Anxiogenic, memory-impairing, pro-oxidant and pro-inflammatory effects of sodium benzoate in the mouse brain

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ABSTRACT
Objective: Sodium benzoate (NaB), a commonly used food additive, is effective in preventing deterioration and/or spoilage of foods and drinks. While there have been reports suggesting its potential use as an adjunct in schizophrenia management; there is a lack of information on its effects on the brain, especially when added to dry foods. This study examined the effects of NaB added to rodent feed on neurobehavior, antioxidant status, anti-inflammatory and apoptotic markers in mouse brain.

Method: Animals were divided into 4 groups of 10 mice each. Groups included normal control (fed with rodent chow) and 3 groups fed with NaB at 125, 250 and 500 mg/kg, respectively, for eight weeks. Open field, elevated plus maze (EPM), Y-maze, and radial-arm maze behaviors were assessed on day 57, following animals were euthanized 24 hours after the behavioral test. Whole-brain homogenate processed for the assessment of antioxidant status, inflammatory/apoptotic markers, and acetylcholinesterase activity.

Results: The NaB diet altered body weight, open-field behaviors, working-memory, and anxiety indices. Brain antioxidant status, tumor necrosis factor-α and interleukin-10 decreased, while the malondialdehyde, caspase-3 level, and acetylcholinesterase activity increased.

Conclusion: The results of this study revealed that the addition of NaB at these concentrations to rodent chow was associated with memory loss, anxiety, oxidative stress and increased inflammatory/apoptotic effects suggesting vigilance in its use.

Keywords: Anxiety, cognition, inflammation, memory, neurobehavior, oxidative stress

INTRODUCTION
Food preservatives containing sodium benzoate (NaB) have been used for decades to prevent deterioration or spoilage of foods and drinks due to the activities of microorganisms and enzymes (1). The use of a chemical agent as a food preservative presupposes that it is readily soluble, exhibits antimicrobial properties across
the pH range of the food, is non-toxic, and does not impart off-flavors (1,2).

NaB, the sodium salt of benzoic acid, is a preservative approved for use in foods (to prevent the growth of microbes such as fungi and bacteria that easily spoil foods) and pharmaceutical (as a preservative for liquid medicines, and lubricant for tablets) industries in several countries (1,3-6). Apart from its use in drinks, the use of NaB to preserve dried foods such as flour, fruits, and vegetables; and in biscuits, cakes and muffins are also widespread. Again, while moisture content is a major determinant of the efficacy of conventional food preservatives such as common salt, NaB had been shown to have a remarkable ability to preserve food or food ingredients regardless of moisture content; hence, foods with high moisture content such as tomato juice can still be well-preserved. However, NaB had also been found to be most suitable for foods and drinks that are in the acidic pH range, and its ability to preserve food in the low pH range contributes to its preservative property.

Different countries have regulations for the acceptable limits of NaB use, such as the United States of America approves its use under the generally accepted as safe status (6,7). In Nigeria, the acceptable limits are set at 250 mg/kg (in drinks) in accordance with the Codex Alimentarius Commission guidelines (8,9). NaB has also been assessed for its possible therapeutic effects with reports of health benefits derived from its pharmaceutical use (1,10-12).

In recent times, however, there has been also a growing body of knowledge highlighting the possible adverse effects of NaB when used as a food preservative (1,5,13,14). In vivo and in vitro studies have associated the use of NaB with the development of oxidative stress, memory deficits, anxiety, motor impairment, testicular inflammation and apoptosis (1,5,13,14). While a number of the studies examined the effects of NaB when added to drinking water (1,5,15); there is a lack of scientific information on the effects of NaB when added to dry food. This study investigated the effect of NaB, at different concentrations, on the brain of mice. We tested the hypothesis that at these concentrations (in food), NaB would have significant effects on neurobehavior, brain levels of oxidative stress, Caspase-3 activity, acetylcholinesterase activity, and inflammatory markers in mice.

**METHOD**

**Materials**
NaB (sourced from the Open market, Osogbo, Osun State, Nigeria). Tumor necrosis factor (TNF)-α and interleukin (IL)-10 assay kits (ENZO Life Sciences, U.S.A), Caspase-3 and Acetylcholinesterase assay kit (BioVision Inc. USA).

**Animals**
Adult mice (20-25 g) obtained from Empire farms in Osogbo, Nigeria were used for this study. Animals housing was a room kept at 23-25°C with 12-hour light-dark cycle. Animal feed was obtained from TOP FEEDS® Nigeria Ltd. Animal care and use complies with protocols approved by the Faculty of Basic Medical Sciences LAUTECH ethical committee and the European Council Directive (EU2010/63).

**Feed**
The animal diet was made up of 11% fat and 58% carbohydrate. NaB was incorporated into standard rodent diet at 125 (0.0125%), 250 (0.025%) and 500 (0.05%) mg/kg feed.

**Experimental Methodology**
Adult male mice (40) were randomly assigned to 4 groups of 10 mice each The groups included: control, fed standard diet (SD), and three groups fed one of 3 concentrations of NaB included in the SD at 125, 250 and 500 mg/kg feed. NaB or SD was administered for eight weeks and body weight was measured weekly. Open field, elevated plus maze (EPM), Y-maze, and radial-arm maze behaviors were assessed on day 57, after which animals were euthanized (24 hours after the last behavioral test) as previously described (16). The brains were excised and homogenized for the assessment of malondialdehyde (MDA) levels, lipid profile, superoxide dismutase, total antioxidant capacity, TNF-α, IL-10, caspase-3 and acetylcholinesterase activity.

**Body Weight and Food Intake**
Weekly body weight and daily food intake measurements were carried out using an electronic weighing scale as previously described (16-18).

**Neurobehavioral Tests**
At the end of the experiment, animals were exposed to the neurobehavioral paradigms (EPM, Y-maze, Open field and radial-arm maze). Protocols for the care of animals before and during the test period are as previously described (16,19).

**Open Field Test**
In the open-field arena, animals were allowed to go exploring for 10 minutes during which locomotor
activity, number of rearing and grooming episodes were observed and scored to assess. The open-field box used for this study was a rectangular box made of white painted wood, measuring 36x36x26 cm as previously described (20-22).

Memory Tests (Y-maze and radial arm maze)
Mice were exposed to the Y-maze and radial-arm maze for 5 minutes, respectively, to assess spatial working memory. Arm entry sequences in the Y-maze were observed and recorded as described previously (23-25). Working-memory in the radial-arm maze is scored as previously described (26-28).

Anxiety Model: Elevated Plus-maze
The elevated plus-maze, a four-arm cross-shaped apparatus placed at right angles to each other, was used to measure anxiety-related behaviors. Anxiety behaviors were scored as previously described (23-25).

Lipid Peroxidation (MDA)
The lipid peroxidation kit was used to determine MDA levels as previously described (21-25).

Antioxidant Activity
Superoxide dismutase activity was assayed as described in a previous study (24). Total antioxidant capacity measures the number of free radicals scavenged by the test solution in any biological sample (29-32). The total antioxidant capacity was based on the Trolox equivalent antioxidant capacity principle (33,34).

TNF-α and IL-10
TNF-α and IL-10 were measured using enzyme-linked immunosorbent assay (ELISA) techniques with commercially available kits designed to measure the ‘total’ (bound and unbound) amount of the respective cytokines.

Acetylcholinesterase Activity and Caspase-3 Levels
Brain acetylcholinesterase and caspase-3 activity were assayed according to the instructions provided by the manufacturers.

Brain Homogenization
Whole brains (5) were removed from the skulls of the animals, weighed and homogenized as described in an earlier study (16).

Statistical Analysis
Data were analyzed using Chris Rorden’s ezANOVA for windows. One-factor ANOVA was used for analysis.

Tukey’s honest significant difference test was used for intragroup and intergroup comparisons. Results were expressed as mean±standard error of mean (SEM) and p values less than 0.05 were considered statistically significant.

RESULTS

NaB on Body Weight and Food Intake
Figure 1 shows the effects of NaB on body weight (upper panel) and food consumption (lower panel). Body weight increased significantly (F [3, 36]=9.970, p=0.00063 SS=0.35 MSe=0.01) with NaB at 125 mg/kg feed compared to mice fed control diet. Intragroup comparisons (NaB vs. NaB) revealed a significant decrease in body weight with NaB at 250 and 500 mg/kg compared to the group fed with NaB at 125 mg/kg feed.

Food consumption increased significantly (F [3, 36]=2450.000, p=0.00001 SS=0.17 MSe=0.01) with NaB at 125 mg/kg of feed in comparison to mice fed control diet. Intra group comparisons (NaB vs. NaB) revealed a significant decrease in food consumption with NaB at 250 and 500 mg/kg compared to group fed NaB at 125 mg/kg feed.

NaB on Line Crossing and Rearing Activity
Figure 2 shows the effect of NaB on the number of line crossings (upper panel) and rears (lower panel). Line crossing increased significantly (F [3, 36]=14.300,
p=0.000003 SS=14151.88 MSE=329.00) with NaB at 125, and decreased at 500 mg/kg of feed compared to mice fed control diet. Intragroup comparisons (NaB vs. NaB) revealed a significant decrease in line crossing with NaB at 250 and 500 mg/kg are significantly different from NaB 125 mg/kg feed, number of mice/group=10, NaB: Sodium benzoate.

Number of rears increased significantly (F [3, 36]=2.36, p=0.0450, SS=481.88, MSE=68.10) with NaB at 125 mg/kg feed compared to mice fed control diet. Intragroup comparisons (NaB vs. NaB) revealed a significant decrease in rearing with NaB at 250 and 500 mg/kg compared to the group fed NaB at 125 mg/kg feed.

NaB on Self-grooming
Figure 3 shows the effect of NaB on the number of self-grooming episodes. Number of grooming episodes increased significantly (F [3, 36]=4.300, p=0.0105, SS=71.48 MSE=5.51) with NaB at 125 mg/kg feed compared to mice fed control diet. Intragroup comparisons (NaB vs. NaB) revealed a significant decrease in self-grooming with NaB at 250 and 500 mg/kg compared to the NaB-fed group at 125 mg/kg feed.

NaB on Working Memory in the Y- and Radial-arm Maze
Figure 4 shows the effects of NaB on memory scores in the Y- (upper panel) and radial-arm (lower panel) maze. A maze-dependent modulation of working memory scores was observed. The data analysed using One-way ANOVA. Each bar is Mean±SEM, *p<0.05 treatment groups significantly different from control, #p<0.05 when NaB 250 and 500 mg/kg are significantly different from NaB 125 mg/kg feed, number of mice/group=10, NaB: Sodium benzoate.

NaB at 125 mg/kg feed and increased with NAB at 250 mg/kg feed compared to mice fed control diet. Intragroup comparisons (NaB vs. NaB) revealed a significant increase in working memory scores with NaB at 250 and 500 mg/kg compared to the NaB-fed group at 125 mg/kg feed.

Memory scores in the radial-arm maze decreased significantly (F [3, 36]=4.590, p=0.0081, SS=1844.35 MSe=93.96) with NaB at 125 mg/kg feed compared to mice fed control diet. Intragroup comparisons (NaB vs. NaB) revealed a significant increase in memory scores with NaB at 250 and 500 mg/kg compared to the NaB-fed group at 125 mg/kg feed.
NaB on Anxiety-Related Behaviors

Figure 5 shows the effects of NaB on time spent in the open (upper panel) and closed (lower panel) arm of the elevated plus-maze. A concentration-dependent decrease in anxiety-related behaviors was observed. Data were analyzed using One-way ANOVA. Each bar is Mean±SEM, *p<0.05 treatment groups significantly different from control, #p<0.05 when NaB 250 and 500 mg/kg are significantly different from NaB 125 mg/kg feed, number of mice/group=10, NaB: Sodium benzoate.

NaB on MDA Levels and Antioxidant Status

Table 1 shows the effect of NaB on lipid peroxidation levels and antioxidant status. Brain MDA levels increased significantly (F [3, 36]=2154, p<0.0001, SS=9668.49 MSe=2.56) with NaB at 125, 250 and 500 mg/kg feed compared to the mice fed control diet. Intragroup comparisons (NaB vs. NaB) revealed a significant increase in lipid peroxidation levels with NaB at 250 and a significant decrease at 500 mg/kg compared to the NaB-fed group at 125 mg/kg feed.

Brain superoxide dismutase activity increased significantly (F [3, 36]=45.000, p=0.001, SS=25.26 MSe=0.06) with NaB at 125 and 250 mg/kg and decreased at 500 mg/kg feed compared to control diet. Intragroup comparisons (NaB vs. NaB) revealed a significant increase in superoxide dismutase activity with NaB at 250 and a significant decrease at 500 mg/kg compared to the NaB-fed group at 125 mg/kg feed.

Brain total antioxidant capacity decreased significantly (F [3, 36]=250.000, p=0.001 SS=388.61 MSe=0.03) with NaB at 125, 250 and 500 mg/kg compared to the mice fed control diet. Intragroup comparisons (NaB vs. NaB) revealed a significant increase in total antioxidant capacity with NaB at 250 and 500 mg/kg compared to the NaB-fed group at 125 mg/kg feed.

Sodium Benzoate on Inflammatory Markers, Brain Acetylcholinesterase and Caspase-3 Levels

Table 2 shows the effect of NaB on IL-10, TNF-α, acetylcholinesterase and caspase-3 levels. Brain TNF-α levels decreased significantly (F [3, 36]=102, p=0.001, SS=6373.86 MSe=0.45) with NaB at 125 and 250 mg/kg feed compared to the mice fed control diet. Intragroup comparisons (NaB vs. NaB) revealed a significant increase in TNF-α level with NaB at 250 and 500 mg/kg compared to the NaB-fed group at 125 mg/kg feed.

Brain IL-10 levels decreased significantly (F [3, 36]=50.54, p=0.0001, SS=278.13 MSe=0.13) with NaB at 125, 250 and 500 mg/kg of feed compared to the mice fed control diet. Intragroup comparisons (NaB vs. NaB) revealed a significant increase in IL-10 levels with NaB at 250 and 500 mg/kg compared to the NaB-fed group at 125 mg/kg feed.

| Groups | MDA nmol/g | SOD U/mg/protein | TAC (TE mg/protein) |
|--------|------------|------------------|---------------------|
| Control | 8.13±0.20  | 1.42±0.03        | 6.30±0.02           |
| NaB 125 | 32.45±0.28*| 3.56±0.04*       | 1.98±0.01*          |
| NaB 250 | 46.3±0.22**| 4.36±0.02**      | 3.15±0.01**         |
| NaB 500 | 12.32±0.11**| 0.98±0.01**   | 3.45±0.02**         |

The data were analyzed using one-way ANOVA. Values are presented as Mean±95% SEM, *p<0.05 treatment groups significantly different from control, **p<0.05 when NaB 250 and 500 mg/kg are significantly different from NaB 125 mg/kg feed, number of mice/group=10, NaB: Sodium Benzoate, MDA: Malondialdehyde, SOD: superoxide dismutase, TAC: Total antioxidant capacity, TE: Trolox equivalent.
Brain acetylcholinesterase (ACHE) (F [3, 36]=32.00, p=0.0001, SS=15.12 MSe=0.04) and caspase-3 (F [3, 36]=25.000, p=0.001, SS=119.47 MSe=2.22) activity increased significantly with NaB at 125, 250 and 500 mg/kg feed compared to the mice fed control diet. Intragroup comparisons (NaB vs. NaB) revealed a significant decrease in ACHe and Caspase-3 activity with NaB at 250 mg/kg compared to the NaB-fed group at 125 mg/kg feed.

**DISCUSSION**

In this study, the effect of sodium benzoate on body weight, food consumption, behavioral parameters, oxidative stress parameters, inflammatory and apoptotic markers were examined in mice. Results showed that sodium benzoate, when incorporated into dry rodent feed was associated with: a) an increase in body weight and food consumption at 125 mg/kg feed, b) a decrease in line crossing and an increase in rearing at 125 mg/kg, d) an increase in self-grooming at 125, and a decrease at 250 mg/kg, d) a decrease in memory scores in both the Y- and radial arm-maze memory, e) anxiogenic response at 125 mg/kg feed, f) increase in brain levels of MDA, acetylcholinesterase and caspase-3 activity, g) increase in SOD activity at 125, 250 and a decrease at 500 mg/kg feed, h) decrease brain TAC and; i) a decrease in brain levels of TNF-α and IL-10.

Feeding mice with NAB for 8 weeks, as observed in this study, was associated with an increase in body weight and food consumption at the lowest concentration. The effects of NAB on body weight have been studied severally (15,35,36) with varying results. Griffith (35) reported that feeding zebrafish with a benzoate diet at 1.5, 2.0 or 2.5% (significantly higher concentrations than those used in this study) did not significantly alter body weight (compared to controls), corroborating the results of this study when sodium benzoate diet was consumed at the higher concentrations. In a study in which t sodium benzoate was administered daily in distilled water at much lower concentrations than those used in this study, a time and concentration-dependent decrease in body weight was observed irrespective of sex (15). The results of our study and the other studies show that the effect of NAB on body weight depends on concentration, mode of administration and duration of administration. Food intake increased at 125mg/kg feed and showed no significant difference from control at the other concentrations. It has been reported that NAB reduces feed intake (37). Although there have been reports of no significant effect when NAB was administered by gavage (36). Overall, the results show that an increase in food consumption corresponds to an increase in weight, suggesting that NAB likely increases the palatability of food at this concentration. There was a visual decrease in food consumption and body weight at higher concentrations, probably suggesting that the inclusion of NAB to diet at these concentrations possibly altered food palatability. While preservation of the freshness and taste of food (due to the presence of NAB) might preserve or maintain its palatability, it is not impossible for NAB to affect palatability by the utilization of other mechanisms.

**Table 2: Tumour necrosis factor-α, Interleukin-10, acetylcholinesterase and caspase-3**

| Groups  | TNF-α ng/g/protein | IL-10 pg/mg/protein | ACHe/mmol/mg | Caspase-3 (ng/mg) |
|---------|--------------------|--------------------|--------------|------------------|
| Control | 40.31±0.23         | 24.12±0.10         | 36.35±1.60   | 0.30±0.02        |
| NaB 125 | 24.22±0.15*        | 14.15±0.20*        | 40.20±1.10*  | 0.35±0.02*       |
| NaB 250 | 29.31±0.18**       | 18.60±0.22**       | 45.35±1.60** | 0.58±0.04**      |
| NaB 500 | 42.15±0.23**       | 17.98±0.01**       | 40.30±1.60*  | 0.39±0.03*       |

Data analysed using one-way ANOVA. Values are presented as Mean±95% SEM, *p<0.05 treatment groups significantly different from control, **p<0.05 when NaB 250 and 500mg/kg are significantly different from NaB 125 mg/kg feed, number of mice/group=10, NaB: Sodium Benzoate, TNF-α: Tumour necrosis factor –alpha, IL-10: Interleukin-10, ACHe: Acetylcholinesterase
in rats administered NAB dissolved in distilled water daily for 4 weeks (39) and a decrease in locomotor activity in the embryo and larvae of zebrafish, although the embryos were more sensitive to NAB (42). The induction of either a central inhibitory or excitatory response is linked to NAB’s ability to pass through the blood-brain-barrier and alter or modulate neurotransmitter response. Studies in zebrafish have demonstrated that in-utero exposure to NAB altered brain development, resulting in a decrease in tactile sensitivity frequencies of touch-related movements (43). This suggests that it either crosses the underdeveloped blood-brain-barrier or damages it to gain access into the brain. Its effects on brain neurotransmitters have also been examined (39,41,42). While there have been reports that it does not affect the levels of brain monoamines (39,41), Chen et al. (42) reported that treatment of zebrafish with NAB, dose-dependently downregulated the expression of dopamine transporter and tyrosine hydroxylase in neurons of the ventral diencephalon, associating this with the decrease in locomotor activity observed. In our study, the increase in open field behaviors observed at 125 mg/kg feed could be due to the increase in dopamine receptor activity, while at the higher concentrations, due to the inhibition of dopamine receptor activity or ocuring neurotransmitter response.

In this study, working-memory impairment was observed with NAB at 125 mg/kg in the Y-maze and at 125 and 500 mg/kg in the radial-arm maze. This confirms the results of a study by Khoshnoud et al. (1) that reported impairment of memory following sub-chronic oral administration of NAB at 0.56, 1.125, and 2.25 mg/mL. Modi et al. (40), on the other hand reported that in-vivo metabolism of cinnamon (after oral administration in mice) results in NAB production, which was suggested to have a memory-enhancing effect (40). In this study, a significant decrease in acetylcholinesterase activity was observed, contrary to the reports of Khoshnoud et al. (1), suggesting that the memory impairment observed in this study could be attributed to an increase in acetylcholinesterase activity in addition to increased brain oxidative stress (evidenced by an increase in lipid peroxidation and a decrease in total antioxidant capacity) which was observed in this study. Since then, brain oxidative stress has been linked to the development of aging-related memory deficits (44,45).

In the study, NAB administration decreased brain levels of TNF-α/IL-10 and increased brain levels of caspase-3. Its effects on the pro-inflammatory marker TNF-α confirms the result of Brahmachari et al (10), who reported the inhibition of lipopolysaccharide-induced expression of proinflammatory cytokines (TNF-α) and inhibition of nuclear factor kappa B (NF-κB), an important transcription factor that regulates innate immunity by NAB. The authors attributed the anti-inflammatory activity of NAB to the inhibition of NF-Kb (10). The effects on caspase-3 confirms the result of a study by El-Shennawy et al. (5) that observed increased apoptotic activity in the testis of rats which were administered NAB dissolved in distilled water.

A limitation of this study is the fact that mice were utilized in conducting the experiments; therefore, caution is required in extrapolating the findings to humans. Also, the duration of the study is such that it may not necessarily reflect the effects of long-term consumption of NAB in humans.

While the therapeutic benefits of NAB have been reported severally, the results of this study revealed that chronic ingestion of NAB at these concentrations was associated with memory loss, anxiety, oxidative stress, increased inflammatory and apoptotic effects suggesting caution in its use.

| Contribution Categories          | Author Initials         |
|----------------------------------|-------------------------|
| Category 1                       | A.T.O., A.Y.O., O.J.O.  |
| Concept/Design                   |                         |
| Data acquisition                 | A.T.O., A.Y.O., O.J.O.  |
| Data analysis/Interpretation      | A.Y.O., O.J.O.          |
| Category 2                       | A.T.O., A.Y.O., O.J.O.  |
| Drafting manuscript              |                         |
| Critical revision of manuscript   | A.T.O., A.Y.O., O.J.O.  |
| Category 3                       | A.T.O., A.Y.O., O.J.O.  |
| Final approval and accountability|                         |
| Other                            | A.T.O., A.Y.O., O.J.O.  |
| Technical or material support     |                         |
| Supervision                      | N/A                     |

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*Conflict of Interest:* None declared.

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