INTRODUCTION

Increased prevalence of neurodevelopmental toxicity implicates the importance of environmental factors in influencing such adverse outcomes (1). Determinants of during fetal and early life development serve as predictors for later health underlying the importance of maternal diet (2) and even gut microbiota (3, 4). Acrylamide (ACR), a chemical known to be formed during heating of food at high temperature is widely recognized as neurotoxic primarily involving oxidative stress, mitochondrial dysfunction and protein adduct formation (5-7). It is proposed that the average dietary intake of ACR in adults range 0.3 – 0.6 µg/kg bw/day (8) with children and adolescents are at greater risk owing to higher consumption of foods such as potato chips, fried potato, biscuits. Importantly, the sensitive sub-populations have estimated mean ACR exposure of 1.80 µg/kg bw/day and young children are at higher risk of developmental neurotoxic actions beginning prenatally with increased risk of obesity (9, 10). ACR's placental and breast milk transfer has shown to produce developmental deficits and motor impairment (11). Studies examining the developmental ACR exposures have shown to have a significant effect on behaviors of offspring (12). Further, maternal ACR treatment was shown to enhance the levels of reelin-immunoreactive GABAergic interneurons (13) and perinatal reduction in liver protein thiol oxidation (14).

Early-life influence has profound effect on neurodevelopment that persists into adolescence. Emerging data have provided a greater understanding of the role of gut bacteria in development and function of the brain. Commensal microbiota has been shown to modulate a variety of behaviors, including affective (depressive and anxiety-like), communicative, sensorimotor, motor and cognition (3, 15-16). Studies in germ-free mice have demonstrated neurodevelopmental deficits including alterations in monoaminergic systems and behavioral abnormalities (17). Prebiotics, often oligosaccharides are naturally occurring high-fiber foods upon consumption promotes proliferation of intestinal carbohydrate-utilizing bacteria. Bacterial fermentation products such as the short chain fatty acids (SCFAs), generated by selective bacteria possess wide array of functions on the host (18). Several studies in humans and animals have described prebiotics to enhance beneficial gut microbes (19, 20). Given the presence of diverse gut bacterial ecosystem, it is likely that promoting resident bacterial growth by combined prebiotic ingestion might offer an important strategy to promote well-being.

Prebiotic intake during pregnancy profoundly influence many aspects of health status, including resistance to allergy,
modulation of immune system and promotes tolerance (21-23). Administration of prebiotics during juvenile period enhances glutamate receptors (GluN1) and N-methyl-D-aspartate receptor (NMDAR) subunit expression, and improves locomotor behavior associated with increase in neuronal activity marker cFOS (24). Previously, we have shown that gestational oligosaccharide supplementation proliferates maternal beneficial gut bacteria and protects fetal brain against neurotoxicity (25, 26). Recent studies with a specific mixture of prebiotics has shown to possess potent behavior modulating capability (27), prevent oxidative dysfunctions (28), and amyloid pathology (29).

The long term, lower intake of dietary ACR during pregnancy imposes a serious risk considering the adverse effects on developing offspring brain. The perinatal development undergoes marked neurodevelopment and hence this period is critical for the establishment of indigenous microbiota of the mother to subsequently impact offspring development. In this context, mitigating neurotoxic consequences with consumption of specific foods during pregnancy associated with higher ACR exposure, assumes importance. Accordingly, in the present study we assessed the protective effect of a combination supplementation of fructo- and xylo-oligosaccharides (FOS + XOS) during perinatal period to mitigate ACR-induced oxidative stress and neurotoxicity in mothers and young pups.

MATERIALS AND METHODS

Chemicals

Acrylamide (electrophoresis grade; 99% purity), 2-thiobarbituric acid, 1,1,3,3-tetramethoxypropane, 2',7'-dichlorofluorescein diacetate, N,N,N',N'-tetramethyl ethylenediamine, acetylthiocholine iodide were obtained from Sigma-Aldrich (St. Louis, MO, USA). Fructo-oligosaccharides (90% purity) and xylo-oligosaccharides (> 95% xylose residue) were procured from Cascade Analytical Reagents and Biochemicals (Corvallis, OR, USA). All other reagents used were of analytical or high-performance liquid chromatography (HPLC) grade.

Group assignment, treatment and tissue collection

All animal procedures followed the guidelines established by the Committee for the Purpose of Control and Supervision of Experiments on Animals (49/1999/CPCSEA), Ministry of Environment, Forests and Climate Change, Government of India, India and were approved by CSIR-CFTRI Institutional Animal Ethics Committee.

Male and virgin female adult Wistar rats were randomly drawn from our institute's animal house facility and housed in temperature and humidity-controlled room. After acclimatized for 1 week, two females were mated with one male in each cage. Females were examined daily for the presence of spermatozooids in the vaginal smears for positive evidence of mating, designated as gestation day (GD) 0 and were separated. The mating period did not exceed 5 days. Food and water was provided ad libitum at all times.

Sperm-positive rats were randomly assigned to four treatment groups as follows; Group I rats served as untreated controls and received regular drinking water. Group II rats received FOS + XOS (3 g/kg bw/day, per os; beginning on GD 0 and continued until weaning at PND 21); Group III rats received ACR in drinking water (100 ppm, GD 6-PND 21), and Group IV rats received ACR in drinking water (100 ppm, GD 6-PND 21) along with FOS + XOS (3 g/kg bw/day, per os; GD 0-PND 21). No prebiotic treatment was made on the day of parturition and dams were left undisturbed. Offspring were nursed with their dams until weaning at PND 21.

Growth and feed/water consumption of dams

Dam measures including gestational (GD 6-21) and lactational (post parturitional day 1 – 21) body weights, food consumption, and water intake were measured daily. Water consumption for each rat was measured daily by measuring the bottles empty, full (200 mL) and then after every day to assess volume intake. Dams were not treated on the day of parturition.

Litter measures

All dams were allowed to deliver naturally and rear their pups (PND 1-21). During the days of expected parturition, the dams were observed daily for completion of parturition. On the day of parturition (designated as PND 0), pups were sexed and litter endpoints were collected such as total pups/litter, live pups/litter and live sex ratio. Gestation duration was calculated using the date delivery was first observed. On the day after parturition (i.e., PND 1), the total number of pups/ litter were counted, weighed, sexed by relative anogenital distance and litters were culled to achieve a litter size of 8 pups (with 4/sex or 5:3 sex ratios/litter) or 7 pups (4:3 sex ratio/litter). Where necessary, immediately cross-fostering of pups within treatment groups was done to create targeted litter size and sex distribution.

Developmental landmarks

Beginning on PND 1, all retained pups (including foster pups) of all treatment groups were observed daily for the occurrence of developmental landmarks viz., fur development (the appearance of fur sufficient to cover the skin on the dorsal surface of the pups), eye-opening (break in the membrane connecting upper and lower eyelids) and pinnae detachment (ear flap to completely detach from the head).

Behavioral assessments

All offspring were subjected to behavioral assessments on PND 21. Open field test was used to assess the exploratory behavior (30). Male pups were placed individually in the corner of the arena (illuminated by ambient fluorescent lights) and allowed free exploration of the field for 10 min during which their movements were recorded. Behavioral indices of exploration such as the frequency of entries into the center zone and total time spent in a pre-defined center zone of the open field.

Elevated plus maze test was used to assess anxiety-like behavior as previously described (25). Briefly, male pups were placed at the junction of the open and closed arms, facing the open arm and permitted free exploration for 5 min. The offspring behaviour was video monitored during which their entries onto the open and closed arms and the time spent in each arm were manually recorded following a four-paw criterion. Each arm was cleaned with 10% ethanol between each trials.

Sample preparation

On PND 22, mothers and male offspring were sacrificed by necropsy. Cortex and cerebellum were homogenized (10%) in ice-cold Tris-Sucrose buffer (0.25 M, pH 7.4) and centrifuged at 1000 × g for 10 min at 4°C to obtain the nuclear pellet. Differential centrifugation (700 and 4500 × g, 10 min, 4°C) was employed to isolate cytosol and mitochondria from different
brain regions (31). The crude mitochondrial pellet was washed and re-suspended using HEPES buffer (10 mM HEPES, 0.1 mM EDTA, 200 mM mannitol and 70 mM sucrose; pH 7.4).

**Measurement of reactive oxygen species**

The cytosolic total reactive oxygen species (ROS) was estimated using fluorescent probe dihydro dichlorofluorescein diacetate (H2DCFH-DA). H2DCFH-DA is actively cleaved by esterases to form 2',7'-dichlorodihydrofluorescein (DCHF) and then oxidized by free radicals to the fluorescent 2',7'-dichlorofluorescein (DCF) (32). Briefly, an aliquot (100 µg protein equivalent) was incubated in Locke’s buffer (pH 7.4; 154 mM NaCl, 5.6 mM KCl, 3.6 mM NaHCO3, 5 mM HEPES, 2 mM CaCl2 and 10 mM glucose) containing H2DCFH-DA (5 µM) for 30 min at room temperature. DCF was measured with excitation and emission wavelengths of 480 nm and 530 nm respectively. The ROS generation was calculated from a DCF standard curve and expressed as pmol DCF/min/mg protein.

**Measurement of lipid peroxidation**

Lipid peroxidation (LPO) was measured by assessing the formation of thiobarbituric acid reactive substances (TBARS) as described previously (33). Briefly, the cytosolic samples (500 µg protein) were mixed with 20% of acetic acid (pH 3.5), 0.8% thiobarbituric acid and 8% sodium dodecyl sulfate (SDS). The mixture was heated in a boiling water bath for 45 min. The colored adduct formed was measured at 532 nm. Results were expresses as malondialdehyde (MDA) equivalents/mg protein using 1,1,3,3-tetramethoxypropane as the standard.

**Measurement of hydroperoxides**

Hydroperoxides (HP) levels were determined as previously described (34). Briefly, tissue samples (100 µg protein) was added to ferrous ion oxidation xylene orange (FOX) reagent (100 mM xylene orange; 250 mM ammonium ferrous sulphate; 100 mM sorbitol; 25 mM H2SO4) and incubated for 30 min at room temperature (protected from light). The formation of ferric ions was detected by measuring the coloured complex at 560 nm (ε = 2.2 X 104 M-1 cm-1).

**Estimation of protein carbonyls**

Protein oxidative damage was estimated by determination of protein carbonyl groups (PC) based on a reaction with dinitrophenylhydrazine as described previously (35). Briefly, proteins were precipitated by addition of 20% trichloroacetic acid. The pellet washed twice with acetone and dissolved in 2 mM acetic acid (EDTA). The mixture was heated in a boiling water bath for 45 min. The absorbance was monitored at 532 nm for 3 min. The enzyme activity was expressed as nmol substrate reduced/min/mg protein (ε = 43.6 mM-1 cm-1).

**Activity of acetylcholinesterase and butyrylcholinesterase**

The activities of acetylcholinesterase (ACHE) and butyrylcholinesterase (ButChE) was determined according to the method as described previously (40). The reaction mixture consisted of an aliquot of cytosol (100 µg protein) in 0.1 M phosphate buffer (pH 7.0) containing 10 mM EDTA, 0.2 mM NADPH. The enzyme activity was expressed as nmol substrate hydrolyzed/min/mg protein (ε = 13.6 mM-1 cm-1).

**Mitochondrial functions**

The activity of NADH-Cyt C reductase (Complex I-III) was estimated by adding an aliquot of mitochondrial sample (equivalent to 10 µg protein) to 0.1 M phosphate buffer (pH 7.4) containing 0.2 mm NADH and 1 mM KCN. The reaction was initiated by addition of 0.1 mM cytochrome C and decrease in absorbance was monitored for 3 min at 550 nm. The activity was expressed as nmol cytochrome C reduced/min/mg protein (ε = 19.6 mM-1 cm-1) (41).

MTT (3-[4,5-dimethylthiazole-2-yl]-2,5-diphenyltetrazolium bromide) reduction was determined following a previously described procedure (42). Briefly, mitochondrial sample (equivalent to 10 µg protein) was added to mannitol-sucrose-HEPES buffer containing 20 mM sodium succinate, 1 mM NADH (pH 7.4). To this mixture, MTT (5 mg/mL) was added and incubated at 37°C for 1 hour. The formazan crystals formed were dissolved in SDS-DMF buffer (mixture of 45% DMF and 10% SDS in distilled water, pH 4.7) and the absorbance was read at 570 nm.

The activity of citrate synthase (CS) was determined by monitoring the oxidation of DTNB (43). Mitochondrial protein (50 µg) was added to 0.1 M Tris-HCl buffer (pH 8.1, 0.1% Triton X-100) containing 0.2 mM DTNB and 0.1 mM acetyl CoA. The reaction was started by the addition of 10 mM oxaloacetate and absorbance was monitored at 412 nm for 3 min and the activity was calculated from the standard curve and expressed as µg GSH/mg protein.

**Activities of antioxidant/detoxifying enzymes**

Superoxide dismutase (SOD) activity was determined by monitoring the inhibition of quercetin auto-oxidation. Mixture consisted of sample (3 – 5 µg protein) with 0.016 M sodium phosphate buffer (pH 7.8), 8 mM N,N,N,N tetramethyl ethylenediamine (TEMED) and 0.08 mM ethylene diamine tetra acetic acid (EDTA). The reaction was started by adding quercetin (1.5 mg dissolved in 1 mL dimethyl formamide). Auto-oxidation was monitored for 3 min at 406 nm, expressed as the amount of protein required to inhibit 50% of quercetin auto-oxidation (37).

Catalase (CAT) activity was determined according to a previously described method (38). Sample (equivalent to 10 µg protein) was added to mixture containing 10 mM H2O2, 0.1 mM sodium-phosphate buffer (pH 7.0) to initiate the reaction. The decrease in H2O2 was monitored for 3 min at 240 nm and expressed as nmol H2O2 decomposed/min/mg protein (ε = 43.6 mM-1 cm-1).

**Measurement of reduced glutathione**

The reduced glutathione (GSH) content was estimated based on a fluorimetric method as described previously (36). The cytosolic samples were mixed with 0.1 M formic acid and centrifuged at 10,000 x g for 10 min. The supernatant was transferred to tubes containing buffered formaldehyde [37% formalin: 0.1 M NaH2PO4, 1:4 (v/v)] with 5 mM EDTA (pH 8.0) followed by addition of O-phthalaldehyde (1 mg/mL in methanol). The mixture was incubated for 45 min at room temperature and the fluorescence was measured at 345/425 (excitation/emission) wavelengths. The concentration of GSH was calculated from the standard curve and expressed as µg GSH/mg protein.
was expressed as nmol thiol oxidized/min/mg protein (ε = 13.6 mM⁻¹ cm⁻¹).

**Dopamine estimation by high-performance liquid chromatography analysis**

High-performance liquid chromatography (HPLC) was performed as previously reported (44) to determine the dopamine (DA) levels. Briefly, an aliquot of cytosol was taken in 5% TCA and centrifuged. Supernatants were then injected at a flow rate of 1 ml/min into HPLC system with UV detector (SPD-10 A vp). Each liter of mobile phase used for separation comprised 0.2% aqueous trifluoroacetic acid and methanol (70:30, v/v) and HPLC-grade water in the cytosol were analyzed using HPLC system with UV visible detector. DA levels were calculated using a standard curve and expressed as µg DA/g tissue.

**Determination of protein**

Protein concentrations were determined by incubating an aliquot of the sample (cytosolic and mitochondrial fraction) with Folin-Ciocalteau’s phenol in an alkaline medium (30 min) and measuring the OD at 750 nm (45). The amount of protein was quantified using bovine serum albumin as a standard.

**Statistical analysis**

All results are represented as mean ± standard error (SE). All analyses were conducted using SPSS (version 17.0) statistical software package. P values less than 0.05 were considered as statistically significant. Dam gestational body weights were analyzed separately from lactational body weights. Gestational or postparturitional body weights were analyzed by multivariate analysis of variance (repeated-measures) ANOVA. Preweaning body weights of both sex offspring were similarly analyzed. Significant interactions were analyzed using Tukey’s comparisons to compare with control (CTR) or acrylamide (ACR) group.

The gestation duration included all littered dams regardless of the day (early and late). Since litters were culled to achieve litter size, PND 1 birth weights which included all pups were analyzed separately from later PND’s body weights which included pups that were retained.

Offspring birth weights were averaged by sex (separately for males and females) and body weights (PND 1-21) of pups were analyzed for each sex separately using one way ANOVA followed by Tukey test. Similarly, all developmental landmark data were analyzed by one-way ANOVA followed by a post hoc Tukey test.

**RESULTS**

**Dam measures: gestation/lactational body weight and feed/water intake**

To determine the effects of ACR exposure and combined prebiotic supplementation on body weight, we assessed gestational and lactational weight gain. Repeated measures ANOVA showed significant effect on body weight with results showing lower gestational weight gain (GD 21) among ACR dams (Fig. 1A). Similarly, postparturitional (day 21) body weight among ACR per se group was significantly reduced (Fig. 1B). However, combined prebiotic supplementation had no effect on gestational or postparturitional body weight among rats provided ACR.

**Litter endpoints**

Litter results are shown in Table 1. No treatment effects were noticed on mean gestation length. Similarly, the total number of pups/litter, the number of live pups/litter, sex ratio (number of males/total live pups) were highly comparable among treated groups.

**Effect on offspring weight at birth**

Analysis of birth weight (PND 1) of male and female pups (pre-culling) indicated that pups of the ACR group weighed significantly less (17.3 and 22.2% respectively). However, ACR induced decrease in body weight was not appreciably influenced with combined FOS + XOS supplementation (Table 2).

**Offspring (PND 1-21) body weight and developmental landmarks**

Repeated measures ANOVA showed a significant reduction in weight gain among male (PND 17 and 21) and female (PND 13, 17 and 21) offspring exposed perinatally to ACR (Fig. 2A and 2B). However, lower weight gain with ACR exposure...
remained unaffected with combined FOS + XOS supplementation through the entire weaning period. Further, ACR exposure slightly delayed developmental landmarks viz., age at body fur appearance, eye-opening and pinna detachment among both the sexes with lactational ACR exposure (data not shown). Combined FOS + XOS supplementation did not affect these morphological developments.

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**Table 1.** Effect of combination supplementation of fructo- and xylo-oligosaccharides (FOS + XOS) on gestation duration, litter outcomes and PND 1 measures among rats provided acrylamide (ACR).

<table>
<thead>
<tr>
<th>Group</th>
<th>Gestation duration (days)</th>
<th>Total pups/litter</th>
<th>% live pups/litter</th>
<th>Males/litter</th>
<th>Females/litter</th>
<th>Live sex ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>CTR</td>
<td>22.0 ± 0.0</td>
<td>10.5 ± 0.9</td>
<td>95.2 ± 2.8</td>
<td>5.2 ± 0.9</td>
<td>4.5 ± 0.5</td>
<td>0.47 ± 0.03</td>
</tr>
<tr>
<td>FOS + XOS</td>
<td>22.1 ± 0.1</td>
<td>8.5 ± 0.6</td>
<td>97.5 ± 2.5</td>
<td>3.8 ± 0.5</td>
<td>4.5 ± 0.6</td>
<td>0.63 ± 0.10</td>
</tr>
<tr>
<td>ACR</td>
<td>22.1 ± 0.1</td>
<td>8.3 ± 0.6</td>
<td>94.4 ± 3.3</td>
<td>4.3 ± 0.3</td>
<td>3.5 ± 0.3</td>
<td>0.49 ± 0.04</td>
</tr>
<tr>
<td>FOS + XOS + ACR</td>
<td>22.0 ± 0.0</td>
<td>8.8 ± 0.6</td>
<td>95.0 ± 5.0</td>
<td>4.0 ± 0.8</td>
<td>4.3 ± 0.8</td>
<td>0.43 ± 0.05</td>
</tr>
</tbody>
</table>

FOS + XOS (3 g/kg bw/day, oral, GD 0-PND 21); ACR (100 ppm in drinking water, GD 6-PND 21). Values are expressed as mean ± SE.

Table 1. Effect of combination supplementation of fructo- and xylo-oligosaccharides (FOS + XOS) on gestation duration, litter outcomes and PND 1 measures among rats provided acrylamide (ACR).

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**Table 2.** Postnatal day 1 (PND 1) measures among rats provided a combination supplementation of fructo- and xylo-oligosaccharides (FOS + XOS) and exposed to acrylamide (ACR).

<table>
<thead>
<tr>
<th>Group</th>
<th>PND 1 male body weight (g)</th>
<th>PND 1 female body weight (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CTR</td>
<td>5.40 ± 0.11</td>
<td>4.96 ± 0.16</td>
</tr>
<tr>
<td>FOS + XOS</td>
<td>5.60 ± 0.20</td>
<td>5.01 ± 0.17</td>
</tr>
<tr>
<td>ACR</td>
<td>4.45 ± 0.10                *</td>
<td>4.50 ± 0.09                 *</td>
</tr>
<tr>
<td>FOS + XOS + ACR</td>
<td>3.90 ± 0.08                *</td>
<td>4.39 ± 0.07</td>
</tr>
</tbody>
</table>

FOS + XOS (3 g/kg bw/day, oral, GD 0-PND 21); ACR (100 ppm in drinking water, GD 6-PND 21). Values are expressed as mean ± SE. Data analyzed by one way ANOVA followed by post hoc Tukey test (P < 0.05). *Significantly different than same-sex CTR; *significantly different than same-sex ACR.

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**Offspring behaviors**

**Effect of combined prebiotics on exploratory behavior and elevated plus maze performance.**

ACR exposed male offspring entered the center zone less frequently and spent the significantly lesser duration of time in the center than did their control counterparts (Table 3). However, among animals receiving combined FOS + XOS supplementation did not appreciably influence the exploratory endpoints of the novel environment. Pups exposed to ACR lactationally had fewer numbers of entries into the open and closed arms of the maze suggesting the anxiogenic response. This effect was also reflected in terms of reduced time spent in the open/ closed arm. However, there were no significant differences into the arm entries and time spent among rats receiving combined FOS + XOS supplementation.

**Effect of combined prebiotics on accelerating rotarod task**

Analysis of motor function showed no significant difference in the latency to fall off the rotating rod among rats provided ACR and combined FOS + XOS supplementation (Table 3).

**Modulatory effect of combined oligosaccharides on oxidative parameters**

**Maternal brain regions:** as shown in Table 4, significant elevation of ROS levels in the in maternal cortex [cytosol (59%), and mitochondria (32%)] with ACR exposure was protected by combined oligosaccharides. Similarly, combined FOS + XOS
supplementation protected against elevated lipid peroxidation in the cytosol (41%). Further, ACR exposure significantly increased the HP levels in the cytosol (46%) was completely protected with FOS + XOS. However, no significant changes were evident in MDA and HP levels in mitochondria. In cerebellum while ROS levels in the cytosol and mitochondria were elevated, with ACR exposure, the levels were significantly diminished among rats given combined prebiotics. Likewise, elevated MDA and HP levels were also prevented with FOS + XOS administration.

**Offspring brain regions:** the elevated ROS level induced by lactational ACR exposure in the cortex (22%) and cerebellum (28%) was alleviated by prebiotics supplementation (Fig. 3A and 3B). Further, ACR exposure did not appreciably affect MDA levels in both the brain regions (Fig. 3C and 3D). Similarly, HP

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**Table 3.** Effect of a combination supplement of fructo-oligosaccharides and xylo-oligosaccharides (FOS + XOS) on male offspring behavior among acrylamide (ACR) exposed rats.

<table>
<thead>
<tr>
<th>Endpoint</th>
<th>CTR</th>
<th>FOS + XOS</th>
<th>ACR</th>
<th>FOS + XOS + ACR</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Open field</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Entries into center</td>
<td>17.5 ± 2.3</td>
<td>17.3 ± 3.5</td>
<td>9.1 ± 3.5</td>
<td>10.9 ± 2.4</td>
</tr>
<tr>
<td>Time spent in center (s)</td>
<td>60.4 ± 4.1</td>
<td>52.8 ± 7.5</td>
<td>55.3 ± 6.6</td>
<td>40.5 ± 8.1</td>
</tr>
<tr>
<td><strong>Elevated plus maze</strong></td>
<td></td>
<td></td>
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<tr>
<td>Open arm entries</td>
<td>5.5 ± 0.4</td>
<td>5.0 ± 0.7</td>
<td>2.0 ± 0.2</td>
<td>2.2 ± 0.2</td>
</tr>
<tr>
<td>Open arm duration (s)</td>
<td>18.5 ± 1.4</td>
<td>22.2 ± 1.5</td>
<td>7.8 ± 0.5</td>
<td>6.5 ± 0.8</td>
</tr>
<tr>
<td>Closed arm entries</td>
<td>12.2 ± 0.8</td>
<td>13.3 ± 0.5</td>
<td>5.6 ± 0.3</td>
<td>4.5 ± 0.5</td>
</tr>
<tr>
<td>Closed arm duration (s)</td>
<td>357.2 ± 11.3</td>
<td>382.5 ± 14.6</td>
<td>518.2 ± 8.1</td>
<td>485.8 ± 15.2</td>
</tr>
</tbody>
</table>

**Rotarod**

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<table>
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<th></th>
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<tbody>
<tr>
<td>Latency to fall (s)</td>
<td>223.5 ± 10.5</td>
<td>219.7 ± 5.5</td>
<td>225.4 ± 9.4</td>
<td>228.6 ± 11.0</td>
</tr>
</tbody>
</table>

FOS + XOS (3 g/kg bw/day, oral, GD 0-PND 21); ACR (100 ppm in drinking water, GD 6-PND 21). Values are expressed as mean ± SE. Data analyzed by one way ANOVA followed by post hoc Tukey test. *Significant against CTR; #significant against ACR (P < 0.05).

**Table 4.** Effect of a combination supplement of fructo-oligosaccharides and xylo-oligosaccharides (FOS + XOS) on oxidative markers in cytosol/ mitochondria of maternal brain regions among acrylamide (ACR) exposed rats.

<table>
<thead>
<tr>
<th>Brain region/parameter</th>
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<tbody>
<tr>
<td><strong>Cortex</strong></td>
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<tr>
<td><strong>Cytosol</strong></td>
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<tr>
<td>CTR</td>
<td>8.32 ± 0.1</td>
<td>6.73 ± 0.4</td>
<td>27.6 ± 1.3</td>
</tr>
<tr>
<td>FOS + XOS</td>
<td>5.05 ± 0.3*</td>
<td>4.62 ± 0.2*</td>
<td>25.8 ± 0.8</td>
</tr>
<tr>
<td>ACR</td>
<td>13.2 ± 0.9*</td>
<td>9.50 ± 0.5*</td>
<td>40.2 ± 1.2*</td>
</tr>
<tr>
<td>FOS + XOS + ACR</td>
<td>7.85 ± 0.3*</td>
<td>7.12 ± 0.2*</td>
<td>27.3 ± 1.6*</td>
</tr>
<tr>
<td><strong>Mitochondria</strong></td>
<td></td>
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</tr>
<tr>
<td>CTR</td>
<td>3.48 ± 0.14</td>
<td>1.86 ± 0.10</td>
<td>12.5 ± 2.0</td>
</tr>
<tr>
<td>FOS + XOS</td>
<td>3.35 ± 0.22</td>
<td>1.80 ± 0.12</td>
<td>10.3 ± 0.9</td>
</tr>
<tr>
<td>ACR</td>
<td>4.60 ± 0.20*</td>
<td>1.98 ± 0.10</td>
<td>14.9 ± 1.7</td>
</tr>
<tr>
<td>FOS + XOS + ACR</td>
<td>3.12 ± 0.18*</td>
<td>1.74 ± 0.10</td>
<td>9.2 ± 0.4*</td>
</tr>
<tr>
<td><strong>Cerebellum</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Cytosol</strong></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>CTR</td>
<td>11.1 ± 0.8</td>
<td>6.65 ± 0.3</td>
<td>24.1 ± 1.8</td>
</tr>
<tr>
<td>FOS + XOS</td>
<td>9.60 ± 0.7</td>
<td>4.58 ± 0.2*</td>
<td>20.3 ± 2.2</td>
</tr>
<tr>
<td>ACR</td>
<td>18.3 ± 1.0*</td>
<td>9.3 ± 0.3*</td>
<td>33.5 ± 0.8*</td>
</tr>
<tr>
<td>FOS + XOS + ACR</td>
<td>13.5 ± 0.4*</td>
<td>7.43 ± 0.2*</td>
<td>25.8 ± 1.1*</td>
</tr>
<tr>
<td><strong>Mitochondria</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CTR</td>
<td>2.96 ± 0.1</td>
<td>1.55 ± 0.2</td>
<td>14.7 ± 0.8</td>
</tr>
<tr>
<td>FOS + XOS</td>
<td>2.50 ± 0.2</td>
<td>1.23 ± 0.2</td>
<td>13.0 ± 1.0</td>
</tr>
<tr>
<td>ACR</td>
<td>3.68 ± 0.3*</td>
<td>1.78 ± 0.1</td>
<td>17.5 ± 0.7</td>
</tr>
<tr>
<td>FOS + XOS + ACR</td>
<td>3.40 ± 0.1*</td>
<td>1.60 ± 0.1</td>
<td>13.2 ± 0.5*</td>
</tr>
</tbody>
</table>

FOS + XOS (3 g/kg bw/day, oral, GD 0-PND 21); ACR (100 ppm in drinking water, GD 6-PND 21). Values are expressed as mean ± SE. Data analyzed by one way ANOVA followed by post hoc Tukey test. *Significant against CTR; *significant against ACR (P < 0.05). ROS, reactive oxygen species: nmol DCF/min/mg protein; MDA, malondialdehyde: nmol MDA/min/mg protein; HP, hydroperoxides: nmol HP/min/mg protein.
levels remained unaltered in the brain tissues of offspring exposed to ACR lactationally (Fig. 3E and 3F).

Effect on glutathione, protein carbonyls and nitric oxide levels

Significant GSH level depletion was evident in maternal (27%) and offspring (22%) cortex with ACR exposure. Combined supplementation of FOS + XOS restored the levels only in the maternal cortex (Fig. 4A and 4D). Similarly, elevated levels of PC (maternal cortex: 41%; offspring cortex: 30%) were restored with prebiotic supplementation only in the maternal cortex (Fig. 4B and 4E). Further, enhanced NO levels were normalized by combined prebiotics only in the maternal milieu (Fig. 4C and 4F).

Effect on activities of antioxidant/detoxifying enzymes

ACR exposure significantly reduced antioxidant enzyme activity in the maternal cortex (SOD: 32%, CAT: 25% and TrxR: 36%) and offspring cortex (SOD: 20%, CAT: 26% and TrxR: 19%) (Fig. 5A-5C). Combined FOS + XOS supplementation offered protection by restoring the activity levels only in the maternal milieu. Further, in the cerebellum of both the milieu, ACR exposure caused reduction in the activity of SOD (maternal brain: 31%; offspring brain: 20%) (Fig. 5D), CAT (maternal brain: 14%; offspring brain: 30%) (Fig. 5E) and TrxR (maternal brain: 19%) (Fig. 5F). However, restoration of SOD and CAT activity was evident only in the maternal cerebellum.

Effect on neuronal markers

Cholinesterase activity: in the maternal brain, ACR enhanced AChE activity in the cortex (44%), and cerebellum (46%) was normalized among rats provided combined oligosaccharides (Fig. 6A). However, enhanced BuChE activity (cortex: 43%; cerebellum: 31%) remained unaffected with combined oligosaccharide feeding (Fig. 6B). Further, enhanced cholinesterase activity in the offspring cortex (AChE: 18%) (Fig. 6C) again remained unaffected among FOS + XOS group. No significant change in BuChE activity was evident in the offspring brain regions (Fig. 6D).

Dopamine levels: while ACR exposure did not deplete DA levels in the offspring brain, significant depletion was evident in the maternal brain regions (cortex: 34% and cerebellum: 25%) (Fig. 7). Further, the levels were marginally restored only in the cortex among FOS + XOS rats.

Effect on MTT, Complex I-III and citrate synthase activity

MTT levels were significantly depleted among ACR rats in the maternal brain (cortex: 37%; cerebellum: 28%). Combined
FOS + XOS supplementation offered protection in both the regions (Fig. 8A). Further, reduced activities of complex I-III (45%, Fig. 8B) and CS (29%, Fig. 8C) in the maternal cortex was improved with FOS + XOS supplementation.

DISCUSSION

Certain sub-populations are at a higher risk of CNS disturbances due to higher dietary intake of thermally processed food during pregnancy. In this regard, dietary ACR exposure would readily cross blood-brain barrier to produce neurotoxicity (46). Substantial evidence suggests that maternal intestinal microbiota is crucial for offspring neurodevelopment (4) and beneficial effects of prebiotic oligosaccharide intake during pregnancy (22). The present study we assessed the effects of perinatal combined prebiotic (FOS + XOS) supplementation on ACR-induced changes in preweaning phenotypes, oxidative stress and neurotoxicity in rats.

Although developmental exposure to ACR did not affect gestation length, a marked reduction in birth weight of offspring along with preweaning body weight reduction among both the sexes was observed. This observation is consistent with an epidemiological study on birth outcome showing small-for-gestational age among children prenatally exposed to ACR (47). Lower birth weight is associated with multiple adverse metabolic outcomes such as increased susceptibility to cardiovascular diseases, insulin resistance and hypertension (48). This result is concurrent with earlier finding with ACR reducing offspring body weights (49). Weaning represents a critical period for survival as pups confront the challenge of independent feeding. However, combined FOS + XOS supplementation to mothers showed no evidence of recovery of offspring body weight gain with previous studies showing lower tendencies for water intake during lactational period when rats are exposed to ACR at 100 ppm (50). Moreover, the general lack of ACR effects on litter outcomes (live pups/litter, sex ratio, etc.) is consistent with results from previous studies involving postnatal ACR administration to offspring (12). Further, in the present study, no developmental delays were observed in any of the treatment groups and this suggests that the maternal ACR exposure or prebiotics administration had no effect on developmental measures. Analysis of offspring preweaning behavior showed ACR exposure caused decreased exploration in an open-field arena, together with lesser open arm entries in elevated plus maze consistent with previous ACR effects on dams (26) which could reflect a number of possible processes being affected by ACR including those related anxiety-like behavior, or general malaise.

High oxygen levels are required to maintain arterial oxygen tension necessary for postnatal life and are considerably higher than those normally present during fetal existence. Newborn pups are exposed to more ROS than they would have been had they remained in utero. In the present study, significant changes...
Fig. 5. Effect of a combination FOS + XOS supplementation on activities of SOD (A, D); CAT (B, E) and TrxR (C, F) in maternal/offspring brain regions of acrylamide (ACR) exposed rats. Data are presented as means (± SEM); n = 6. *P < 0.05 compared to control (CTR); #P < 0.05 compared to ACR; one way ANOVA followed by post hoc Tukey test.

Fig. 6. Effect of a combination FOS + XOS supplementation on acetylcholinesterase (A, C); and butyrylcholinesterase (B, D) activities in maternal and offspring brain regions of acrylamide (ACR) exposed rats. Data are presented as means (± SEM); n = 6. *P < 0.05 compared to control (CTR); #P < 0.05 compared to ACR; one way ANOVA followed by post hoc Tukey test.
elicited by ACR exposure were evident in maternal brain regions for markers involved in the oxidative stress response (viz., ROS and MDA) with corresponding enhanced levels of ROS in the offspring brain regions. Additionally, evidence suggests that endogenous unsaturated aldehydes are known to interact with type-2 alkenes to cause cytotoxicity (51). In vitro studies have demonstrated the antioxidant property of oligosaccharides (52, 53). In the present model, combined FOS + XOS

![Fig. 7. Effect of FOS + XOS supplementation on dopamine levels in maternal brain regions (cortex and cerebellum) of acrylamide (ACR) exposed rats. Data are presented as means (± SEM); n = 6. *P < 0.05 compared to CTR; #P < 0.05 compared to ACR; one way ANOVA followed by post hoc Tukey test.](image)

![Fig. 8. Effect of a combination FOS + XOS supplementation on NADH cytochrome C reductase (A); MTT reduction (B) and citrate synthase (C) activity in maternal and offspring brain regions of acrylamide (ACR) exposed rats (D). Data are presented as means (± SEM); n = 6. *P < 0.05 compared to CTR; #P < 0.05 compared to ACR; one way ANOVA followed by post hoc Tukey test.](image)
supplementation reduced the enhanced oxidative responses, which is attributable to the antioxidant property, concurrent with our findings in prenatal model (25) and other reports (54, 55). Previous data has suggested that bacterial fermentation of oligosaccharides in the gut can produce antioxidants (56). While ACR exposure decreased GSH levels, the PC levels were significantly enhanced in the maternal brain regions (cortex and cerebellum), the redox balance was maintained with combined prebiotic supplementation. It has been shown that ACR conjugates with GSH stores leading to change in the redox status of the cell leading to apoptosis (57). From initial high GSH concentrations in erythrocytes and plasma of preterm infants, during the postnatal period, the concentrations of GSH decreases rapidly due to enhanced oxidative stress (58). In fact, it has been reported that protein carbonylation precedes neuronal apoptosis concurrent with GSH depletion (59).

Apart from the antioxidant capacity of the oligosaccharides, the protective effect of oligosaccharides could also be linked to microbial degradation of dietary carbohydrates into SCFAs. This view is supported by the fact that acetate acid affects availability of HAT substrate (acetyl CoA), leading to higher levels of histone acetylation in neurons thus facilitating neuroprotection (60). Furthermore, the capacity of combined FOS + XOS to promote normalization of perturbed enzymatic antioxidant activities in brain regions of mothers and PND 21 offsprings could be important in its ability to enhance antioxidant defenses in response to oxidative toxicity (61). Moreover, the modulation of endogenous amino acid levels in the brain regions by prebiotics might also account for the mitigation of oxidative stress. Consistent with such a view, lowering oxidative stress by prebiotics potentially lead to enhanced neurotrophic factor (62), which has earlier been shown to be neuroprotective. However, the present investigation warrants gut microbiota analysis by gene sequencing technique to identify the specific bacterial genera that can influence the antioxidant defenses.

In the present study, ACR exposure enhanced activity levels of cholinesterases in both maternal and offspring brain regions. Reports have documented that the cholinergic system plays an important role in the brain development, and the role of AChE has been implicated in axonal guidance and neurite outgrowth (63). Moreover, experimental evidence shows that ACR impairs cholinergic synaptic transmission centrally (hypothalamus) and...
peripherally on target organs (heart and skeletal muscle) associated with oxidative stress (64). Although scant data exists regarding the impact of prebiotic oligosaccharides on cholinergic functioning, the results presented herein show that combined prebiotics normalized AChE activity in the maternal cortex suggesting its cholinergic neuronal activity. DA levels in the maternal cortex were restored with combined FOS + XOS feeding that were reduced with ACR exposure. Combined FOS + XOS supplementation prevented ACR-induced reduction in DA levels which might be due to the likelihood of intestinal commensals in influencing central neurochemicals (65). Although the underlying mechanisms leading to regulation of neurotransmitter levels are not well understood, it has been suggested that signals from the gut microbiota to neurotransmitter-producing cells (such as the enterochromaffin or even to glia), may regulate neurotransmitter production. While such signaling to neurotransmitter-producing cells is one way in which the microbiota could influence neurotransmitter levels, gut-harbourenced bacteria are capable of releasing certain molecules with potential to act as neurotransmitters (66). In turn, these molecules may act as signals to the brain, and ultimately influence neurological function (67). However, the exact mechanism how gut bacteria alter the neurotransmitter levels needs further investigation and such studies open up possibilities in understanding how the microbiome may affect the function of the CNS through the creation of bioactive metabolites via several modes of gut-brain connection. Further, the beneficial effect by combined oligosaccharide feeding may provide for a phenotypic shift in the cecal microbial numbers towards more beneficial and reduction in the oxidative damage. The results from histomorphometric studies of the guinea pig small intestine has suggested that prenatal ACR exposure damaged villi, decreased epithelium thickness and formation of crypts along with with enlarged myenteric and submucosal plexus (68). This finding essentially provides for understanding the effect of maternal ACR exposure on intestinal dysfunction and consequently on gut microbiota. The specific effect of perinatal ACR exposure on brain mitochondrial dysfunction was evident with diminished complex I-III activity, MTT reduction and citrate synthase activity in maternal brain milieu. The combined prebiotic oligosaccharides supplementation protected against mitochondrial dysfunction and normalized citrate synthase activity in the maternal cortex. These results reconfirm the previous results with prenatal study that FOS + XOS combination offer benefits in restoring the mitochondrial protection in the maternal and offspring brain modulating oxidative stress, mitochondrial function and neurotransmission changes under ACR exposure (Fig. 9). Clearly, mechanistic understanding of how prebiotic mediated effect during pregnancy that governs basic developmental processes in the brain hold promise.

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Author’s address: Dr. Gokul Krishna, Department of Child Health, University of Arizona College of Medicine-Phoenix, 425 N. 5th St., ABC-1 Building, Phoenix, AZ 85004, USA
E-mail: gokul2411@gmail.com