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# Methanol-induced neurotoxicity in pups exposed during lactation through mother Role of folic acid

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#### Abstract

Role of folic acid on methanol-induced neurotoxicity was studied in pups at Postnatal Day (PND) 45 exposed to methanol (1%, 2% and 4%, v/v) during lactation through mothers maintained on folic acid-deficient (FD) and folic acid-sufficient (FS) diet. A gradual loss in the body weight gain was observed in the pups exposed to 2% and 4% methanol in the FD group, while FS group exhibited this alteration only at 4% exposure. The assessment of spontaneous locomotor activity (SLA) showing a significant increase in the distance travelled was observed in the 2% and 4% methanol-exposed groups in both the FS and FD animals when compared with their respective controls, but the effect was more marked in the FD group. A significant decrease in the conditioned avoidance response (CAR) was observed in pups exposed to 2% and 4% methanol in the FD group at PND 45. The results also suggest that disturbances in dopaminergic and cholinergic receptors were more pronounced in the FD group as compared to the FS group. A significant decrease in striatal dopamine levels was also observed in the FD group at 2% and 4% methanol exposure, while in the FS group, a significant decrease was exhibited only at 4% methanol exposure. An aberrant increase in the expression of Growth-Associated Protein (GAP-43), a neuron-specific growth-associated protein was observed in pups in the FD group exposed to 2% and 4% methanol, while an increase in the expression of GAP-43 in the FS group was found only at 4% methanol exposure in the hippocampal region as compared to their respective controls. Results suggests that methanol exposure during growth spurt period adversely affects the developing brain, the effect being more pronounced in FD rats as compared to FS rats, suggesting a possible role of folic acid in methanol-induced neurotoxicity. © 2002 Elsevier Science Inc. All rights reserved.

Keywords: Methanol; Dopaminergic receptor; Cholinergic (muscarinic) receptor; Spontaneous locomotor activity; Condition avoidance response; Dopamine; Growth-Associated Protein (GAP-43); Western blotting; Immunohistochemistry

#### 1. Introduction

Methanol (wood alcohol) is widely used as a solvent, antifreeze and, in particular, as a solvent for shellac in varnishes and has recently attracted attention worldwide as an important alternative motor fuel in many countries [27,37]. In recent years, methanol has been reported to cause ocular toxicity, metabolic acidosis, mucosal irritation, nausea, vomiting, abdominal pain and acute renal failure [13,32,39]. In India, several cases of human poisoning leading to blindness and even death have been reported time and again due to consumption of illicit country liquor or hooch, which is adulterated with methanol. Many epidemics of human intoxication of methanol have been described worldwide [29,31,34].

Central nervous system effects of methanol exposure have been attributed both to methanol itself and its metabolites [8,41]. Effects have been observed in both human [30] and nonhuman primates [9,46]. Nonprimate species are less susceptible to formate accumulation because they have higher levels of hepatic tetrahydrofolate and dependent enzymes, which helps in formate oxidation. Folic acid is known to play an important role in methanol metabolism via

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catalase, the peroxidase enzymes system in rat or alcohol dehydrogenase in monkey and human [22], because activities of these enzymes are dependent on the folic acid as a cofactor [7,25]. The lack of an adequate animal model for studying methanol toxicity is a major hindrance in establishing the comprehensive adverse effects of methanol in humans. In experimental rodents, methanol toxicity is more pronounced if the animals are maintained on a folate-deficient diet or subjected to biochemical modification [12,17,32,36].

In the present investigation, an attempt has been made to understand the role of folic acid in methanol-induced toxicity. Developing rats were exposed to methanol through their mothers maintained on folic acid-deficient (FD) or folate-sufficient (FS) diet. Neurochemical and neurobehavioral studies were undertaken to ascertain the extent and degree of neurotoxicity associated with methanol exposure. In addition, Western blotting and immunohistochemistry of the Growth-Associated Protein (GAP-43), a neuron-specific phosphoprotein, which is most prominently localized in growth cone membranes [44,45] and is strongly expressed during developmental and regenerative neurite outgrowth, were also conducted [22,48].

#### 2. Methods

# 2.1. Chemicals

Methanol (HPLC grade) was obtained from Spectrochem, Mumbai, India, Monoclonal anti-GAP-43 was obtained from Sigma, USA. [<sup>3</sup>H]-Quinuclidinyl benzylate ([<sup>3</sup>H]-QNB; 47 Ci/mmol) and 1-Phenyl-4-[<sup>3</sup>H] spipeperidol (29 Ci/mmol) were purchased from New England Nuclear, UK. Biotinylated secondary antibody was obtained from Vector Laboratories. All other chemicals and reagents used in the study were from Sigma.

# 2.2. Animals and treatment

Weaned albino female rats (n = 80 in each group) of Wistar strain (weighing  $30 \pm 2$  g) were obtained from the animal breeding colony of the Industrial Toxicology Research Centre (ITRC) and were maintained on folic acid-deficient (FD) and folic acid-sufficient (FS) diets separately. Along with the FD diet, 1% succinvlsulphathiazole was also mixed to inhibit folic acid biosynthesis from intestinal bacteria [21,32,53]. Animals were maintained on this diet for 14-16 weeks. Liver folate level was estimated in both groups. The female rats exhibiting significant low folic acid level were allowed to mate overnight in a ratio of 3:1 (female/male). The pregnant rats were housed individually in unit plastic cages using rice bran as bedding in a temperature-controlled room with a 12/12-h light/dark cycle. The dams of both the FD and FS groups had a free access to drinking water containing different concentrations

of methanol (1%, 2% and 4%, v/v). Pups were exposed to methanol via the lactating mother throughout the lactation period from Postnatal Day (PND) 1 to PND 21. Control groups received only drinking water in an identical manner. Liver folate levels in the pups were determined at PND 21. Litter size was culled to 8 with equal male/female ratio as far as possible. Neurobehaviour, neurochemical, Western blotting and immunohistochemical parameters were monitored at PND 45.

#### 2.3. Preweaning evaluation

During the preweaning period, the developmental parameters such as body weight, eye opening, pinna detachment, fur growth, incisor eruption, surface righting and forward locomotion (body and head movement) were evaluated in the pups using functional teratology test battery [51].

#### 2.4. Folate estimation

Liver tetrahydrofolate levels were randomly measured from different litters in FD and FS rats (n = 5 pups in each group) following the method of Jaenicke [23] to ascertain folic acid deficiency before mating and in pups with FD mother at PND 21.

# 2.5. Behavioural studies

#### 2.5.1. Motor performance

Eight animals from each group (FD and FS) were randomly monitored at PND 45 for locomotor activity (LA) using the microprocessor-based Optovarimex Activity monitor (Columbus Instruments, Ohio, USA) as described by Ali et al. [4]. In brief, the rats (n=8) were placed individually in the measuring chamber and after a 1-min acclimation period, distance travelled was assessed. Each rat was subjected to one activity session of 5 min in duration.

# 2.5.2. Conditioned avoidance behaviour

The cognitive ability of the animals were assessed by a two-way conditioned avoidance behaviour using a shuttle box (Techno, India) as described by Ali et al. [3] at PND 45 using six rats from each group. In brief, the naive rats, placed individually in one of the chambers, were subjected to 20 trials/day for 5 days, keeping the time constant for both groups. A trial consists of the presentation of a continuous 90 dB, 1500 Hz tone as the conditioning stimulus (CS) for 10 s during which time the animal has to cross to the nonshock chamber (conditioned avoidance response, CAR). If the animal failed to cross (avoid), it was shocked by giving a 30-40-V foot shock (unconditioned stimulus, US) for 10 s to induce it to cross to the nonshock chamber (unconditioned response, UR). This is followed by a 40-s rest period. The CS remains activated when the US is presented. The number of CARs is recorded for each rat and compared with respective control rats for each day of trial.



Fig. 1. Effect of FD diet on the status of liver folic acid levels in rats (n = 5 each group) on 16 weeks postweaning rats and PND 21 in the lactationally exposed pups.

#### 2.6. Neurochemical studies

# 2.6.1. Neurotransmitter receptors: dopaminergic $(D_2)$ and cholinergic (muscarinic) receptor binding

2.6.1.1. Membrane preparation and binding assay. An assay of dopamine (D<sub>2</sub>) receptors (n=5) in the corpus striatum and cholinergic receptors (n = 5) in the hippocampus were carried out by the method of Agarwal et al. [1]. Briefly, crude synaptic membranes were prepared from corpus striatum and hippocampus brain regions by homogenizing in 19 volumes of 0.32 M sucrose followed by centrifugation at 50,000  $\times$  g for 10 min at 4 °C. The pellet was washed twice with deionized water and suspended in cold 40 mM Tris-HCl buffer, pH 7.4, in the same volume and stored at -20 °C till binding assay. Binding incubation were carried out in triplicate in a final volume of 1.0 ml containing 40 mM Tris-HCl (pH 7.4) with labeled and unlabelled pharmacological agents and 100-µl synaptic membrane preparations per tube equivalent to  $250-300 \ \mu g$ protein [<sup>3</sup>H]-QNB (47 Ci/mmol, 1 nM) and 1-Phenyl-4-[<sup>3</sup>H] spipeperidol (29 Ci/mmol, 1 nM) were used as the specific radioligand for cholinergic (muscarinic) and dopamine (D<sub>2</sub>) receptor. Atropine sulfate (1  $\mu$ M) and haloperidol (1  $\mu$ M) were used as unlabelled competitive ligands for cholinergic (muscarinic) and dopamine receptors, respectively, for determining nonspecific binding. After 15 min of incubation at 37 °C, the contents were filtered through glass fiber discs (Whatman GF/C) and were washed twice with 5 ml cold Tris-HCl buffer. The filters were dried, and radioactivity was counted in 5 ml of scintillation mixture in Liquid Scintillation Analyzer, Tri-Carb 2000 TR, Packard, USA, at an efficiency of 50% for tritium. Specific binding was calculated by subtracting nonspecific binding obtained in

the presence of 1  $\mu$ M unlabelled ligands from total binding obtained in absence of unlabelled ligands. Results are expressed in terms of picomoles of ligand bound per gram of protein.

#### 2.7. Assay of dopamine levels by HPLC

Dopamine levels in the corpus striatum were estimated following the method of Kim et al. [28] using HPLC (Merck) with electrochemical detector. For neurotransmitter analysis, rats (n = 5) were decapitated by cervical dislocation, and their brains were rapidly removed and placed on ice-chilled petri dishes, and striatum was dissected over ice. The striatum was weighed and placed in ice-water bath in polypropylene tubes and was homogenized in 1.0 ml of perchloric acid containing 125 ng of 3,4-dihydroxybenzylamine hydrobromide (DHBA) as an internal standard for 30 s using a polytron homogeniser. Homogenates were centrifuged at 4 °C for 20 min at  $31,550 \times g$ , and the supernatants were separated and either analysed immediately or stored at -70 °C till assay. The supernatants were filtered through a 0.2-µm filter and were injected into the HPLC column (RP-C18). The mobile phase was 15% (v/v) methanol in a solution of (pH 4.2) 32 mM citric acid, 12.5 mM disodium hydrogen orthophosphate, 0.5 mM octyl sodium sulfate and 0.05 mM EDTA. Standard solutions (0.1 mg/ml) were prepared in 0.17 M PCA. The peak was detected by using Ag/AgCl as the reference electrode, and the data were calculated on the basis of standard run each time.



Fig. 2. Effect of methanol (1%, 2% and 4%) exposure during lactation (PNDs 1–21) on distance travelled in the FD and FS groups on PND 45 in rats. Values are means $\pm$ S.E. of eight rats. Multifactorial ANOVA followed by Dunnett's test for all possible pair comparisons. a=versus FSC; b=versus FDC; c=versus FS1; d=versus FS2; e=versus FS4; f=versus FD1; g=versus FD2; h=versus FD4. \*P<.05; \*\*P<.01; \*\*\*P<.001, ns=not significant.

Table 1

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Effect of methanol (1%,	2% and 4%) exposure during lactation (PNDs 1-21) on learning ability (% CAR) of the FD and FS groups on PND 45 in rats
FD group	FS group

	FD group				FS group			
	Control (FDC)	Methanol				Methanol		
		1% (FD1)	2% (FD2)	4% (FD4)	Control (FSC)	1% (FS1)	2% (FS2)	4% (FS4)
PND 45	$98\pm5^{ns}\!a$	$80\pm9$	$51 \pm 4$ **b,d,f	47 ± 3***b,e,f	$91\pm 6$	$88\pm7$	$80\pm10$	71 ± 3**a; *c

Values are means  $\pm$  S.E. of the % CAR on Day 5 of testing of six rats. Multifactorial ANOVA followed by Dunnett's test for all possible pair comparisons. a = versus FSC; b = versus FDC; c = versus FS1; d = versus FS2; e = versus FS4; f = versus FD1; ns = not significant.

\*\*\* P<.001.

# 2.8. Immunohistochemistry of GAP-43

GAP-43 expression was estimated following the modified method of Goslin et al. [18] with slight modification. Control and methanol-treated rats (n=5) were anaesthetized and perfused transcardially with 100 ml of phosphate-buffered saline (PBS), followed by 250 ml of ice-cold 4% paraformaldehyde in 0.1 M sodium phosphate buffer, pH 7.4. Brains were excised out and postfixed overnight in the same fixative at 4 °C. Further, tissue was cryopreserved in 10%, 20% and 30% sucrose. Coronal sections 45 µm thick were cut on a freezing microtome (Slee, Mainz, Germany) and were collected in PBS and stored at 4 °C. Tissue sections were incubated with 0.5% H<sub>2</sub>O<sub>2</sub> (w/v) in methanol to quench endogenous peroxidase. Nonspecific binding sites were blocked by incubating with 0.5% BSA and 0.1% Triton X-100 in PBS for 2 h prior to incubation with GAP-43 antibodies diluted (1:1000) in PBS containing 0.5% BSA and 0.1 Triton X-100 at 4 °C with agitation for 28 h. The sections were washed several times with PBS and then incubated with a biotinylated secondary antibody (1:2000, Vector Laboratories) for 2 h at 4 °C. After several washes with PBS, the product was visualized with the streptavi- din peroxidase and diaminobenzidine as chromogen. Sections were mounted on gelatin-coated slides, dehydrated and coverslipped.

#### 2.9. Western blots

In order to confirm the expression of GAP-43, Western blot analysis was conducted following the method of Schreyer and Skene [43] in the hippocampal region of the brain. The samples were homogenized in 1 ml of 50 mM Tris-HCl, pH 7.5, 5 mM EDTA and 5 mM dithiothreitol; the debris was removed by low-speed centrifugation; and membrane pellets were prepared by centrifugation at  $100,000 \times g$  for 30 min. The pellets were extracted by sonication in 1 ml of 0.2% SDS and were again centrifuged at  $100,000 \times g$  for 30 min. Supernatant was separated out, and protein content of the supernatant were estimated by Lowry et al. [35]. Proteins were separated by SDS-PAGE, 5% stacking gel and 10% separating gel [30]. For the time course analysis, each sample contained 200  $\mu g$  protein. Gels were electroblotted on Immobilon-NC transfer membrane (Millipore, Bedford, MA, USA) according to the method of Towbin et al. [49]. The membrane was incubated with the GAP-43 antibody (1:1000 dilution in 5 ml of PBS containing 0.02% Tween-20 and 0.02% sodium azide, in PBST) for 16-18 h at 4 °C, followed by incubation with 1:30,000 dilution in Tris-buffered saline (TBS) of rabbit antimouse IgG-alkaline phosphatase complex. The membranes were washed with TBS (three times, 10 min each) and then processed for colour development with 5-bromo-4-chloro-3-indolyl-phosphate/nitoblue tetrazolium (BCIP/NBT).

Table 2

Effect of methanol (1%, 2% and 4%) exposure during lactation (PNDs 1-21) on dopaminergic receptor binding (pmol <sup>3</sup>H-Spiperone bound/g protein) in the striatum region of the FD and FS groups on PND 45 in rats

	FD group				FS group				
		Methanol				Methanol			
	Control (FDC)	1% (FD1)	2% (FD2)	4% (FD4)	Control (FSC)	1% (FS1)	2% (FS2)	4% (FS4)	
PND 45	$525\pm16^{ns}a$	706 ± 43**b,c	418 ± 13**b; ***d,f	305 ± 20***b,e,f,g	$507 \pm 17$	$556\pm31$	621 ± 13**a; *c	780 ± 21***a; **c; *d	

Values are means  $\pm$  S.E. of five rats. Multifactorial ANOVA followed by Dunnett's test for all possible pair comparisons. a = versus FSC; b = versus FDC; c = versus FS1; d = versus FS2; e = versus FS4; f = versus FD1; g = versus FD2; ns = not significant.

\* *P* < .05.

\*\* *P* < .01.

\*\*\* P < .001.

<sup>\*</sup> P < .05.

<sup>\*\*</sup> P<.01.

Table 3

	FD group				FS group				
	Methanol				Methanol				
	Control (FDC)	1% (FD1)	2% (FD2)	4% (FD4)	Control (FSC)	1% (FS1)	2% (FS2)	4% (FS4)	
PND 45	$690\pm24^{ns}\!a$	$592\pm94$	$827 \pm 23$ *b	980 ± 71***b,e,f,g	$673\pm31$	$642\pm33$	$604\pm63$	815 ± 21**a,c;*d	

Effect of methanol (1%, 2% and 4%) exposure during lactation (PNDs 1–21) on cholinergic (muscarinic) receptor binding (pmol [<sup>3</sup>H]-QNB bound/g protein) in the hippocampus region of the FD and FS groups on PND 45 in rats

Values are means  $\pm$  S.E. of five rats. Multifactorial ANOVA followed by Dunnett's test for all possible pair comparisons. a = versus FSC; b = versus FDC; c = versus FS1; d = versus FS2; e = versus FS1; g = versus FD2; ns = not significant.

\*\* P<.01.

\*\*\* P<.001.

# 2.10. Protein estimation

Protein was measured following the method of Lowry et al. [35] using bovine serum albumin (BSA) as standard.

# 2.11. Statistical analysis

Mean significant difference in the treatment groups was determined using a two-way analysis of variance (ANOVA). The means of the treatment groups from the controls were compared separately using Dunett's test and P values < .05 were considered as statistically significant [57].

#### 3. Results

#### 3.1. General observation

No significant difference in food and water intake was observed among the different treatment groups. A gradual loss in body weight gain was observed on PNDs 7, 14 and 21 in the animals exposed to 2% (11%, 15% and 19%) and 4% (17%, 24% and 29%) methanol in the FD group and only at 4% (9%, 14% and 17%) methanol in the FS group, respectively, when compared to their respective controls. There was no difference in latency in the retrieval of pups after they had been weighed and replaced in their maturity cage, indicating that methanol exposure did not disrupt maternal behaviour indicating that crossfostering was not crucial to the experimental design. The pups exposed to 2% (24% and 31%) and 4% (37% and 42%) methanol in the FD and only at 4% (25% and 36%) in FS groups exhibited a significant delay in the development of surface righting and forward locomotion, respectively, in comparison to their respective controls.

#### 3.2. Liver folate assay

Liver tetrahydrofolate level was measured prior to mating and on PND 21 in the pups exposed to methanol lactationally. Folic acid level was decreased by 63% in rats prior to mating and 67% in pups on PND 21 (Fig. 1).

# 3.3. Behavioural studies

#### 3.3.1. Spontaneous locomotor activity (SLA)

Significant increase in the distance travelled was observed in the 2% (13% and 33%) and 4% (39% and 66%) methanol-exposed groups in both the FS and FD animals, respectively, when compared with their respective controls, but the effect was more marked in the FD group (Fig. 2).

#### 3.3.2. Conditioned avoidance behaviour

Percent CAR was significantly decreased by 48% and 52% at 2% and 4% methanol-exposed rats, respectively, in the FD group at PND 45. By contrast, the FS group exhibited a significant 22% decrease in the 4% methanol-exposed animals only in CAR as compared to their respective controls (Table 1).

Table 4

Effect of methanol (1%, 2% and 4%) exposure during lactation (PNDs 1-21) on dopamine level (ng dopamine/g tissue) in the striatum region of the FD and FS groups on PND 45 in rats

	FD group					FS group			
		Methanol				Methanol			
	Control (FDC)	1% (FD1)	2% (FD2)	4% (FD4)	Control (FSC)	1% (FS1)	2% (FS2)	4% (FS4)	
PND 45	$5817\pm265^{ns}a$	$5620\pm174$	3956 ± 137*b,f; *d	2851 ± 129***b,f; **e,g	$5934\pm304$	$5760\pm254$	$5184 \pm 198$	3859 ± 147**a,c; *d	

Values are means  $\pm$  S.E. of five rats. Multifactorial ANOVA followed by Dunnett's test for all possible pair comparisons. a = versus FSC; b = versus FDC; c = versus FS1; d = versus FS2; e = versus FD1; g = versus FD2; ns = not significant.

\* P < .05.

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*** P<.001.
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<sup>\*</sup> P < .05.

<sup>\*\*</sup> P<.01.



Fig. 3. Photomicrograph of the FD control. (a) <u>Hippocampus</u> showing unstained pyramidal neurons cell bodies. Photomicrographs of 2% (b) and 4% (c) methanol-exposed rats show increased GAP-43 immunoreactivity in pyramidal and granular cells (arrow) in the hippocampus region at PND 45. Scale bar represents 200  $\mu$ m.

#### 3.4. Effect on neurotransmitter receptors

#### 3.4.1. Dopamine receptor binding

Rats maintained on FD diet exhibited significant increase (34%) at 1% methanol exposure and decrease in the dopamine  $D_2$  type receptor at 2% and 4% methanol-exposed groups by 20% and 42%, respectively, when compared with control. Whereas FS rats showed significantly increased binding by 22% and 54% in the 2% and 4% methanol-

exposed groups, respectively, as compared to the control group (Table 2).

# 3.4.2. Cholinergic (muscarinic) receptor binding

The results of cholinergic muscarinic receptor binding are summarized in Table 3. It is evident from the results that there is a significant increase in cholinergic binding (20% and 42%) in 2% and 4% methanol exposure, respectively, in FD animals. However, the FS group animals exhibit an increase in cholinergic binding (21%) only at 4% methanol exposure that is significantly less than the FD group. No significant effect could be observed at 2% methanol exposure in animals in the FS group at PND 45 (Table 3).

# 3.5. Effect on dopamine levels

The effect of methanol on dopamine levels in the striatum of rats maintained on FD diet was a significant decrease of 32% and 51% at 2% and 4% methanol exposure, respectively, as compared to the control group. While the FS group did not exhibit any significant effect on DA level



Fig. 4. Photomicrograph of the FS control. (a) Hippocampus also showing unstained pyramidal neurons cell bodies. Photomicrographs of only 4% (b) methanol exposed rats show increased GAP-43 immunoreactivity in pyramidal and granular cells (arrow) in the hippocampus region at PND 45. Scale bar represents 200  $\mu$ m.



Fig. 5. Western blot analysis of GAP-43 by using monoclonal anti-GAP-43 clone GAP-7B10 mouse ascites fluid of synaptosomal fraction of hippocampal tissues from rats treated with methanol (1%, 2% and 4%) in the FS and FD groups at PND 45. Hippocampal protein (200  $\mu$ g) was loaded in each lane. Lanes (a) FS control; (b) FD control; (c) FS with 1% methanol treated; (d) FD with 1% methanol treated; (e) FS with 2% methanol treated; (f) FD with 2% methanol treated; (g) FS with 4% methanol treated; (h) FD with 4% methanol.

in 2% methanol-exposed animals, the rats exposed to 4% methanol showed a significant decrease (32%) on PND 45 (Table 4).

# 3.6. Immunohistochemistry of GAP-43

Coronal sections of the hippocampus exhibited a significantly high expression of GAP-43 in the dentate granular and pyramidal cells in 2% and 4% methanol-exposed FD group rats (Fig. 3a,b,c). While the FS group exhibited higher expression of GAP-43 only at 4% methanol exposure as compared to the respective control group (Fig. 4a,b). Furthermore, the expression in the FS group was less as compared to the FD group.

# 3.7. Western blot analysis

Results of Western blot analysis of the hippocampus region further confirm the high expression of GAP-43 at 2% and 4% methanol in the FD group rats. In addition, the expression of GAP-43 at 2% and 4% methanol was less in FS rats when compared with the FD group (Fig. 5).

#### 4. Discussion

Earlier studies have revealed that methanol and its metabolites induce alterations in retinal functions and motor behaviour in rats and humans [19,52,55]. Eells et al. [13] also showed that cerebellum, cortex, striatum and hippocampus are the target brain regions for toxicity to methanol and its metabolites. The aim of our study was to assess the neurotoxic effect of methanol during the preweaning period and ascertain the role of folic acid in methanol toxicity. No overt significant effect of folic acid deficiency as such could be observed in neurochemical and neurobehavioral parameters. This observation is supported by Lee et al. [32] who has reported that physiological state of rats were neither altered by folic acid deficiency nor by succinylsulphathiazole. Several investigators have shown that a

significant increase in the blood formate level occurs in methanol-exposed rats under FD condition [42]. The decrease in folic acid concentration may cause retardation in oxidation of formic acid leading to high accumulation of this metabolite, resulting in potentiation in the neurotoxic effects of methanol [9,17,20,32]. The present study also showed that pups exposed to methanol (2% and 4%) during lactation had altered dopamine level in striatum under FD condition. In FS rats, this decrease was observed only at 4% methanol exposure, and the decrease was less as compared to FD rats revealing a possible role of metabolites in methanol toxicity. Our results are consistent with the data of Jeganathan and Namasivayam [24], who reported a decrease in dopamine levels in adult rats exposed to a single-dose administration of methanol. Several clinical studies reported that survivors of severe methanol poisoning developed a Parkinson-like extrapyramidal syndrome apart from blindness [33,38], suggesting a significant depletion in the dopamine level and degeneration of the dopaminergic nigrostriatal pathway.

The rapid growth and differentiation of the brain neurotransmitter receptors take place during the prenatal and early postnatal period in rats, and the period has been considered vulnerable to the insult of drugs and chemicals. Pronounced effects in neurobehaviour and neurotransmitter receptors have been reported after exposures to drugs and chemicals during the prenatal and postnatal exposure period [2,56]. A significant alteration in dopamine receptor in FD pups, as well as FS pups, at 2% and 4% methanol exposure may result in the disturbance in the nigrostriatal dopamine system, suggesting disruption in neurotransmitter availability, which may be the cause of altered locomotor activity [33]. The specific alterations in the number of cholinergic (muscarinic) receptor proteins in the hippocampal region may lead to impairment in CAR in 2% and 4% methanolexposed pups maintained on FD diet [14,47]. Alteration in DA-ACh balance by the compound could result in the observed changes in behaviour [56]. However, rats in the FS group exhibited the impairment in the cholinergic system only at 4% methanol exposure, and this could be attributed to the fact that high concentration of methanol may overwhelm the body's ability to remove the toxic metabolites (especially formate) [9].

A direct correlation of muscarinic receptor stimulation and GAP-43 phosphorylation in the isolated nerve growth cone has been shown by Van Hooff et al. [50]. The involvement of GAP-43 in transmitter release and neuronal plasticity and increased expression due to the exposure to different toxic compounds have also been reported earlier [10,11,54]. Convergent evidence from many labs indicate that GAP-43 is maximally synthesized during the process of developmental outgrowth and regeneration of neurites and the formation of new connections and neuronal remodeling during injury [5,6,11,16,26]. Our immunohistochemical and Western blotting results also showed an increase in the expression of GAP-43 due to methanol exposure at 2% and 4% in the FD group in the hippocampal region of rat brain. However, FS rats also exhibited GAP-43 expression at 4% methanol exposure in the hippocampal region, which could be the effect of high dose of methanol per se [9,11,40]. Upregulation of GAP-43 in the hippocampal region may be associated with axonal growth or protection of the nervous system from methanol toxicity, a process that supports a role of GAP-43 in regulating learning and memory processes [15]. Our results also indicate that the FD rats are more susceptible to methanol in comparison with FS rats.

In conclusion, our results indicate that hepatic tetrahydrofolate is an important contributing factor in methanolinduced neurotoxicity. The vulnerability of the developing brain to methanol toxicity could be attributed to poor development of the blood-brain barrier, as well as lack of an efficient drug-metabolizing enzyme system.

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