Epigallocatechin-3-gallate ameliorates alcohol-induced cognitive dysfunctions and apoptotic neurodegeneration in the developing rat brain

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Abstract
Clinical and experimental evidence has demonstrated that ethanol is a teratogen, and its consumption during pregnancy induces harmful effects on the developing fetus that leads to mental retardation and long-term cognitive and behavioural deficits in offspring. The brain growth spurt period is highly sensitive to the neurotoxic effects of ethanol and it corresponds to the last trimester in humans and the first two postnatal weeks in rodents. This study was designed to evaluate the effect of epigallocatechin-3-gallate (EGCG) on alcohol-induced behavioural, biochemical and molecular changes in rat pups. Pups were administered alcohol (5 g/kg, 12% v/v) by intragastric intubation on postnatal days (PD) 7, 8, and 9. Ethanol-exposed pups showed impaired spatial navigation in the Morris water maze test and poor retention in the elevated plus maze task conducted from PD 24 to 28 which was coupled with enhanced acetylcholinesterase activity, increased oxidative-nitrosative stress, cytokines (TNF-α and IL-1β), NF-κB and caspase-3 levels in both the cortex and hippocampus of pups sacrificed at PD 28. Apart from this, the mean weight of the whole brain, cortex and hippocampus of ethanol-treated pups was decreased by 34.48%, 39.09% and 34.30%, respectively. EGCG (50 and 100 mg/kg) significantly attenuated all the behavioural, biochemical and molecular changes in the different brain regions of ethanol-treated pups. The current finding demonstrates the activation of oxidative-nitrosative stress-mediated apoptotic signalling in cognitive deficits associated with fetal alcohol spectrum disorders (FASDs) and suggests that EGCG may have potential in prevention of the cognitive impairment in children with FASDs.

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Introduction
Alcohol consumption during pregnancy is a significant public health problem resulting in a wide range of adverse outcomes for the child. Clinical and experimental evidence has demonstrated that ethanol is a teratogen, and its consumption during pregnancy induces harmful effects on the developing fetus that leads to fetal alcohol spectrum disorders (FASDs).

FASDs are estimated to affect between 0.33 and 3/1000 live births and are now recognized as the leading non-genetic cause of mental retardation in the Western world (Abel & Sokol, 1991). In the last decade, the percentage of women who consumed alcohol during pregnancy increased at an alarming rate. Binge drinking during pregnancy has increased in recent years, from 1.3% in 1996–1997 to 3.1% in 2003 (CDC, 2003). The cost of caring for children with FASDs has been estimated at approximately US$ 74.6 million per year, with three quarters of this cost associated with the care of FASD cases with mental retardation (Abel & Sokol, 1991). Therefore, understanding how prenatal alcohol exposure produces behavioural and cognitive deficits is of great medical and economic importance.
Central nervous system (CNS) dysfunctions are the most severe and permanent consequence of maternal alcohol intake and can occur in absence of gross morphological defects associated with fetal alcohol syndrome (FAS). Mental retardation and long-term cognitive and behavioural deficits are some of the problems commonly found in children of women who were moderate or heavy drinkers during pregnancy. Experimental evidence demonstrates that alcohol interferes with many molecular, neurochemical and cellular events occurring during the normal development of the brain. Some brain areas are more affected than others and, even within a given region, some cell populations are more vulnerable than others. The neocortex, hippocampus and cerebellum are especially susceptible to alcohol and have been associated with behavioural deficits (Guerri, 2002). These findings are of clinical interest because humans exposed to ethanol in utero display a cluster of physical, cognitive, behavioural and neurological impairments known as FASDs (Fast & Conry, 2009).

Ethanol is converted into acetaldehyde via intracellular oxidation, eventually generating reactive oxygen species (ROS) such as superoxide anions, hydrogen peroxide, and hydroxyl radicals (Sagara et al. 1998). In addition, ethanol suppresses antioxidant enzymes such as glutathione peroxidase/glutathione reductase (Siler-Marsiglio et al. 2004). Thus, the deleterious effects of ethanol on neuronal cells have been associated with oxidative stress (Pirlich et al. 2002), and many studies have shown that oxidative stress in neuronal cells, including that due to ethanol, causes both apoptosis and necrosis (Oberdoerster et al. 1998; Pirlich et al. 2002; Watts et al. 2005). The developing brain, which has only a fraction of the antioxidant enzyme activity of the adult brain, is perhaps even more vulnerable to the neurotoxic effects of oxidative stress than the adult brain (Henderson et al. 1999). In addition, certain regions of the CNS, such as the hippocampus and cerebellum, may be particularly sensitive to oxidative stress because of their low endogenous levels of vitamin E, an important biochemical antioxidant, relative to other brain regions (Abel & Hannigan, 1995). Such a depressed defence system may be adequate under normal circumstances. However, in pro-oxidative conditions, such as during alcohol exposure, these low antioxidant defences can predispose the fetal brain to oxidative damage.

Epigallocatechin-3-gallate (EGCG), the main polyphenolic constituent of green tea, is being extensively studied primarily because of its reported anti-carcinogenic effects (Moyers & Kumar, 2004). EGCG pretreatment effectively ameliorated amyloid β-induced neurotoxicity and oxidative-nitrosative cell death via augmentation of antioxidant defence capacity (Kim et al. 2009). EGCG also showed a neuroprotective effect against cerebral ischaemia-induced oxidative stress and resulting neuronal damage in gerbils (Lee et al. 2004). Additionally, EGCG has been shown to inhibit various activities of pro-inflammatory cytokines (Han, 2003; Li et al. 2004), activation of transcription 1 and NF-κB responses (Aktas et al. 2004; Han, 2003).

Thus, the present study was designed to investigate the protective potential of EGCG, against cognitive dysfunction in rat pups postnatally exposed to ethanol.

Materials and methods

Animals

Wistar male rat pups (5-d-old neonates) housed in the Central Animal House facility of Panjab University were used in the study. The neonatal rats were housed under standard laboratory conditions, maintained at 22 °C on a 12-h light/dark cycle (lights on 06:00 hours). Animals were acclimatized to laboratory conditions before all behavioural tests. All experiments were performed between 09:00 and 17:00 hours. The experimental protocols were approved by the Institutional Animal Ethics Committee (IAEC) of Panjab University and performed in accordance with the guidelines of Committee for Control and Supervision of Experimentation on Animals (CPCSEA), Government of India on animal experimentation.

Drugs

EGCG was obtained as gift sample from DSM Nutritional Products Ltd (Switzerland). Tumour necrosis factor (TNF)-α and interleukin (IL)-1β ELISA kits were purchased from R&D Systems (USA). While NF-κB and caspase-3 ELISA kits were obtained from Imagenex (USA) and Biovision (USA), respectively. All other chemicals used for biochemical estimations were of analytical grade.

Treatment schedule

The pups were randomly divided into five experimental groups (n = 5–8 per group). Group 1 was the control group in which pups were administered double-distilled water in place of ethanol. Pups in group 2 were administered only ethanol (5 g/kg; oral gavage). Animals in groups 3 and 4 were administered EGCG (50 and 100 mg/kg; oral gavage) along with ethanol (5 g/kg; oral gavage). While animals in
group 5 received EGCG (100 mg/kg; oral gavage) but without ethanol administration (per se group). EGCG (50 and 100 mg/kg) treatment was started on PD 6 and was continued until the end of the study. Ethanol (12% v/v) was administered at a dose of 5 g/kg from PD 7 to PD 9 (Marino et al. 2004), 1 h after EGCG administration (Fig. 1). From PD 24–28, pups were tested for learning and memory task in the Morris water maze and elevated plus maze tests. At the end of PD 28, the pups were sacrificed under deep anaesthesia and brains were rapidly removed and placed on dry ice for isolation of the cerebral cortex and hippocampus. Brain tissues were incubated with 1 ml of ice-cold 1 × hypotonic buffer supplemented with 1% DTT and 1% detergent solution for 30 min on ice. After incubation, the samples were centrifuged for 10 min at 10 000 rpm at 4 °C. The supernatant (cytoplasmic fraction) was transferred into a separate tube and stored at −80 °C until processed for biochemical estimations.

**Behavioural tests**

*Morris water maze test*

Pups were tested in a spatial version of the Morris water maze test (Morris et al. 1982; Tuzcu & Baydas, 2006) from PD 24 to PD 28. The apparatus consisted of a circular water tank (180 cm diameter, 60 cm high). A platform (12.5 cm diameter, 38 cm high) invisible to the pups, was set 2 cm below the water level inside the tank with water maintained at 28.5 ± 2 °C at a height of 40 cm. The tank was located in a large room where there were several brightly coloured cues external to the maze; these were visible from the pool and could be used by the pups for spatial orientation. The position of the cues remained unchanged throughout the study. The pups received four consecutive daily training trials in the following 5 d, with each trial having a limit of 90 s and a trial interval of approximately 30 s. For each trial, each animal was placed into the water at one of four starting positions, the sequence of which was selected randomly. During test trials, pups were placed into the tank at the same starting point, with their heads facing the wall. The animal had to swim until it climbed onto the platform submerged beneath the water. After climbing onto the platform, the animal remained there for 20 s before commencement of the next trial. The escape platform was kept in the same position relative to the distal cues. If the animal failed to reach the escape platform within the maximally allowed time of 90 s, it was guided with the help of a rod and allowed to remain...
on the platform for 20 s. The time to reach the platform (escape latency in seconds) was measured.

**Memory consolidation test**

A probe trial was performed (Tiwari et al. 2009; Tuzcu & Baydas, 2006) at the end of day 28 wherein the extent of memory consolidation was assessed. The time spent in the target quadrant indicates the degree of memory consolidation that has taken place after learning. In the probe trial, the pups were placed into the pool as in the training trial, except that the hidden platform was removed from the pool. The total time spent in the target quadrant in a 90-s time period was recorded.

**Elevated plus maze test**

Memory acquisition and retention was tested using the elevated plus maze test at day 27. The apparatus consisted of two crossed arms, one closed and the other open. Each animal was placed on the open arm, facing outwards. The time taken by the animal to enter the closed arm in the first trial (acquisition trial) was noted and was designated the initial transfer latency. Cut-off time was fixed as 90 s and in case an animal could not find the closed arm within this period, it was gently pushed in to one of the closed arms and allowed to explore the maze for 30 s. The second trial (retention trial) was performed 24 h after the acquisition trial and retention transfer latency was noted (Kumar & Gupta, 2002; Sharma & Gupta, 2002; Tiwari et al. 2009). The retention trial latency was expressed as percentage of initial trial latency.

**Blood alcohol concentration (BAC) quantification**

Blood samples were collected at 0, 2 and 4 h after ethanol administration by decapitation on PD 9. Blood samples from all the groups sent to Dr Lal Path Labs (India) for estimation of BACs. BAC was determined by radiative energy attenuation assay. Radiative energy attenuation assay involves the linked enzyme-catalytic reactions of alcohol dehydrogenase and diaphorase (NAD). Reduced Iodonitrotetrazolium Violet (formazan-INT) yields a red colour with an absorbance peak at 492 nm. Because the chromogen’s absorbance overlaps the excitation and emission spectrum of fluorescein (a fluorescent indicator added to the reaction), the fluorescence intensity decreases logarithmically with increasing concentrations of reduced INT. The concentration of ethanol is therefore proportional to the degree of inner filter effect on the fluorophore.

**Biochemical estimations**

**Acetylcholinesterase activity**

Cholinergic dysfunction was assessed by measuring acetylcholinesterase levels in cytoplasmic fractions of cerebral cortex and hippocampus according to the method of Ellman et al. (1961). Results were calculated using molar extinction coefficient of chromophore \((1.36 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1})\) and expressed as percentage of control.

**Estimation of lipid peroxidation**

The malondialdehyde content, a measure of lipid peroxidation, was assayed in the form of thiobarbituric acid-reactive substances by the method of Wills (1965). Briefly, 0.5 ml of cytosolic fraction of both brain regions and 0.5 ml of Tris–HCl were incubated at 37 °C for 2 h. After incubation 1 ml of 10% trichloroacetic acid was added and centrifuged at 1000 g for 10 min. Then 1 ml of 0.67% thiobarbituric acid was added to 1 ml of supernatant and the tubes were kept in boiling water for 10 min. After cooling, 1 ml double-distilled water was added and absorbance was measured at 532 nm. Thiobarbituric acid-reactive substances were quantified using an extinction coefficient of \(1.56 \times 10^5 \text{ M}^{-1} \text{ cm}^{-1}\) and expressed as nmol of malondialdehyde per mg protein. Tissue protein was estimated using the Biuret method and the malondialdehyde content expressed as nmol/mg protein.

**Estimation of reduced glutathione**

Reduced glutathione was assayed by the method of Jollow et al. (1974). Briefly, 1.0 ml of cytosolic fraction of both brain regions was precipitated with 1.0 ml of sulphosalicylic acid (4%). The samples were kept at 4 °C for at least 1 h and then subjected to centrifugation at 1200 g for 15 min at 4 °C. The assay mixture contained 0.1 ml supernatant, 2.7 ml phosphate buffer (0.1 M, pH 7.4) and 0.2 ml 5,5-dithiobis-(2-nitrobenzoic acid) (Ellman’s reagent, 0.1 mM, pH 8.0) in a total volume of 3.0 ml. The yellow colour developed was read immediately at 412 nm and the reduced glutathione levels were expressed as \(\mu\text{mol/mg protein.}\)

**Estimation of superoxide dismutase**

Superoxide dismutase activity was assayed by the method of Kono (1978). The assay system consisted of 0.1 mM EDTA, 50 mM sodium carbonate and 96 mM of Nitro-blue tetrazolium (NBT). In a cuvette, 2 ml of the above mixture was taken and 0.05 ml of
cytosolic fraction of both brain regions and 0.05 ml of hydroxylamine hydrochloride (adjusted to pH 6.0 with NaOH) were added to it. The auto-oxidation of hydroxylamine was observed by measuring the change in optical density at 560 nm for 2 min at 30-/60-s intervals.

**Estimation of catalase**

Catalase activity was assayed by the method of Claiborne (1985). Briefly, the assay mixture consisted of 1.95 ml phosphate buffer (0.05 M, pH 7.0), 1.0 ml hydrogen peroxide (0.019 M) and 0.05 ml cytosolic fraction of both brain regions in a final volume of 3.0 ml. Changes in absorbance were recorded at 240 nm. Catalase activity was calculated and expressed in terms of k min⁻¹.

**Nitrite estimation**

Nitrite was estimated in the cytosolic fraction of different brain regions using the Greiss reagent and served as an indicator of nitric oxide production. A measure of 500 μl of Greiss reagent (1:1 solution of 1% sulphanilamide in 5% phosphoric acid and 0.1% naphthylamine diamine dihydrochloric acid in water) was added to 100 μl of post-mitochondrial supernatant and absorbance was measured at 546 nm (Green et al. 1982). Nitrite concentration was calculated using a standard curve for sodium nitrite and nitrite levels were expressed as μg/ml. Although, the Griess spectrophotometric assay is not a leading methodology for the quantification of nitric oxide, it employs an indirect measure of nitric oxide content.

**Rat TNF-α and IL-1β ELISA**

Quantifications of TNF-α and IL-1β (R&D Systems Quantikine Rat TNF-α and IL-1β immunoassay kit, USA) were done in the cytosolic fractions of different brain regions according to the manufacturer’s instructions. The Quantikine Rat TNF-α and IL-1β immunoassay is a 4.5-h solid phase ELISA designed to measure rat TNF-α and IL-1β levels. The assay employs the sandwich enzyme immunoassay technique. A monoclonal antibody specific for rat TNF-α or IL-1β is pre-coated in the microplate. Standards, control and samples are pipetted into the wells and any rat TNF-α or IL-1β present is bound by the immobilized antibody. After washing away any unbound substance, an enzyme-linked polyclonal antibody specific for rat TNF-α or IL-1β is added to the wells. Following a wash to remove any unbound antibody-enzyme reagent, a substrate solution is added to the wells. The enzyme reaction yields a blue product that turns yellow when the stop solution is added. The intensity of the colour measured is in proportion to the amount of rat TNF-α or IL-1β bound in the initial steps. The sample values are then read off the standard curve. Values are expressed as mean ± S.E.M.

**Quantification of NF-κB p65 unit**

The NF-κB/p65 ActivELISA kit (Imgenex, USA) was used to measure NF-κB-free p65 in the nuclear fraction of different brain regions. The nuclear levels of p65 may correlate positively with the activation of the NF-κB pathway. The NF-κB ActivELISA is a sandwich ELISA in which free p65 was captured by anti-p65 antibody-coated plates and the amount of bound p65 was detected by adding a second anti-p65 antibody followed by alkaline phosphatase conjugated secondary antibody using colorimetric detection in an ELISA plate reader at 405 nm. The results were expressed as ng/mg of protein.

**Caspase-3 colorimetric assay**

Caspase-3, also known as apopain, is an intracellular cysteine protease that exists as a pro-enzyme and becomes activated during the cascade of events associated with apoptosis. The tissue lysates/homogenates can then be tested for protease activity by the addition of a caspase-specific peptide that is conjugated to the colour reporter molecule p-nitroaniline (pNA). The cleavage of the peptide by the caspase releases the chromophore pNA, which can be quantitated spectrophotometrically at a wavelength of 405 nm. The level of caspase enzymatic activity in the cytoplasmic fraction of different brain regions is directly proportional to the colour reaction. The results were expressed as percentage of control.

**Statistical analysis**

Results were expressed as mean ± S.E.M. The inter-group variation was measured by one-way analysis of variance (ANOVA) followed by Tukey’s test using Jandel SigmaStat statistical software version 2.0 (Jandel Corporation, USA). Two-way ANOVA followed by Tukey’s test was employed to discover the inter-group variation in escape latency data of the Morris water maze using SPSS Statistical Software version 16 (SPSS Inc., USA). A value of p < 0.05 was considered statistically significant.
Results

Behavioural observations

Effect of EGCG on alcohol-induced cognitive deficits in the Morris water maze task

The cognitive function was assessed in the Morris water maze test. The mean escape latency did not differ between any of the groups on the first day of testing in the Morris water maze but from day 2 onwards, there was significant difference in transfer latency. Alcohol-treated animals showed a lower ability to find the platform and learn its location in day 5 of training (Fig. 2a). This poor performance was mitigated by the chronic treatment with EGCG and decreased latency to find the platform from day 2 of training ($F_{5,20} = 8.76, p < 0.001$; day $\times$ treatment interaction: $F_{5,20} = 2.27, p < 0.05$).

In the probe trial, which measures how well the animals had learned and consolidated the platform location during the training, animals showed a significant difference. The time spent in the target quadrant was significantly less in ethanol-treated rats compared to the control group. The rats chronically treated with EGCG (50 and 100 mg/kg) spent more time in the target quadrant than the ethanol-treated group in the probe test ($p < 0.05$). However, EGCG per se had no effect on cognition (Fig. 2b).

Memory assessment in elevated plus maze test

Initial transfer latency (ITL) did not differ significantly in any of the groups. Retention transfer latency (RTL) of the control and per se groups was significantly less than that of the ethanol-treated group (Fig. 3). Treatment with EGCG (50 and 100 mg/kg) significantly ($p < 0.05$) lowered the RTL in ethanol-treated pups signifying improvement in learning and memory.

Effect of EGCG treatment on BACs

We measured the BAC only up to 4 h after ethanol administration as this corresponds to the time-point which had previously been reported to represent peak BAC following ethanol intubation (Han et al. 2005). Administration of ethanol to 7-d-old rat pups resulted in blood ethanol concentrations of 159 and 301 mg/dl at 2 h and 4 h of ethanol administration, respectively (Fig. 4). The blood levels of alcohol remained unaltered on treatment with EGCG at different
time-points suggesting that EGCG does not interfere with the absorption of ethanol.

**Effect on mean weight of different brain regions**

The mean weight of whole brain, cerebral cortex and hippocampus in ethanol-treated pups was decreased by 34.48% (Fig. 5a), 39.09% (Fig. 5b) and 34.30% (Fig. 5c), respectively. Chronic treatment with EGCG (50 and 100 mg/kg) significantly \((p < 0.05)\) prevented the decrease in weight of different brain regions of ethanol-treated pups.

**Biochemical and molecular observations**

**Effect of EGCG on chronic ethanol-induced changes in acetylcholinesterase activity**

Acetylcholinesterase activity was increased 2.85-fold in cerebral cortex (Fig. 6a) and 2.36-fold in hippocampal region (Fig. 6b) of ethanol-treated pups. Although the EGCG treatment decreased cholinesterase activity in both brain regions, these levels were still significantly elevated compared to controls. However, EGCG per se had no effect on brain acetylcholinesterase activity.

**Effect of EGCG on chronic ethanol-induced nitrosative stress**

Nitrite levels were significantly elevated in cerebral cortex [2.71-fold (Fig. 7a)] and hippocampus [2.81-fold (Fig. 7b)] of ethanol-treated pups. EGCG (50 and 100 mg/kg) treatment significantly \((p < 0.05)\) inhibited this increase in nitrite levels in different brain areas of ethanol-treated rats (Fig. 7). However, EGCG per se had no effect on brain nitrite levels.

**Effect of treatment on chronic ethanol-induced changes in lipid peroxidation**

Lipid peroxide levels were increased significantly in cerebral cortex [4.26-fold (Fig. 8a)] and hippocampus [3.51-fold (Fig. 9a)] of ethanol-treated neonatal rats compared to the control and per se groups. Chronic treatment with EGCG (50 and 100 mg/kg) produced a significant reduction in lipid peroxide levels in different brain areas of ethanol-treated pups \((p < 0.05)\).
Although the EGCG treatment reduced lipid peroxide levels at both doses (50 and 100 mg/kg) in the two CNS regions (cortex and hippocampus), these levels were still significantly elevated compared to controls except for EGCG (100 mg/kg) in the hippocampus.

Effect of EGCG on chronic ethanol-induced changes in the antioxidant profile

The reduced glutathione (Figs 8b, 9b) and enzyme activity of superoxide dismutase (Figs 8c, 9c) and catalase (Figs 8d, 9d) significantly decreased in the cerebral cortex and hippocampus of ethanol-treated pups compared to the control and per se groups. This reduction was significantly improved by treatment with EGCG (50 and 100 mg/kg) in both brain areas of ethanol-treated neonatal rats (p < 0.05). EGCG enhanced the reduced activities of endogeneous antioxidants (glutathione, superoxide dismutase, catalase) when co-administered with ethanol, but these levels were still far below control levels.

Effect of EGCG on TNF-α and IL-1β

TNF-α [cerebral cortex (7.41-fold) (Fig. 10a), hippocampus (7.19-fold) (Fig. 11a)] and IL-1β [cerebral cortex (12.41-fold) (Fig. 10b), hippocampus (21.90-fold) (Fig. 11b)] levels were significantly elevated in ethanol-treated neonatal rats. EGCG (50 and 100 mg/kg) treatment significantly (p < 0.05) inhibited both TNF-α and IL-1β levels in different brain areas of ethanol-treated neonatal rats but these levels were still significantly (p < 0.05) elevated compared to the control group.

Effect of EGCG on NF-κB

NF-κB p65 subunit was significantly elevated in cerebral cortex [6.73-fold (Fig. 10c)] and hippocampus [4.04-fold (Fig. 11c)] of ethanol-treated pups. EGCG (50 and 100 mg/kg) treatment significantly (p < 0.05) inhibited enhanced NF-κB p56 subunit expression in the nuclear fraction of different brain areas of ethanol-treated pups.
ethanol-treated pups but these levels were still significantly ($p < 0.05$) elevated compared to the control group.

**Effect of EGCG on caspase-3 activity**

Caspase-3 levels were significantly elevated in cerebral cortex [3.25-fold (Fig. 10d)] and hippocampus [3.77-fold (Fig. 11d)] of ethanol-treated neonatal rats. Chronic treatment with EGCG (50 and 100 mg/kg) significantly ($p < 0.05$) inhibited apoptosis in different brain areas of ethanol-treated pups.

**Discussion**

The clinical condition, FAS, represents the most severe manifestation of heavy maternal alcohol consumption during pregnancy among surviving offspring (Jones & Smith, 1973) and includes somatic growth deficiencies, microcephaly (small head for body size), craniofacial...
dysmorphology, and CNS deficits such as behavioural and cognitive problems (Stratton et al. 1996). Less specific, but consequential deficits fall under the umbrella term ‘fetal alcohol spectrum disorders’, and include deficits in somatic growth, brain growth and CNS function. Although factors contributing to the spectrum of findings in individuals with FAS/fetal alcohol effects are uncertain, recent studies indicate that ethanol and CNS depressant drug exposure on a single day during the first postnatal week in rodents, a time corresponding to the third trimester of pregnancy, greatly increases apoptotic neuronal death in many brain regions (Ikonomidou et al. 1999, 2000). In cases of severe damage, this developmental neuronal loss has adverse effects on brain function and development as animals mature (Jevtovic-Todorovic et al. 2003). Wozniak et al. (2004) suggest that ethanol treatment on PD 7 in C57BL/6 mice produces profound impairment in place learning and memory in the water maze at PD 30.

In the present study, ethanol (5 g/kg.d) treated pups from PD 7–9 showed significant memory impairment in both the memory assessment paradigms,
i.e. Morris water maze and elevated plus maze. Our results are supported by the findings of Goodlett & Peterson (1995), who administered 4.5 g/kg.d ethanol to different groups of male and female neonatal rats at different ages to find out the difference due to sex vulnerability and age, on neurotoxic effects of ethanol. They administered ethanol on PD 4–6, PD 7–9, or PD 4–9 and found that both male and female rats given alcohol on PD 4–9 had significant deficits in acquisition and probe trial performance in the Morris water maze. Males receiving PD 7–9 exposure had significant place learning deficits which were as severe as with the full 6-d exposure. Females were not significantly affected by either (PD 4–6 or PD 7–9) 3-d exposure. These findings form the rationale of selecting the 5 g/kg.d dose of ethanol from PD 7–9 in male rat pups in our study.

Recently, numerous lines of evidence have demonstrated that many antioxidants can improve cognitive function (Calabrese et al. 2003; Kontush & Schekatolina, 2004). Many studies have suggested that reversals in age-related memory declines might be accomplished by increasing dietary intake possessing high antioxidant activity (Andres-Lacueva et al. 2005; Jabeen et al. 2007) and this reversal effect has been claimed to be associated with antioxidant activity (Raghavendra & Kulkarni, 2001).

Apart from acting as an antioxidant, EGCG, the main catechin in green tea, also has potent anti-atherosclerotic and anti-inflammatory properties (Katiyar et al. 2007). Williamson et al. (2006) found that EGCG treatment reduced plaques related to AIDS-related dementia in the laboratory.

In our study, chronic treatment with EGCG significantly improved the impaired memory of ethanol-treated pups in both behavioural tests. In the probe trial also, the time spent in the target quadrant is significantly decreased in ethanol-treated pups which was significantly reversed on treatment with EGCG. While there were EGCG-mediated improvements, these cognitive measures were still below those found in the control and EGCG per se groups.

It is known that maintaining blood ethanol concentration >200 mg/dl for four consecutive hours is the minimum condition for triggering apoptotic neurodegeneration (Ikonomidou et al. 2000). We therefore examined BAC to confirm that the apoptotic cell death and resulting cognitive deficits were due to ethanol. Administration of ethanol to 7-d-old rat pups resulted in blood ethanol concentration of 159 and 301 mg/dl at 2 h and 4 h of ethanol administration, respectively. The blood levels of alcohol remained unaffected on treatment with EGCG at different time-points suggesting that EGCG does not interfere with the absorption of ethanol.

A significant increase in lipid peroxide and nitrite levels and marked decrease in the activity of reduced glutathione, superoxide dismutase and catalase in the cerebral cortex and hippocampal region of ethanol-treated pups was also found. Marino et al. (2004) also found impairment of spatial navigation task in the Morris water maze in neonatal rats exposed to ethanol (5.25 g/kg) on PD 7, 8, and 9 along with increased oxidative stress in the hippocampus of pups. In our findings, while ethanol-mediated alterations in the levels of lipid peroxides and these antioxidants were mitigated by EGCG, the ethanol-induced deficits nonetheless persisted, with levels still markedly different from controls. In our study, the markers of oxidative stress remained elevated at the end of PD 28 in different brain regions of pups exposed to ethanol from PD 7–9 suggesting sustained oxidative stress and inflammation. Our findings are supported by results from Marino et al. (2004) who used the same dosing paradigm for ethanol administration and found a significant increase in protein carbonyl, a marker of oxidative stress in the hippocampus of ethanol-exposed pups at PD 29.

Acetylcholine can induce proliferation of glial cells by activating muscarinic receptors coupled to phospholipid metabolism, and act as a trophic factor in developing neurons by preventing apoptotic cell death (Mount et al. 1994). Ethanol has been shown to inhibit both actions of acetylcholine in vitro (Balduini & Costa, 1989). These effects of ethanol may lead to a decreased number of glial cells and to a loss of neurons, which have been observed following in-vivo alcohol exposure (Guerri 1998). In turn, these may be the basis of microencephaly and cognitive disturbances in children diagnosed with FAS (Costa & Guizzetti, 1999). In our findings, we also found significantly enhanced levels of acetylcholinesterase in different brain regions of ethanol-treated pups which was significantly inhibited on treatment with EGCG. Recently, Zhang et al. (2009) also found that oral administration of EGCG enhanced the acetylcholinesterase inhibiting activity of huperzine A. In that study, the authors speculated that the enhancement and complementary effect of EGCG on huperzine A activity may partly be due to the antioxidant property of EGCG.

In addition to oxidative and nitrosative stress, chronic alcohol administration is also associated with enhanced inflammatory response. Very recently Qin et al. (2008) found that ten daily doses of ethanol exposure results in persistent alterations of cytokines
and significantly increases the magnitude and duration of central and peripheral pro-inflammatory cytokines and microglial activation suggesting a role of cytokines in alcohol-induced neuroinflammation. In the present study, we observed a significant elevation in the levels of TNF-α and IL-1β in the cerebral cortex and hippocampus of ethanol-treated rats which is indicative of enhanced neuroinflammation in the two main regions of brain involved in learning and memory. Treatment with EGCG significantly reduced both the cytokines (TNF-α and IL-1β) in different brain regions of ethanol-treated pups and these findings are in accord with results from Yuan et al. (2006), who also found that 6-wk treatment with EGCG prevented alcohol-induced liver injury in rats by blunting the elevated expressions of CD14, TNF-α, COX2 and iNOS.

Apart from increased cytokine levels, we also found significant enhancement in levels of NF-κB and caspase-3 in the cerebral cortex and hippocampus of ethanol-treated pups suggesting a role of apoptotic pathway in alcohol-induced developmental neurotoxicity. Our findings are supported by observations from Jung et al. (2005) who found that chronic exposure to ethanol results in increased amounts of oxidative damage; activation of PKC and NF-κB, which results in DNA fragmentation; and ultimately increased neuronal death through apoptosis or other mechanisms that are responsible for the behavioural deficits including dementia (Jung et al. 2005). Chronic ethanol administration induces inflammatory mediators such as IL-1β, COX2, and iNOS suggesting that ethanol-induced ROS in the brain may be related to NF-κB activation of inflammatory enzymes (Crews et al. 2006). Izumi et al. (2005) also demonstrated that a single day of ethanol exposure in rats at PD 7 results in significant apoptotic neuronal damage throughout the forebrain after 24 h of ethanol administration. In the present study, treatment with EGCG significantly inhibits both NF-κB and caspase-3 in cerebral cortex and hippocampus of ethanol-treated pups.

In addition to increased oxido-nitrosative stress and pro-inflammatory cytokines, the weights of cerebral cortex and hippocampus, the two areas involved in learning and memory, were also found to be significantly decreased in ethanol-treated pups. Our findings are in accord with Shirpoor et al. (2009) who also found a significant decrease in weight of cerebellum and hippocampus of rat offspring with developmental neurotoxicity due to ethanol administration prenatally. Treatment with EGCG significantly prevented the decrease in weight of both the brain regions of ethanol-treated pups.

The findings from the present study suggests that oxidative stress-mediated inflammatory cascade results in activation of apoptotic signalling pathways and may contribute to the learning and memory deficits in an animal model of fetal alcohol spectrum disorders. Treatment with EGCG prevented behavioural deficits induced by postnatal ethanol exposure not only by modulating oxido-nitrosative stress but also by attenuating the elevated levels of pro-inflammatory cytokines (TNF-α and IL-1β), NF-κB and caspase-3 in different brain regions of ethanol-treated pups. Therefore, mechanism underlying the neuroprotective effects of EGCG observed in our study may be due to its antioxidant, anti-inflammatory and neuromodulating activities.

Thus, the present study concluded that EGCG prevents developmental neurotoxicity associated with postnatal alcohol exposure by inhibiting oxido-nitrosative stress-mediated activation of apoptotic signalling pathways and thus has the potential to be a useful therapeutic option against children with fetal alcohol spectrum disorders.

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Statement of Interest
None.

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