups, while in the brain, the CAT enzyme activity significantly decreased in the NaF group compared to control and CAR groups ($p < 0.05$). Moreover, the levels of MDA, an important marker of lipid peroxidation, were significantly higher in the liver and brain tissues of the NaF group compared to the control group ($p < 0.05$). The behavioral study results indicated no significant difference in the EPM test but showed an increase in the number of rearing behaviors in the NaF group compared to the NaF+CAR group in the OFT, and a decrease in the grooming time in the NaF group compared to the CAR group ($p < 0.05$).

Conclusions: In the brain, kidney, and liver tissues, NaF exerted oxidative stress, as seen via the examined parameters (CAT, MDA, and SOD), while the CAR seemed to have an ameliorative effect against NaF-induced oxidative stress. The administration of NaF did not induce significant anxiety; on the contrary, the concomitant administration of NaF and CAR caused increased anxiety-like behaviors compared to the lone NaF exposure.

Key-words: *Sodium Fluoride; Carvacrol; Phytochemicals; Anxiety; Elevated Plus Maze; Open Field Test; Oxidative stress*

INTRODUCTION

Fluoride is one of the elements commonly found in the Earth's crust[1]. Additionally, fluoride can be found in the air and in waste from various industrial activities, especially electroplating, semiconductors, glass, steel, ceramics, and chemical fertilizer production[2] or toothpaste. It leaches into drinking water from some underground sources where the rocks contain fluoride-rich minerals in significant amounts[3]. Fluoride concentrations in groundwater can reach up to 67 mg/L[4]. Sodium fluoride (NaF) is one of the forms through which fluoride mixes into drinking water from these underground sources[5]. The World Health Organization (WHO) has set the permitted limits for fluoride intake into the human body at 0.6 mg/L to 1.5 mg/L[6,7]. In studies conducted in areas where underground water sources, which are higher in fluoride content recommended by the WHO, are consumed as drinking water, structural abnormalities in teeth have been emphasized due to fluorosis[8]. The reduction of fluoride levels in drinking water with methods such as electrochemical processes to decrease excess fluoride has been highlighted[9]. There are also studies indicating potential fluoride exposure from different sources. In a study related to beverages consumed as drinks other than drinking water, it has been emphasized that energy drinks contain more fluoride compared to other processed beverages, although they do not pose a standalone risk of fluoride toxicity[10]. The daily consumption of milk can also be among the consumption sources that increase daily fluoride exposure[11]. Numerous studies have demonstrated the toxicity of NaF to several organs, including bone, kidneys, and the brain[7,12-14]. While there are numerous studies in the literature focusing on metabolic and behavioral changes after chronic exposure such as 90 days[14-17], there are fewer studies on short-term exposure to NaF[14]. There is limited research on the administration of NaF in a subacute duration, concomitant with phytochemicals possessing antioxidant properties to alleviate the negative effects of NaF[18,19]. Additionally, in the literature, animals have been administrated very high doses of fluoride in short-term NaF exposure[3,20]. However, these doses are extremely high concentrations not even found in underground waters worldwide[4,8,21]. The current study will contribute to the literature by elucidating changes in behavior and oxidative stress parameters during fluoride administration at levels that may be encountered in underground waters during the subacute period (14 days).

Phytochemicals are biologically significant chemicals found in plants that possess various activities[22,23,15]. One of these is carvacrol (CAR), a monoterpenoid phenolic compound present as a volatile oil in various aromatic medicinal plants, exhibiting antioxidant properties[24,25]. CAR's potent antioxidant capability is known to play a protective role in the brain and other tissues[26,27]. There are studies indicating that phytochemicals, particularly their antioxidant properties, might be effective against fluoride toxicity[18,19,28-30]. Fluoride can induce behavioral and biochemical changes in the brain by causing oxidative stress. Phytochemicals are considered a potential therapeutic group against oxidative stress due to their strong antioxidant properties. However, there is insufficient research on the protective effects of phytochemicals during subacute exposure to NaF. No studies have been found demonstrating the antioxidant properties of CAR, a phytochemical, against NaF.

Thus, the aim of this study was to investigate the effect of CAR on oxidative stress induced by NaF in the brain, liver, and kidneys, as well as evaluate its impact on behavioral parameters.

MATERIAL AND METHODS

Animals

In the experiments, male Balb/c mice weighing 25–40 g were housed in appropriate-sized plexiglass cages under a 12-hour light/dark cycle at 22 ± 2 °C. All of the mice had access to food and water. A total of 32 mice were used in the study, with 8 animals (n = 8) in each group, and the treatments continued for 14 days. The CAR was obtained from Glentham Life Sciences (GP9460) and the fluoride was obtained from Sigma. The drinking water was changed every two days and NaF was added at a concentration of 50 mg/L.

Control group: 0.2% Tween80 in saline solution injected i.p.

CAR group: CAR (20 mg/kg) dissolved in 0.2% Tween80 and injected i.p.

NaF group: 50 ppm NaF added to tap water and 0.2% Tween80 injected i.p.

NaF+CAR group: 50 ppm NaF added to tap water, in addition to 20 mg/kg CAR dissolved in 0.2% Tween80 injected i.p.

On day 14, behavioral tests including the OFT, EPM test, and Barnes Maze Test were sequentially conducted 1 hour after the treatment administrations. Once the tests were completed, the animals were euthanized appropriately. Brain, liver, and kidney tissues were rinsed with saline solution and preserved in a deep freezer (–80 °C) until analysis of the CAT, MDA, SOD levels. The study was carried out following ethical approval from the relevant Animal Experiments Ethical Committee. Experimental design is given in figure 1.

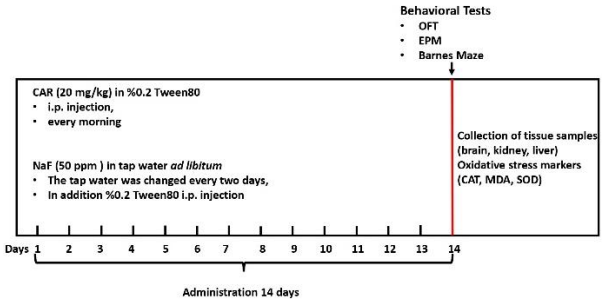

Figure 1: Experimental design representing the model used in the study

Behavioral Tests

Elevated Plus Maze

The EPM is one of the most commonly used models for rodents to evaluate anxiety-like behavior. This test consists of two open arms and two closed arms that intersect each other in the shape of a plus sign, elevated at sufficient height above the ground. The animals are placed in the center of the maze, facing an open arm, and are allowed to explore the maze freely for 5 min. An increase in the time spent in the open arms indicates less anxiety[31]. Parameters such as the number of entries into the open and closed arms, the time spent or percentage of time spent in the open or closed arms are frequently used to assess the anxiety level[32]. These parameters serve as indicators of anxiety-like behavior and the effects of anxiolytic substances.

Open Field Test

The OFT is another test utilized in evaluating anxiety-like behavior. Although there are different setups for the OFT, the OFT apparatus is typically a 40 × 40 × 40 cm^3 box containing a 20 × 20 cm^2 center square field at the bottom. The test duration allows the mice to freely explore for a period of 5 min. Rodents tend to avoid spending time in the center of the open field. An increase in the time spent in the center and the number of rearing behaviors (vertically standing on the hind legs) indicates a decrease in anxiety-like behavior. Parameters compared between groups in the OFT include the number or percentage of entries into the center as well as the number of rearing behaviors. These parameters serve as indicators of anxiety-like behavior and the effects of substances or treatments on reducing anxiety levels[33].

Barnes Maze Protocol

This is a test commonly used to evaluate rodents' spatial learning and memory functions. Although different designs are present, it generally involves a circular apparatus containing 20 evenly spaced holes, positioned on a platform high enough above ground to induce fear of height in rodents. An opaque escape box is placed directly under one of the evenly spaced holes. To facilitate learning, the walls of the quadrant where the escape box is located are marked with different colors and patterns. Following a 3 to 4-day training period, on day 4 or 5, a probe test is conducted. The parameters assessed across all groups include the time spent by the rodent in the correct quadrant, percentage of time spent in the correct quadrant, time taken to reach the target, and number of visits to incorrect holes before and after finding the correct hole[34,35].

Oxidative Stress Markers

Brain, Kidney, and Liver Tissue Preparation for Oxidative Stress Parameter Determination

Brain, kidney, and liver tissues were obtained from the mice to perform an evaluation of the oxidative stress parameters. Samples weighing 0.5 g from each tissue type were precisely measured. To buffer the tissues, a cold Tris buffer (1 mmol/L EDTA, 0.32 mol/L sucrose, and 10 nmol/L Tris-HCl, pH 7.4) in an amount ten-fold of their weight was added. Subsequently, the tissues were homogenized using an Ultra Turrax T25 homogenizer (IKA, Staufen, Germany). The samples, transferred to porcelain crucibles, underwent treatment in an ultrasonic disruptor at a frequency of 20 KHz to break down the cell membranes. Afterward, centrifugation was performed at 1600 rpm for 30 min to obtain clear supernatants, which were then transferred into Eppendorf tubes. All of the procedures were conducted at 4 °C. The samples were stored in a deep freezer at –80 °C for biochemical analysis. Assessment of the antioxidant enzyme activity levels, including CAT (EC 1.11.1.6)[36] and SOD (EC 1.15.1.1)[37], was carried out. Additionally, the MDA level, which is an indication of lipid peroxidation and so cell damage, was also measured[38]. The total protein content in the liver, kidney, and brain tissue homogenates was spectrophotometrically measured using the method described by Lowry et al., using bovine serum albumin solution as a standard solution[39].

Statistical analysis

Statistical analysis of the data was conducted using the Kruskal–Wallis test, Mann–Whitney U test, and Bonferroni correction ($p < 0.05$). IBM SPSS Statistics for Windows 23.0 (IBM Corp., Armonk, NY, USA) was used to perform the statistical analyses.

RESULTS

Behavioral Results

Open Field Test

The number of rearing behaviors in the NaF group (41.0 ± 12.7) was significantly higher compared to the NaF+CAR group (21.0 ± 11.7) ($p < 0.01$). Additionally, the grooming duration in the NaF group (1.57 ± 1.2) was significantly lower compared to the CAR group (4.87 ± 2.3) ($p < 0.01$) (Table 1).

Elevated Plus Maze Test

No significant differences were found in the investigated EPM parameters ($p > 0.05$) (Table 2). The number of entries and time spent (s) in the open arms in the CAR (3.25 ± 2.6 and 239.62 ± 80.7, respectively) and NaF (4.50 ± 2.6 and 215.62 ± 78.5, respectively) groups increased compared to the control group (3.0 ± 2.3 and 70.62 ± 91.6, respectively). The number of entries and time spent (s) in the closed arms in the NaF+CAR group (1.62 ± 2.1 and 52.0 ± 89.4, respectively) increased compared to the NaF group (1.0 ± 2.4 and 17.12 ± 45.2, respectively), but there were no significant differences ($p > 0.05$). Anxiety-like behaviors were reduced in the CAR and NaF groups compared to the control. However, in the NaF+CAR group, these behaviors were increased compared to the NaF group, but the difference was insignificant ($p > 0.05$).

Barnes Maze

The time spent in the correct quadrant and the percentage of time spent in the correct quadrant were lower in the NaF+CAR group (11 ± 18.8 and 6.10 ± 10.4, respectively) compared to the control group (73.75 ± 43.6 and 40.96 ± 24.2, respectively) ($p < 0.05$). However, the number of visits to the incorrect hole after finding the correct hole was lower in the NaF+CAR group (0.75 ± 1.3) compared to the control group (10.25 ± 6.0) ($p < 0.05$) (Table 3).

Biochemical Results

The enzyme activity values in the liver and kidneys for SOD (2129.72 ± 95.9 and 1016 ± 65, respectively) and CAT (26.15 ± 1.2 and 806.0 ± 13.7, respectively), and in the brain for CAT (15.19 ± 1.5), significantly decreased in the NaF group compared to the control group [2527.21 ± 64.51 (liver SOD), 1241.8 ± 8.72 (kidney

SOD), 40.15 ± 1.4 (liver CAT), 917.26 ± 47.3 (kidney CAT), and 23.7 ± 4.2 (brain CAT)] and CAR group [2637.89 ± 132.3 (liver SOD), 1311.32 ± 42.51 (kidney SOD), 37.69 ± 2.87 (liver CAT), 908.16 ± 45.2 (kidney CAT), and 23.74 ± 1.25 (brain CAT)] ($p < 0.05$). Moreover, the MDA level, an important indicator of lipid peroxidation, significantly increased in the liver (327.66 ± 19.5) and brain (711.2 ± 87.9) tissues in the NaF group compared to the control group (257.25 ± 13.2 and 539.11 ± 22.0, respectively) ($p < 0.05$). However, this increase was not significant in the kidney tissue. When the NaF group (5.09 ± 0.44) was compared with the NaF+CAR group (3.39 ± 0.5), it was noted that the increased MDA level in the kidneys decreased significantly ($p < 0.05$). Additionally, in the NaF+CAR group (1159.96 ± 94.4), the kidney SOD activity significantly increased compared to the NaF group (1016 ± 65) ($p < 0.05$). However, no significant increase was observed in the CAT enzyme activity in the liver, kidney, and brain tissues between these same groups (Table 4).

Table 1: Results of the OFT following the different treatment administrations.

OFT	Control	CAR	NaF	NaF+CAR
Time spent in the central square (s)	78.0 ± 20.9	82.12 ± 30.2	85.62 ± 24.7	74.37 ± 25.1
Number of entries into the central square	21.87 ± 4.4	23.37 ± 6.3	23.12 ± 6.0	18.75 ± 6.5
Percentage of entries into the central square (%)	48.97 ± 0.4	48.86 ± 0.3	49.23 ± 0.65	49.12 ± 1.0
Duration of grooming (s)	4.25 ± 2.1^{ab}	4.87 ± 2.3^{a}	1.57 ± 1.2^{b}	3.25 ± 2.7^{ab}
Number of rearing behaviors	36.62 ± 12.1^{ab}	40.37 ± 8.2^{ab}	41.0 ± 12.7^{a}	21.0 ± 11.7^{b}

CAR: Carvacrol, NaF: Sodium fluoride. Groups: Control, CAR (20 mg/kg), NaF (50 ppm), and NaF+CAR (20 mg/kg+50 ppm) (n = 8 in each group). Different letters (a, b, c) indicate a statistically significant difference. $p < 0.05$.

Table 2: Results of the EPM following the different treatment administrations.

EPM	Control	CAR	NaF	NaF+CAR
Number of entries into the open arms	3.0 ± 2.3	3.25 ± 2.6	4.50 ± 2.6	1.75 ± 0.7
Number of entries into the closed arms	2.87 ± 3.2	1.50 ± 1.5	1.0 ± 2.4	1.62 ± 2.1
Percentage of entries into the open arms (%)	72.01 ± 31.4	77.4 ± 21.8	90.14 ± 19.8	70.62 ± 32.3
Time spent in the open arms (s)	196.62 ± 116.5	239.62 ± 80.7	215.62 ± 78.5	226.25 ± 112.9
Time spent in the closed arms (s)	70.62 ± 91.6	27.0 ± 47.2	17.12 ± 45.2	52.0 ± 89.4
Percentage of time spent in the open arms	70.7 ± 37.4	87.66 ± 22.7	91.12 ± 23.3	78.47 ± 37.7

CAR: Carvacrol, NaF: Sodium fluoride. Groups: Control, CAR (20 mg/kg), NaF (50 ppm), and NaF+CAR (20 mg/kg+50 ppm) (n = 8 in each group). Different letters (a, b, c) indicate a statistically significant difference. $p < 0.05$.

Table 3: Results of the Barnes test following the different treatment administrations.

Barnes Maze	Control	CAR	NaF	NaF+CAR
Time spent in the correct quadrant (s)	73.75 ± 43.6^{a}	38.87 ± 24.0^{ab}	47.85 ± 33.9^{ab}	11 ± 18.8^{b}
Percentage of time spent in correct quadrant (%)	40.96 ± 24.2^{a}	21.59 ± 13.3^{ab}	26.58 ± 18.8^{ab}	6.10 ± 10.4^{b}
Latency to reach the target quadrant (s)	48.0 ± 39.9	61.37 ± 60.0	48.75 ± 48.9	34.50 ± 65
Checking the incorrect hole after the correct hole	10.25 ± 6.0^{a}	4.25 ± 5.1^{ab}	4.28 ± 5.5^{ab}	0.75 ± 1.3^{b}

CAR: Carvacrol, NaF: Sodium fluoride. Groups: Control, CAR (20 mg/kg), NaF (50 ppm), and NaF+CAR (20 mg/kg+50 ppm) (n = 8 in each group). Different letters (a, b, c) indicate a statistically significant difference. $p < 0.05$.

Table 4: Results of the biochemical analyses of different oxidative parameters found in the brain, kidney, and liver tissues following the different treatment administrations.

	Biochemical Parameters	Control	CAR	NaF	NaF+CAR
Liver	**SOD (EU/mg protein)**	2527.21 ± 64.51^{ab}	2637.89 ± 132.3^{a}	2129.72 ± 95.9^{c}	2301.36 ± 51.3^{b}
	CAT (EU/mg protein)	40.15 ± 1.4^{a}	37.69 ± 2.87^{ab}	26.15 ± 1.2^{c}	28.23 ± 27.2^{bc}
	MDA (nmol/mg protein)	257.25 ± 13.2^{b}	231.21 ± 12.4^{b}	327.66 ± 19.5^{a}	309.64 ± 11.2^{ab}
Kidney	**SOD (EU/mg protein)**	1241.8 ± 8.72^{a}	1311.32 ± 42.51^{ab}	1016 ± 65^{c}	1159.96 ± 94.4^{ab}
	CAT (EU/mg protein)	917.26 ± 47.3^{a}	908.16 ± 45.2^{ab}	806.0 ± 13.7^{c}	879,1 ± 29,1^{abc}
	MDA (nmol/mg protein)	3.65 ± 0.75^{abc}	2.98 ± 0.9^{c}	5.09 ± 0.44^{a}	3.39 ± 0.5^{b}
Brain	**SOD (EU/mg protein)**	1921.62 ± 93.7^{a}	2246.01 ± 308.5^{a}	1953.78 ± 110.3^{a}	2066.77 ± 127.9^{a}
	CAT (EU/mg protein)	23.7 ± 4.2^{a}	23.74 ± 1.25^{a}	15.19 ± 1.5^{b}	16.39 ± 0.58^{ab}
	MDA (nmol/mg protein)	539.11 ± 22.0^{b}	567.84 ± 61.8^{b}	711.2 ± 87.9^{a}	590.64 ± 17.9^{ab}

CAR: Carvacrol, NaF: Sodium fluoride. Groups: Control, CAR (20 mg/kg), NaF (50 ppm), and NaF+CAR (20 mg/kg+50 ppm) (n = 8 in each group). Different letters (a, b, c) indicate a statistically significant difference. $p < 0.05$.

DISCUSSION

The results of the current study indicated that the NaF group exhibited significantly increased oxidative stress compared to the CAR and control groups, but when administered in combination with CAR, this negative effect decreased. NaF did not show a significant negative impact on anxiety-like behaviors and learning during subacute exposure, and CAR did not exhibit a corrective effect on any potential adverse outcomes. Although NaF+CAR demonstrated more pronounced negative effects than NaF in both learning and memory as well as anxiety tests, statistical significance was not reached. The results of the current study contribute to the literature on the effects of subacute NaF administration and the potential impact of CAR.

This study was conducted to investigate the effects of subacute NaF exposure on behavioral and oxidative stress parameters (SOD, CAT, and MDA) in concomitant administration with CAR. The reason for conducting both behavioral and biochemical analyses in this current study was to investigate the interaction between NaF, which has been shown to trigger oxidative stress in various studies in the scientific literature, and the antioxidant properties of CAR.

Fluoride is one of the harmful chemicals that causes oxidative stress and inflammation in the brain and causes structural damage in cells and tissues proportional to the magnitude of these effects. It is

known that substances that initiate oxidative stress cause behavioral changes. The strong relationship between oxidative stress and diseases with neuronal and behavioral aspects, such as Alzheimer's, dementia, and Parkinson's, has been shown by numerous studies conducted to date[40,41]. In this study, it was aimed to examine oxidative stress and behavioral changes with the combination of NaF and antioxidant CAR, since NaF has been shown in different studies to trigger oxidative stress. The results revealed that NaF, administered both alone and in combination with CAR, significantly altered certain parameters. The most important findings of this current study were the augmented oxidative stress due to NaF administration in a subacute duration on the brain, kidney, and liver and the ameliorative effect of CAR on the investigated parameters (SOD, CAT, and MDA). These research results are important for showing the potent antioxidant effect of phytochemicals during oxidative stress due to NaF exposure.

Studies have indicated that both natural and synthetic phytochemicals ameliorate the damage caused by reactive oxygen species (ROS) mediated by fluoride in *in vivo* and *in vitro* models [12,42-44]. The current research focused on examining the behavioral and antioxidant effects of CAR, during high-dose NaF subacute exposure.

Herein, the subacute administration of NaF did not result in the expected anxiety effects, and similarly, the CAR did not demonstrate potential anxiolytic effects. It is known that some antidepressants used in anxiety treatment can initially increase anxiety levels[45]. Similarly, the administration of CAR during short-term NaF exposure might have shown a negative effect on anxiety, as observed in the NaF+CAR group. However, confirmation of this requires assessment through chronic studies.

In this study, 50 ppm of NaF was chosen to model the concentration found in some underground water sources, avoiding much higher doses[4]. In the scientific literature, anxiety-like effects have been observed with much higher concentrations, such as 600 ppm, which humans are not commonly exposed to in various regions of the world[46-48]. However, in the present study, we deliberately avoided selecting excessively high concentrations not typically observed in underground water sources. One possible reason for the discrepancy between our findings and the existing literature could be this approach in dose selection. While the NaF toxicity significantly reduced the SOD and CAT activities compared to the control and CAR groups herein, this subacute NaF toxicity might not have had a pronounced neuronal effect reflected in behaviors.

In the scientific literature, a single study that could serve as an example in exploring the protective role of CAR against NaF-induced toxicity was conducted by Shanmugam *et al.*, who administered NaF at a dose of 10 mg/kg i.p. for 60 days and administered *Ocimum sanctum* (OS) ethanol extract orally at a dose of 25 mg/kg and investigated their effects on the liver[49] They argued that the bioactive compound CAR in OS protected hepatic tissue from NaF toxicity. However, it is known that the antioxidant activities of plant extracts are not solely limited to phenolics like CAR. Their study highlighted that the observed antioxidant effect might be associated with other antioxidant secondary metabolites in the plant, such as volatile oils, carotenoids, and vitamins[50]. Although their study is valuable in demonstrating CAR's ability to modulate antioxidant enzymes and liver biomarkers against NaF toxicity, as emphasized in the article itself, solely emphasizing CAR's role might not be accurate. As mentioned earlier, thyme species do not solely consist of CAR as their active ingredient[50]. Additionally, their research was not a direct study showing its impact on oxidative stress parameters in the brain or behavioral parameters. Therefore, it did not contain data directly comparable with those in the current study.

Oyagbemi *et al.* administered 300 ppm of NaF (in tap water) to male Wistar rats for 7 days. Their study indicated that NaF increased the time spent in the center area and the number of cross movements in the OFT compared to the control and other groups, indicating an increase in mobility. However, it should be noted that this administered dose is considerably high. Additionally, similar to our study, their study showed a significant increase in rearing behaviors in the NaF group compared to the control when NaF was administered with drugs thought to have neuroprotective effects. NaF significantly increased the oxidative stress and neuroinflammation parameters compared to the control and other treatment + NaF groups. Moreover, the NaF group exhibited a significant inhibition of acetylcholinesterase (AChE)

activity compared to the control and other groups[3]. The increase in rearing behaviors and stretched posture movement in the NaF group was highlighted as an indicator of the potential anxiogenic effect of NaF. However, in several studies, an increase in rearing behaviors has been emphasized as an indicator of a decrease in anxiety[51,52]. The increase in rearing behaviors in the NaF group and the simultaneous decrease in grooming time in the current study suggest the potential anxiolytic effect of NaF in the subacute period. The findings from a study by Oyagbemi et al. were similar to the behavioral outcomes in the present study. Additionally, their study argued that NaF's inhibition of AChE activity during the subacute period is one of the key points of neurotoxicity[3,53]. However, it is worth noting that since the study did not conduct any tests related to learning and memory in behavioral parameters, this argument lacks support. Moreover, in some studies, the ameliorative effects of acute AChE inhibitors on short-term spatial memory in rats have been confirmed through the Barnes Maze test when exposed to neurotoxic substances[54]. In summary, based on articles in the literature, NaF's neurotoxic effects, observed alongside increased anxiety, and its cholinesterase inhibitory property may yield different behavioral outcomes in learning and memory. In the present study, while there was evidence of NaF-induced oxidative stress in the brain tissue, there was no worsening in the observed behavioral parameters compared to the control or CAR groups.

Studies have emphasized that AChE inhibitors increase ACh levels and are approved as the first choice for treating mild to moderate Alzheimer's disease[54]. Drugs with such effects include donepezil[55] and rivastigmine[56]. In the current study, the subacute administration of NaF did not significantly worsen the time or percentage of time spent in the correct quadrant on the Barnes Maze test compared to the control group. Therefore, the potential AChE inhibitory effect of NaF during the subacute period can be speculated by assessing the increase in learning and memory parameters in the Barnes Maze test.

Fuxin Lu *et al*. conducted a study administering NaF at a dose of 50 ppm in drinking water to 3-week-old C57BL/6J mice for 7 and 42 weeks. It was demonstrated that at weeks 7 and 42, the time spent, total distance traveled, and number of entries into the open arms significantly increased in the NaF group compared to the control group. The study indicated a decrease in anxiety in both NaF-administered groups[57]. Their study also mentioned an increase in serotonin levels following NaF exposure. This could be considered as a contributing factor to the observed changes related to anxiety. It has been argued that behavioral changes associated with fluoride intake in mice and rats could depend on the dosage, sex of the animal, and exposure duration. Although the duration in their study was much longer compared to the subacute period herein, it supports our findings that NaF does not induce anxiety.

Shambudi *et al*. administered 20 ppm of NaF i.p. to rats for 15 days. Additionally, they orally administered 120 mg/kg of *Allium sativum* ethanol extract. The results indicated a significant decrease in motor coordination and pain response in the NaF group. There was a substantial reduction in the SOD and CAT activities, while an increase in lipid peroxidation and glutathione peroxidase was observed. It was suggested that *Allium* extract might have a protective effect against fluorosis in terms of both antioxidant activity and behavioral parameters[58]. Their study, showing an increase in toxicity due to NaF in the subacute period, supports our oxidative stress data. However, the effects of NaF on learning, memory, and anxiety were not investigated in their study. Additionally, differences in species and the method of NaF administration may contribute to disparities between the current study and theirs.

CONCLUSION

In conclusion, in the three examined tissues (brain, kidney, and liver), NaF induced oxidative stress, while CAR exhibited an ameliorative effect on the assessed indicators (CAT, MDA, and SOD). In addition, the administration of NaF did not lead to significant anxiety, while the co-administration of NaF with CAR resulted in a particularly negative impact on the anxiety test compared to NaF group.

High-dose NaF, as reported in the literature, could be further investigated in subacute models in combination with CAR to explore its effects on various biochemical and behavioral parameters in future studies. We believe that due to the increasing

popularity and usage of phytochemicals, a more detailed investigation is necessary on their protective effects, particularly against fluorosis.

FUNDING

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CONFLICT OF INTEREST

The authors have no conflicts of interest to declare.

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