PRENATAL BEE POLLEN TREATMENT IMPROVES THE NEUROTOXICITY IN NEWBORN RATS DURING CHRONIC FLUORIDE EXPOSURE IN RELATION TO PROPIONIC ACID-INDUCED RODENT MODELS OF AUTISM

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ABSTRACT: Although fluoride is considered to neurotoxic, very few investigations are available on fluoride exposure as a possible risk factor for autism. The present study compared the neurotoxicity in propionic acid (PA)-induced rodent models of autism with fluoride in prenatal bee pollen-protected and unprotected newborns. The study was performed on five groups of neonatal male Western albino rats. Rats in group I were a control group and received only phosphate-buffered saline, rats in group II received a neurotoxic dose of PA (250 mg/kg for 3 days) and served as an autistic model, group III received a chronic dose of sodium fluoride (NaF) (3–5 mg/kg for 30 days), group IV were prenatal bee pollen-treated neonates (250 mg/kg bw from 0–23 days of gestation + 7 days postpartum), and finally group V were prenatal bee pollen-treated neonates (250 mg/kg bw from 0–23 days of gestation + 7 days postpartum) toxicated with NaF (3–5 mg/kg for 30 days). The results of the PA acid and NaF treatments showed almost the same trend with a significant increase ($P \leq 0.001$) in lipid peroxides and significant decreases ($P \leq 0.001$) in glutathione, catalase, and vitamin C as compared to the control group. Potassium was significantly decreased ($P \leq 0.010$) in the PA group with no outstanding effects in the other groups. The bee pollen-protected neonatal group showed perfection in all the tested parameters. Our results argue for further investigation of fluoride as a risk factor for autism.

Keywords: Autism; Bee pollen; Fluoride; Neurotoxicity; Propionic acid.

INTRODUCTION

Fluorine is a highly electronegative element with the least van der Waals radius of the halogens in group VII of the periodic table and is able to react with almost all the elements with the exception of helium and neon.[1,2] Although fluoride (F), the ion of fluorine, can stimulate osteoblastic activity when combined with calcium and can form fluorapatite crystals with calcium and phosphate resulting in a decrease of solubility and an increase of bone crystallinity in the human body, F is neither an essential trace element for humans nor necessary for the development of healthy teeth and bones.[3,4] Although the topical use of fluoride is helpful in reducing the rate of dental caries, high intakes of F can result in adverse effects with dental, skeletal, and non-skeletal fluorosis including an increased rate of bone fractures and urolithiasis, and decreased thyroid and brain function in children.[5,6] The human body is exposed to fluorine mainly through drinking water but it is also present in meat, fish, cereals, fruits, vegetables, tea, and in some baby dietary supplements.[7,8] Only 10% of ingested fluoride is excreted in feces and almost 90% is absorbed in the gastrointestinal tract and then transported to all the organs through the blood.[9] The brain, liver, and kidney are the most affected if fluoride intake exceeds the

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recommended level. Fluoride can cross the blood-brain barrier and is able to amass in the brain. It can lead to neurotoxicity by increasing oxidative stress in brain tissue and can result in a lowering of intelligence and an impairment in memory in children in addition to delaying brain development in infants.[10,11] Many studies have reported on the neurotoxicity of F and its ability to damage the central nervous system by excitotoxicity which is the central mechanism of autism.[12,13] Usually cases of autism are found less frequently in rural areas, as compared to cities,[14] as some cities add fluoride to their water supply.[15] Also a meta-analysis of fluoride exposure agreed that the high fluoride exposure can result in many neurodevelopment disorders, like autism, in children.[16]

Bee pollen has been used as dietary supplement and natural medicine throughout the world for centuries, as it is rich in nearly all the nutrients which are required by the human body.[17,18] Bee pollen is well known for its detoxification activity and has the capacity to remove the toxic heavy metals and drugs from the body.[19] However, high doses of bee pollen are not recommended during pregnancy.[20] Some recent findings have shown that bee pollen has the capacity to ameliorate the oxidative stress involved in the etiology of autistic features in rodent models.[21]

Collectively, these discoveries prompted our interest to investigate and compare the neurotoxic effects of fluoride to those induced by orally administered PA in a rodent model of autism.[22] Our study also scrutinized the healing power of bee pollen by exposing prenatal pollen-treated newborns to a chronic dose of sodium fluoride.

MATERIAL AND METHODS

Animals: Twenty-five healthy female Western albino rats (180–200 g) were mated overnight and the detection of spermatozoa was taken as the first day of gestation. The pregnant females were randomly divided into two sets. Set I were allowed to grow with normal conditions and set II was treated with bee pollen (250 mg/kg bw) starting from day 0 of gestation for 30 days (0–23 days of gestation + 7 days postpartum). Three groups (6 neonatal rats each) were organized from set I as follows: Group I received phosphate buffered saline throughout the experiment and served as a control group; group II was treated with oral neurotoxic doses of propionic acid (PA) (250 mg/kg bw) for three days to induce autistic features.[22] Group III were treated with 3 mg/kg bw of sodium fluoride for one week followed by an increase in the dose to 5 mg/kg bw for three more weeks (dose was increased to induce the neurotoxicity). Newborns from set II were further divided into two groups of six pups each as follows: Group IV received phosphate buffered saline and served as a control group to interpret the effect of sodium flouride on a bee pollen-protected group and Group V were treated with the same dose of sodium fluoride as was given to group III. A schematic presentation of the designed groups is shown in Figure 1.

Ethics approval: King Saud University approved all the animal experiments conducted in this study.

Brain tissue collection: Whole brain was collected and washed with cold normal saline and then homogenized in 1:10 weight/volume in double distilled water followed by centrifugation at 3,500 rpm for 15 min. Supernatant was collected and used for various biochemical analyses.

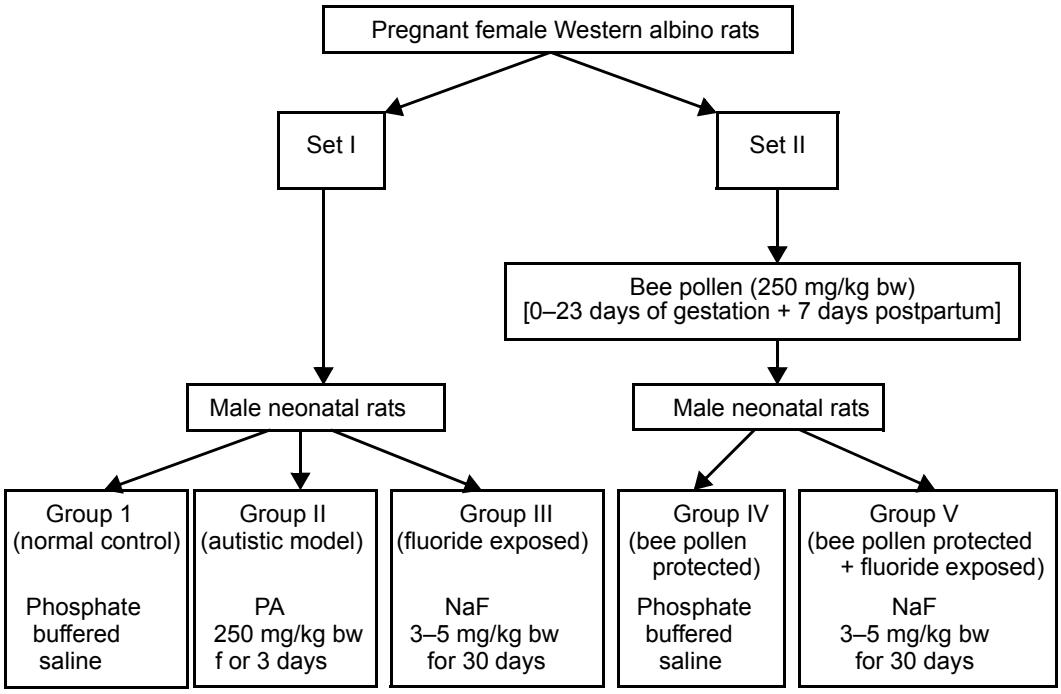

Figure 1. Schematic presentation of the designed experiment.

Biochemical analyses: The method described by Ruiz-Larrea et al.[23] was used to measure lipid oxidation by the formation of thiobarbituric acid reactive substances (TBARS). The method of Beutler et al.[24] was used to measure glutathione by using 5,5'-dithiobis 2-nitrobenzoic acid (DTNB) and sulfhydryl compounds. An assay kit from Biovision, USA using GST-catalyzed reaction between glutathione and 1-chloro-2, 4-dinitrobenzene (CDNB) was used to measure glutathione S-transferase activity.[25] Vitamin C level was estimated according to the method described by Jagota and Dani.[26] The activity of catalase was measured by the method given by Chance.[27] Potassium levels were measured by producing a colloidal suspension by reaction with sodium tetraphenyl boron.[28]

Statistical analysis: Results are presented as mean±standard deviation (SD) using SPSS and evaluated using One-Way ANOVA test between all groups and with Multiple Comparisons Dunnett test with $P \leq 0.05$ considered as significant. Receiver Operating Characteristic (ROC) analysis was performed as a comprehensive way to measure the effectiveness of the studied parameters in terms of either the neurotoxicity of PA and sodium fluoride or the effects of the bee pollen.

RESULTS

Tables 1A and 1B and Figure 2 represent the levels and percentage change relative to the control group for all the measured parameters in brain homogenate of all the five groups under study. Table 1A clearly shows that both PA and sodium fluoride induce a significant increase ($P \leq 0.001$) in lipid peroxides in the rat pup brains as compared to the control group.

Table 1A. Mean±SD of all the measured parameters in the brain homogenates of all the treated groups compared to control

Parameter	Group	Min.	Max.	Mean ±SD	Percent Change	P value*	P value†
Lipid peroxides (µmoles /mL)	Control	0.50	0.57	0.532 ±0.028	100.00		0.001
	PA-treated newborns	0.68	0.80	0.745 ±0.048	140.12	0.001	
	NaF-treated newborns	0.51	0.61	0.578 ±0.038	108.76	0.117	
	Newborn (pollen-treated dams)	0.44	0.57	0.495 ±0.045	93.10	0.274	
	NaF-treated newborns (pollen-treated dams)	0.48	0.52	0.502 ±0.015	94.36	0.441	
Glutathione (µg/mL)	Control	60.00	69.00	64.50 ±3.73	100.00		0.001
	PA-treated newborns	50.00	55.00	52.33 ±2.34	81.14	0.001	
	NaF-treated newborns	50.00	65.00	56.17 ±5.08	87.08	0.004	
	Newborns (pollen-treated dams)	64.00	77.00	71.17 ±5.56	110.34	0.024	
	NaF-treated newborns (pollen-treated dams)	85.00	88.00	86.33 ±1.21	133.85	0.001	
GST (U/mL)	Control	76.50	102.00	89.95 ±9.40	100.00		0.020
	PA-treated newborns	69.50	78.00	74.82 ±3.40	83.18	0.013	
	NaF-treated newborns	66.60	83.80	74.63 ±7.13	82.97	0.012	
	Newborns (pollen-treated dams)	58.80	85.40	78.07 ±9.74	86.79	0.061	
	NaF-treated newborns (pollen-treated dams)	71.70	90.80	81.02 ±9.45	90.07	0.206	

*P value between each group and the control group
†P value between all groups

Table 1B. Mean±SD of all the measured parameters in the brain homogenates of all the treated groups compared to control

Parameter	Group	Min.	Max.	Mean ±SD	Percent Change	P value*	P value†
Vitamin C (µg/mL)	Control	20.00	26.18	22.47	100.00		0.001
	PA-treated newborns	12.00	15.00	13.12	58.36	0.001	
	NaF-treated newborns	16.10	18.33	17.41	77.46	0.001	
	Newborns (pollen-treated dams)	14.00	18.22	16.13 ±1.41	71.78	0.001	
	NaF-treated newborn s (pollen-treated dams)	11.00	19.33	14.97 ±2.98	66.62	0.001	
Catalase (U/dL)	Control	6.30	9.38	7.68 ±1.31	100.00		0.001
	PA-treated newborns	4.40	6.55	5.61 ±0.85	73.07	0.001	
	NaF-treated newborns	5.80	7.00	6.46 ±0.39	84.15	0.067	
	Newborns (pollen-treated dams)	6.70	8.70	7.95 ±0.77	103.58	0.947	
	NaF-treated newborn (pollen-treated dams)	6.00	8.10	7.12 ±0.67	92.73	0.623	
Potassium (mmol/L)	Control	15.83	18.90	17.25 ±1.06	100.00		0.001
	PA-treated newborns	13.47	16.32	14.94 ±1.01	86.64	0.010	
	NaF-treated newborns	16.00	19.50	18.24 ±1.28	105.73	0.440	
	Newborns (pollen-treated dams)	17.71	21.30	19.41 ±1.25	112.51	0.017	
	NaF-treated newborn (pollen-treated dams)	18.33	22.23	19.83 ±1.42	114.98	0.004	

*P value between each group and the control group
†P value between all groups

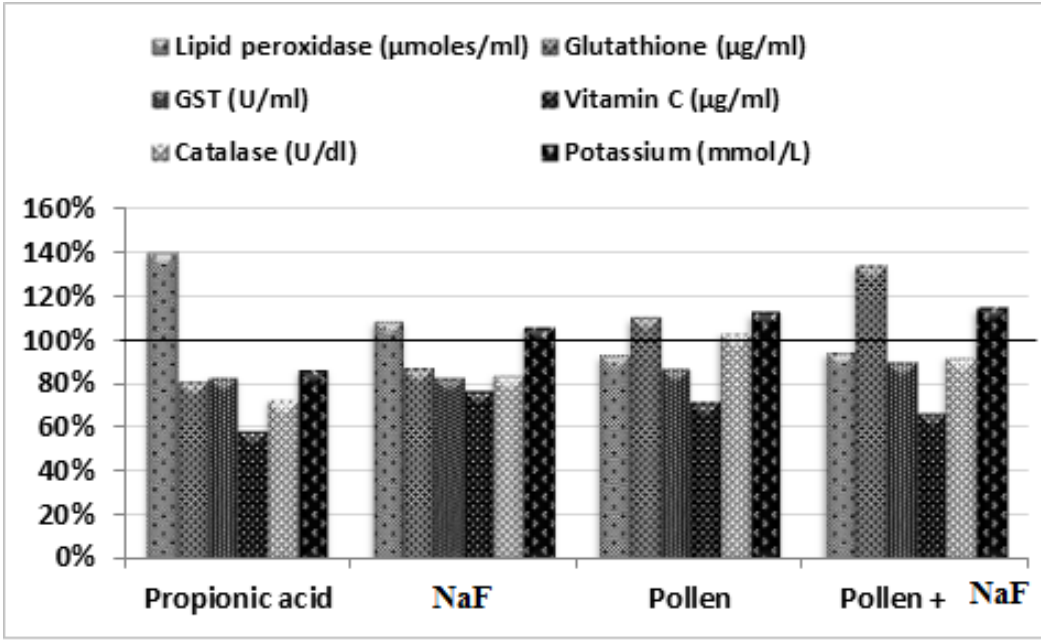

Figure 2. Percentage change of all the parameters in all the groups compared to the control group.

Significant reductions were found in glutathione (P≤0.001), catalase (P≤0.001), vitamin C (P≤0.001), and GST (P≤0.020) in both the PA and NaF groups when compared to the control group. The brain homogenates of the bee pollen-protected neonatal group showed perfection in all the tested parameters. In addition, the sodium fluoride treatment given to the pollen-protected neonatal rats was found to be less toxic which indicates the ameliorative effect of pollen against NaF toxicity. On the other hand, the level of potassium was found almost same as that of the control group in all the treated groups except for the PA group which showed a significant decrease (P≤0.001) (Tables 1A and 1B).

Table 2 presents Pearson's correlations between the measured parameters. There was a significant positive correlation between glutathione~catalase (Pearson's R=0.391), between glutathione~potassium (Pearson's R=0.616), GST~vitamin C (Pearson's R=0.367) between GST~catalase (Pearson's R=0.409), and between catalase~potassium (Pearson's R=0.462). There were also significant negative correlations between lipid peroxidase~glutathione (Pearson's R= –693), lipid peroxidase~vitamin C (Pearson's R= –0.385), lipid peroxidase~catalase (Pearson's R= –0.663), and lipid peroxides~potassium (Pearson's R= –0.757).

Tables 3A, 3B and 3C represent the ROC analysis with the area under the curve (AUC), and the specificity and sensitivity of all the measured parameters.

Table 2. Pearson's correlations between the measured parameters

Parameter	R (Pearson correlation)	Level of significance	Direction of correlation
Lipid peroxidase (µmoles/mL) with glutathione (µg/mL)	-0.693†	0.001	Negative
Lipid peroxidase (µmoles/mL) with vitamin C (µg/mL)	-0.385*	0.036	Negative
Lipid peroxidase (µmoles/mL) with catalase (U/dL)	-0.663†	0.001	Negative
Lipid peroxides (µmoles/mL) with potassium (mmol/L)	-0.757†	0.001	Negative
Glutathione (µg/mL) with catalase (U/dL)	0.391*	0.033	Positive
Glutathione (µg/mL) with potassium (mmol/L)	0.616†	0.001	Positive
GST (U/mL) with vitamin C (µg/mL)	0.367*	0.046	Positive
GST (U/mL) with catalase (U/dL)	0.409*	0.025	Positive
Catalase (U/dL) with potassium (mmol/L)	0.462*	0.010	Positive

*Correlation is significant at the 0.05 level.
†Correlation is significant at the 0.01 level.

Table 3A. ROC-Curve of parameters in all groups

Parameter	Group	Area under the curve	Cut-off value	Sensitivity (%)	Specificity (%)	P value
Lipid peroxides (µmoles/mL)	PA-treated newborns	1.000	0.625	100.0 %	100.0 %	0.004
	NaF-treated newborns	0.833	0.580	66.7 %	100.0 %	0.055
	Newborns (pollen-treated dams)	0.778	0.495	66.7 %	100.0 %	0.109
	NaF-treated newborn (pollen-treated dams)	0.833	0.515	83.3 %	66.7 %	0.055

Table 3B. ROC-Curve of parameters in all groups

Parameter	Group	Area under the curve	Cut-off value	Sensitivity (%)	Specificity (%)	P value
Glutathione (µg/mL)	PA-treated newborns	1.000	57.500	100.0 %	100.0 %	0.004
	NaF-treated newborns	0.917	58.500	83.3 %	100.0 %	0.016
	Newborns (pollen-treated dams)	0.833	63.500	100.0 %	50.0 %	0.055
	NaF-treated newborns (pollen-treated dams)	1.000	77.000	100.0 %	100.0 %	0.004
GST (U/mL)	PA-treated newborns	0.917	81.150	100.0 %	83.3 %	0.016
	NaF-treated newborns	0.917	84.050	100.0 %	83.3 %	0.016
	Newborns (pollen-treated dams)	0.833	86.150	100.0 %	66.7 %	0.055
	NaF-treated newborns (pollen-treated dams)	0.750	91.200	100.0 %	50.0 %	0.150
Vitamin C (µg/mL)	PA-treated newborns	1.000	17.500	100.0 %	100.0 %	0.004
	NaF-treated newborns	1.000	19.165	100.0 %	100.0 %	0.004
	Newborns (pollen-treated dams)	1.000	19.110	100.0 %	100.0 %	0.004
	NaF-treated newborns (pollen-treated dams)	1.000	19.665	100.0 %	100.0 %	0.004

Table 3C. ROC-Curve of parameters in all groups

Parameter	Group	Area under the curve	Cut-off value	Sensitivity (%)	Specificity (%)	P value
Catalase (U/dL)	PA-treated newborns	0.889	6.825	100.0 %	66.7 %	0.025
	NaF-treated newborns	0.722	7.050	100.0 %	66.7 %	0.200
	Newborns (pollen-treated dams)	0.556	7.300	83.3 %	50.0 %	0.749
	NaF-treated newborns (pollen-treated dams)	0.597	7.695	83.3 %	50.0 %	0.575
Potassium (mmol/L)	PA-treated newborns	0.972	16.490	100.0 %	83.3 %	0.006
	NaF-treated newborns	0.750	17.335	83.3 %	66.7 %	0.150
	Newborns (pollen-treated dams)	0.917	17.415	100.0 %	66.7 %	0.016
	NaF-treated newborn (pollen-treated dams)	0.944	18.120	100.0 %	83.3 %	0.010

DISCUSSION

We evaluated the role of prenatal exposure to bee pollen in preventing the neurochemical alterations in brain caused by NaF (Table 2). Our findings revealed that prenatal bee pollen exposure ameliorates some of the neurochemical changes that are induced by NaF. Even low doses of fluoride can affect the function of the developing brain as compared to the mature brain.[29] The current results discovered a significant increase in lipid peroxide in addition to notable decreases of GSH, GST, vitamin C, and catalase in the brain tissue of the NaF-treated neonates and the trend was almost the same as that found in the PA model of autism. Hence NaF treatment can be connected with the development of the oxidative stress related to autism.[22] These findings are coherent with the report that fluoridated water may act as an environmental risk factor for many neurodevelopmental disorders like Attention-Deficit Hyperactivity Disorder (ADHD) and autism in children.[14-16] Recently, it has been found that prolonged exposure of fluoride is strongly associated with inflammatory bowel disease which is also reported in autistic children and adults.[30,31] Likewise, the ability of vitamin D to improve the core symptoms of both

autism and fluoride toxicity,[32,33] suggests a relationship between fluoride intoxication and vitamin D deficiency in autistic patients.[34] These studies can be related to the outcomes shown in the PA- and NaF-treated groups in Tables 1A and 1B.

Potassium was significantly lower in the PA-intoxicated pups, while NaF did not induce a significant alteration in its level. Large ion imbalances in brain tissue, mostly with potassium, has been found in many neurodegenerative diseases.[35] In addition, a low level of potassium has also been reported recently in the PA-induced autistic model.[36] Acute fluoride toxicity can also inhibit membrane sodium potassium ATPase resulting in an increased level of potassium in the blood. However the chronic dose of NaF used in the present study resulted in only a slight alteration in potassium level.[37] All of the prenatal pollen-treated groups showed a remarkable increase in the potassium level in the brain tissue ($P<0.001$) and this strong effect can be attributed to the high concentration of potassium in bee pollen.[38]

In the present study, treatment with bee pollen alone did not show any adverse effect on the markers of oxidative stress in the prenatal pollen-treated pups' brains. However, it significantly attenuated the NaF-induced oxidative damage (Tables 1A and 1B). Bee pollen has good nutritional value and is rich in polyphenol which acts as a potent antioxidant. Pollen is very rich in rutin which is a flavone glycoside with the capacity to suppress free radical generation.[39,40] Recently, El-Ansary et al.[21] discovered that bee pollen can ameliorate the oxidative stress in PA-intoxicated rats which lends support to our findings that NaF- and PA-intoxicated rat pups show almost the same trend of toxicity (Tables 1A and 1B, and Figure 1).

The positive and negative correlations shown in Table 2 indicate that oxidative stress is an etiological factor in the neurotoxicity of PA and NaF. Moreover, AUC, and the specificity and sensitivity values listed in Tables 3A, 3B, and 3C demonstrate the possibility that all the parameters used in the study can be used as markers of PA- and NaF-induced neurotoxicity.

CONCLUSIONS

As an environmental toxin, fluoride displays similar effects on the brain to PA acid and hence it may be considered as a possible etiological factor in the occurrence of developmental neurotoxicity and of autism. Also, the prenatal use of bee pollen can reduce the oxidative stress in the brains of NaF-intoxicated newborns. However further investigations are needed to confirm the role of fluoride as a risk factor for autism.

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