Effect of early natal supplementation of paracetamol on attenuation of exotoxin/endotoxin induced pyrexia and precipitation of autistic like features in albino rats

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Abstract
The present study was aimed to test the hypothesis that paracetamol (PCM) can precipitate autistic like features when used to counteract vaccine-induced fever using experimental rat pups. The pups were treated with measles mumps rubella (MMR) vaccine, diphtheria tetanus and pertussis (DPT) vaccines and lipopolysaccharide (LPS) with subsequent PCM treatment. The pups were evaluated for postnatal growth (weight gain, eye opening) and behavior alterations (swimming performance, olfactory discrimination, negative geotaxis, nociception, and locomotor activity) by performing battery of neurobehavioral test. Significant correlation was observed between social behavioral domains (nociception, anxiety and motor coordination) and pro-inflammatory load in the pups when treated with MMR/LPS along with PCM. A significant change in pro and anti-inflammatory (IL-4, IL-6, IL-10) markers were observed in rats treated with PCM, MMR, LPS, DPT alone or in combination with MMR, LPS and DPT (5128.6 ± 0.000, 15,488 ± 0.000 ***, 9661.1 ± 157.29***, 15,312 ± 249.29***, 10,471 ± 0.00***a, 16,789 ± 273.34*** and 12,882 ± 0.00***a). Pups were also scrutinized for the markers of oxidative stress, inflammation and histopathologically. All the treatment groups showed significant alteration in the behavioral changes, oxidative markers (TBARS-in control-4.33 ± 0.02, PCM-9.42 ± 0.18 ***, MMR-5.27 ± 0.15***, MMR + PCM-8.57 ± 0.18***a, LPS-6.84 ± 0.10***, LPS + PCM-4.51 ± 0.30***a, DPT-5.68 ± 0.12***, DPT + PCM-7.26 ± 0.18***a) and inflammatory markers without following any specific treatment. These observation could be accorded to variable phenotypes of autistic spectrum disorders (ASDs).

Keywords Autism · Lipopolysaccharide · Cyclooxygenase · Interleukin · Lipoxygenase · Paracetamol

Introduction
Autism is neurodevelopmental disorder characterized by deficit social interaction, impaired communication along with restricted and repetitive behavior with symptoms usually appearing in the first 2 years of age (Kumar and Bhagat 2012). Worldwide 1–2 per 1000 peoples are diagnosed with autism, with males five times more than girls. A 30% increase in the cases of autism has been reported between 2012 and 2014 in United States (Falcão et al. 2008; Gregory et al. 2009). Neuroanatomical studies have suggested that autism is alteration in the brain development soon after conception and large range of teratogens/drugs have been implicated in the development of this condition. However, the pathophysiology underlying these events is not well understood (Ghanizadeh 2012; Kota et al. 2012). A decade ago, the scientific community agreed that there
is need to study the link between vaccines and autism, as the use of vaccine and the number of children being diagnosed with autism were on the rise. This has lead to several number of scientific theories hypothesizing “vaccine overload”, “vaccine preservative” or measles mumps rubella (MMR)/diphtheria tetanus and pertussis (DPT) vaccine are the leading cause of autism (Kumar et al. 2011). When scrutinized by various researchers no link was established between MMR/DPT vaccines, and autism (Andrade 2016; Saurabh et al. 2012; Raja et al. 2009). Later the association between thiomersal, vaccines and autism was also questioned (Schultz 2010; Verma 2010). However, the growing number of incidence of autism has always been a concern for the parents and a challenge for the scientific community. In last few years, use of paracetamol (PCM) has also been implicated to be associated with autism in children subjected to PCM after vaccination. Evidence now suggests an association between the use of PCM and autism (Ramasubramaniaraja and Niranjandabu 2011; Ding et al. 2012). A plausible hypothesis is the impaired sulphation in the infants with subsequent formation of toxic N-acetyl p-benzoquinone imine (NAPQI) from PCM (Brunton et al. Brunton et al. 2011). In fact a recent population-based study identified country level correlation between indicators of prenatal and perinatal exposures of PCM with autism. The study could not find strong evidence of causality; however, the authors have suggested the biological plausibility of linking between PCM and autism through growing body of experimental and clinical evidence. The US federal vaccine injury compensation program (vaccine court) has awarded millions of dollars of compensation to two children’s with autism for pain and suffering. However, the US government has not admitted that the vaccine caused autism, at least in one of the children. Altogether the association between “vaccines”, “thiomersal”, “paracetamol” and autism appears to be a riddle (Dorsinville 2015).

The vaccines have exotoxin or sometimes endotoxin (with or without thiomersal). Vaccines can initiate an Arthus reaction (type III hypersensitivity reaction) involving the deposition of antigen/antibody complexes in the vascular walls, pleura, pericardium, synovium and glomeruli. This leads to the activation of complement system resulting in cleavage of soluble complement proteins with subsequent activation of polymorphonuclear leukocytes and mast cell degranulation. Subsequently, this leads to the generation of pyrogens who could be endotoxin or exotoxin. The role of endotoxin (as a vaccine or produced after vaccination) has been poorly studied to date. It is our opinion that the poor sulfation/diminished glutathione after PCM therapy and the endotoxin (endogenous or exogenous) could have a role to play in the precipitation of autism and the same is been explored through piece of work.

Materials and methods

Animals

Pregnant albino rats (Wistar strain) were obtained from central animal house, United Institute of Pharmacy, United Group of Institution, Naini, Allahabad, (UP), India. The animals were housed in polypropylene breeding cages, under standard condition of temperature (25 ± 1 °C) with 12 h light/dark cycle and free access to commercial pellets diet and water ad libitum. The experimental protocol was endorsed by “Institutional Animal Ethical Committee (IAEC)” (UIP/IAEC/2014/FEB./03). The study was supervised as per the guidelines laid by Department of Animal Welfare, Government of India.

Drugs and chemicals

PCM was received as gift sample from Yash Pharma Laboratories Private Limited, Baddi, India. MMR vaccine (Tresivac: Serum Institute of India Limited, Pune, India) and DPT vaccine (Comvac 3: Bharat Biotech International Limited, Hyderabad, India) were procured from the local market. Bacterial lipopolysaccharide (LPS) from Genetix Biotech Asia Private Limited. New Delhi, India. All other chemicals used were of analytical grade.

Experimental protocol

All the female rats were transferred to laboratory 1 week before the experiment to acclimatize in the laboratory condition. After 1 week, the animals were divided into eight groups. Each group consisted of four female rats placed in separate cages for delivering pups and treatment started just after the delivery of the pups. Each group should have at least eight pups. The grouped animals were treated in the following way:

- **Group I:** received normal saline (3 mg/kg s.c.).
- **Group II:** was treated with analgesic dose of PCM (50 mg/kg sc.) on postnatal day (PND) 5th.
- **Group III:** received the MMR (0.5 ml i.p.).
- **Group IV:** received the MMR (0.5 ml) with PCM (50 mg/kg s.c.).
- **Group V:** received the LPS (50 µg/kg i.p.).
- **Group VI:** received the LPS (50 µg/kg i.p) with PCM (50 mg/kg s.c.).
- **Group VII:** received DPT (0.5 ml i.p.).
- **Group VIII:** received DPT (0.5 ml i.p) with PCM (50 mg/kg s.c.).
After induction of fever the body temperature of the pups was measured using digital thermometer and was compared with the earlier one (result not included). The postnatal growth and behavioral alterations were observed up to 45 days at different postnatal days (PNDs) as specified in following section. On the 46th day animals were killed using light anaesthesia followed by heart perfusion for the purpose of removal of total blood from the brain. Brains were extracted out without damage. Collected brains were subjected to the biochemical estimations while a brain from a particular group was kept in 20% formalin for the purpose of histopathological studies. All tests were performed on particular PND to avoid tolerance in rat pups.

Postnatal growth and maturation development

Pups were evaluated for weight gain and for eye opening once daily during prepubertal period, i.e., PND 45, 18, respectively. Eye opening scores were rated as: 0 for both eyes closed, 1 for one eye open, 2 for both eyes open (Umashanker and Shruti 2011).

Behavioral tests

Negative geotaxis

Negative geotaxis was contemplated once on PND 11. Pups were timed for completing a 180° turn when placed in a head down position on a 25° inclined surface. Negative geotaxis reflects vestibular function and motor development (Nikhat et al. 2012).

Swimming performance

An aquarium filled with water (28–29 °C) was used for swimming test on PND 25. Each animal was put at the centre of the aquarium and perceived for 5–10 s. The swimming performance was determined according to the position of nose and head (angle) on the surface of water. The angle of swimming was appraised as: 0—head and nose below the surface; 1—nose below the surface; 2—nose and top of head at or above the surface but ears still below the surface; 3—same as two except that water line was at mid-ear level; and 4—same as three except that water line was at the bottom of ears. Swimming is a combination of motor development and consolidation of coordinated reflex responses (Kosif et al. 2008).

Locomotor activity

This was recorded for each animal by the numbers of photo beams crossing in a period of actophotometer in 10 min of interval (Gupta and Shaw 2009).

Olfactory discrimination

The test was conducted on PND 15. The apparatus comprised of a plastic container 20 × 8 × 8 cm³ (l × w × h), two small bins, and a clear plastic cover, which was placed over the bins and container. One end of the container comprised a bin filled with new bedding, while the other end had a bin filled with home cage bedding. A 3 cm² of area was demarcated at the center of the screen. A line was drawn on the screen above each bin. Each pup was allocated in the centrally marked off region on the screen, and the latency to enter the home bedding side by crossing the divided line with the front paws and head was timed. Central placement of the pup was balanced by altering the pup facing to or away the experimenter. Age of home bedding was balanced across the groups and averaged 3 days old at testing. The test reflects a nest-seeking response by the olfactory system (Borrelli and Izzo 2000).

Nociception

Nociceptive effects were recorded using tail flick test on PND 25 using a tail flick analgesiometer. The animal was gently restrained, and radiant heat was focused onto its tail. The cutoff time was 9 s. Tail flick measurements were taken three times at 30 s intervals (Gadekar et al. 2010).

Elevated plus maze test (EPM)

EPM was implemented on PND 30. The apparatus consist of two open arms (30 × 5 cm) and two closed arms (30 × 5 × 25 cm) in perpendicular position. The open and closed arms were connected by a transparent acrylic. The floor was made of black acrylic. The maze was 45 cm above the floor. The rat was placed at the center of the plus maze with its nose in the direction of one of the closed arms, and contemplated for 5 min for the measurement of the following parameters: number of entries in the open (NEOA) and closed (NECA) arms (Ahmad et al. 2013).

Biochemical changes

The animals were killed using light ether anesthesia superseded by heart perfusion on PNDs 45. The brains were surgically removed, rinsed with ice-cold saline, dried on filter paper and then tissue homogenates prepared in 10% ice-cold KCl using a tissue homogenizer. The post nuclear fraction obtained by centrifuging the homogenate at 10,000 rpm for 10 min at 4 °C in a refrigerated centrifuge were used for the spectrophotometric estimation of acetylcholinesterase (AchE), thiobarbituric acid reactive substances (TBARS), catalase, superoxide dismutase (SOD), protein carbonyl and tissue glutathione using the methods previously established.
Effect of early natal supplementation of paracetamol on attenuation of exotoxin/endotoxin…

at our laboratory (Kamble et al. 2010; Maury et al. 2012; Priya and Rao 2012; Ahmad et al. 2013).

Histopathological evaluation

Brain tissues were evaluated histopathologically using haematoxylin and eosin staining. The tissues were fixed in paraformaldehyde overnight, succeeded by 70% isopropanol overnight. Tissues were then placed in increasing concentration of isopropanol (70, 90, and 100%), followed by dehydration with 100% xylene. The tissues were embedded in paraffin wax and 5 µm sections were prepared using microtome followed by staining with haematoxylin and eosin (Alqaoumi, Al-Dosari et al. 2011).

Results

Postnatal growth and maturation

Increases in the post natal growth (weight gain) and maturation development (eye opening) was observed in all the groups except in the LPS and PCM treatment, when related to sham control (Fig. 1a, b).

Behavioral tests

A significant decrease in the locomotor activity was observed in all the groups when compared to control. However, the pups treated with LPS and PCM were found to have pronounced decreased locomotor activity when used either alone or in combination (Fig. 1c). A significant reduction was observed in the pups treated with vaccines/LPS and PCM on the negative geotaxis prototype when compared with control (Fig. 1d). The LPS alone and in combination with PCM accorded momentous increase in the thermal nociception time (Fig. 1e). The ontogeny of the swimming behavior was least affected in all the test subjects (Fig. 1f) except in the LPS-treated group who demonstrated slight reduction in score of the swimming performance in early postnatal life. Diminished open arms entries were manifested in the LPS treatment group when perceived for the anxiety like behavior using plus maze paradigm (Fig. 1g). The nest-seeking behavior when arbitrated through the olfactory discrimination was adversely affected in all the test group. Significant reduction in the number of entries toward the home bedding with increased exploration for the new bedding was observed (Fig. 1h). The olfactory clue was more markedly affected in the LPS and PCM treatment groups.

Biochemical assays

Following behavioral assay, the brain tissues were subsequently analyzed for the antioxidant defense by measurement of oxidant and enzymatic antioxidant defense. When compared with the lipid (TBARs) and protein per oxidation (Protein carbonyl), all the test groups demonstrated significant increase in the formation of hydroperoxides and carbonyls with more significant increase by PCM alone and its combination with MMR. Similar pattern of increase occurred in the acetylcholinesterase activity. A significant reduction in the enzymatic activity of SOD with consequent reduction in catalase. The LPS and PCM treated groups showed the most significant reduction in catalase in comparison to control. Similar pattern
of decline was noted in the tissue glutathione level after vaccine and subsequent PCM administration (Table 1).

**Inflammatory markers**

The brain tissues were further analyzed for the presence of proinflammatory, anti-inflammatory and immunoregulatory cytokines. The tissue levels of IL-10 and IL-2 were increased. The tissue levels of IL-2 (immunoregulatory), IL-10 (anti-inflammatory), IL-6 were significantly inflated, whereas IL-1β was reduced in most of the treatments, no significant change was observed in the IL-4 level except in case of LPS associated decline (Table 2). Significant correlation was observed between social behavioral domains (nociception, anxiety and motor coordination) and pro-inflammatory load in the pups when treated with MMR/LPS along with PCM (Table 2). When compared with the enzymatic level of COX and LOX enzymes no significant variation was accorded in the enzymatic level of COX-1, on the contrary significant upsurge was accorded in the enzymatic level of COX-2 in the treatment groups except for the DPT treatment. The concomitant treatment of PCM with LPS was acknowledged with momentous upregulation in the enzymatic activity of 15-LOX (Table 3).

**Histopathology**

When observed histopathologically, no significant variation could be observed between control and PCM treatment. However, MMR, LPS with PCM and DPT with PCM treatment afforded significant neuronal degeneration. No

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**Table 1** Effect on analgesic dose of paracetamol in combination with LPS, MMR and DPT on oxidative stress markers

<table>
<thead>
<tr>
<th>Group</th>
<th>Treatment</th>
<th>TBARs (nanomoles of MDA/mg of protein)</th>
<th>GSH $\times 10^{-2}$ (mg %)</th>
<th>SOD (units of SOD/mg of protein)</th>
<th>Catalase (nanomoles of H$_2$O$_2$/min/mg of protein)</th>
<th>Protein carbonyl (nanomoles/ml)</th>
<th>AchE (nanomoles/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>Control</td>
<td>4.33 ± 0.02</td>
<td>0.65 ± 0.53</td>
<td>1.62 ± 0.14</td>
<td>46.70 ± 1.03</td>
<td>64.40 ± 1.46</td>
<td>0.51 ± 0.00</td>
</tr>
<tr>
<td>II</td>
<td>PCM</td>
<td>9.42 ± 0.18***</td>
<td>0.17 ± 0.01*</td>
<td>1.43 ± 0.11</td>
<td>5.63 ± 0.49***</td>
<td>175.00 ± 1.41***</td>
<td>0.74 ± 0.03***</td>
</tr>
<tr>
<td>III</td>
<td>MMR</td>
<td>5.27 ± 0.15***</td>
<td>0.22 ± 0.02*</td>
<td>0.73 ± 0.52**</td>
<td>2.30 ± 0.39***</td>
<td>149.85 ± 6.57***</td>
<td>0.63 ± 0.04***</td>
</tr>
<tr>
<td>IV</td>
<td>MMR + PCM</td>
<td>8.57 ± 0.18***</td>
<td>0.14 ± 0.001*</td>
<td>1.21 ± 0.05</td>
<td>6.33 ± 0.44****</td>
<td>171.67 ± 0.57***</td>
<td>0.73 ± 0.02***</td>
</tr>
<tr>
<td>V</td>
<td>LPS</td>
<td>6.84 ± 0.10***</td>
<td>0.21 ± 0.003*</td>
<td>1.39 ± 0.06</td>
<td>3.61 ± 0.18***</td>
<td>147.20 ± 2.29***</td>
<td>0.66 ± 0.02***</td>
</tr>
<tr>
<td>VI</td>
<td>LPS + PCM</td>
<td>4.51 ± 0.30***</td>
<td>0.13 ± 0.02*</td>
<td>1.51 ± 0.01</td>
<td>1.62 ± 1.32***</td>
<td>171.14 ± 1.59***</td>
<td>0.73 ± 0.02***</td>
</tr>
<tr>
<td>VII</td>
<td>DPT</td>
<td>5.68 ± 0.12***</td>
<td>0.23 ± 0.01***</td>
<td>1.31 ± 0.05</td>
<td>3.53 ± 0.37***</td>
<td>146.89 ± 3.31***</td>
<td>0.67 ± 0.02***</td>
</tr>
<tr>
<td>VIII</td>
<td>DPT + PCM</td>
<td>7.26 ± 0.18***</td>
<td>0.13 ± 0.01*</td>
<td>1.28 ± 0.03</td>
<td>2.61 ± 0.32***</td>
<td>103.56 ± 6.87***</td>
<td>0.74 ± 0.02***</td>
</tr>
</tbody>
</table>

Values are expressed as Mean ± SD, Each group contains 8 animals
Statistical Significance compared using one-way ANOVA followed by Student–Newman–Keuls test
All groups were compared to the control (***P < 0.001, **P < 0.01, *P < 0.05)
Group III, V, VII compared to Group IV, VI, VIII, respectively (**P < 0.001, *P < 0.01)

**Table 2** Effect on analgesic dose of paracetamol in combination with LPS, MMR and DPT on Interleukins

<table>
<thead>
<tr>
<th>Group</th>
<th>Treatment</th>
<th>IL-1β (pg/ml)</th>
<th>IL-2 (pg/ml)</th>
<th>IL-4 (pg/ml)</th>
<th>IL-10 (pg/ml)</th>
<th>IL-6 (pg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>Control</td>
<td>602.56 ± 0.000</td>
<td>586.36 ± 22.90</td>
<td>589.41 ± 36.44</td>
<td>1778.3 ± 0.00</td>
<td>5128.6 ± 0.000</td>
</tr>
<tr>
<td>II</td>
<td>PCM</td>
<td>616.59 ± 0.000</td>
<td>933.50 ± 30.39**</td>
<td>408.79 ± 0.67***</td>
<td>1949.8 ± 0.00***</td>
<td>10,968 ± 357.08***</td>
</tr>
<tr>
<td>III</td>
<td>MMR</td>
<td>555.94 ± 9.051**</td>
<td>1189.2 ± 58.06***</td>
<td>585.84 ± 29.56</td>
<td>1659.6 ± 0.00***</td>
<td>15,488 ± 0.000***</td>
</tr>
<tr>
<td>IV</td>
<td>MMR + PCM</td>
<td>295.12 ± 0.00***</td>
<td>1373.2 ± 64.82***</td>
<td>540.96 ± 21.13</td>
<td>966.11 ± 15.73***</td>
<td>9661.1 ± 157.29***</td>
</tr>
<tr>
<td>V</td>
<td>LPS</td>
<td>732.88 ± 11.93***</td>
<td>841.90 ± 41.11***</td>
<td>237.97 ± 3.49***</td>
<td>1862.1 ± 0.00**</td>
<td>15,312 ± 249.29***</td>
</tr>
<tr>
<td>VI</td>
<td>LPS + PCM</td>
<td>367.89 ± 29.99***</td>
<td>857.04 ± 2.79***</td>
<td>531.52 ± 7.79***</td>
<td>3311.3 ± 0.00***</td>
<td>10,471 ± 0.00***</td>
</tr>
<tr>
<td>VII</td>
<td>DPT</td>
<td>441.60 ± 7.19</td>
<td>1175.4 ± 93.68***</td>
<td>787.10 ± 12.82***</td>
<td>1084.6 ± 52.96***</td>
<td>16,789 ± 273.34***</td>
</tr>
<tr>
<td>VIII</td>
<td>DPT + PCM</td>
<td>609.57 ± 9.93***</td>
<td>787.99 ± 11.55***</td>
<td>451.56 ± 41.41***</td>
<td>1429.0 ± 23.27***</td>
<td>12,882 ± 0.00***</td>
</tr>
</tbody>
</table>

Values are expressed as Mean ± SD, each group contains 8 animals
Statistical Significance compared using one-way ANOVA followed by Student–Newman–Keuls’s test
All groups were compared to the control (***P < 0.001, **P < 0.01, *P < 0.05)
Group III, V, VII compared to Group IV, VI, VIII, respectively (**P < 0.001, *P < 0.01)
significant neuronal loss was observed in any of the test groups (Fig. 2).

Discussion

Autism is a pervasive neurodevelopmental disorder with an alarming increase in last four decades (Jain et al. 2010; Thirunavukkarasu et al. 2010; Venkatalakshmi and Senthamaraiselvi 2012). A wide range of definitions/descriptions are available with most of them describing it as an neurodevelopmental disorder associated with impaired social interaction, restricted and repetitive stereotype behavior, abnormalities in verbal and nonverbal communications (Patankar et al. 2011). The present work is an attempt to elucidate the underlying pathology beneath the ASD by performing series of tests during prepubertal period. (1) We observe continued weight gain in all the experimental groups except for the LPS group (which is well known for causing stress like conditions) and its combination with PCM during prepubertal period (E 45 after the birth). (2) Significant delay in the eye opening (maturation development) was accorded in the pups subjected to LPS and PCM combination. (3) When related to the locomotor activity, hyperlocomotor activity (compulsive non goal behavior) was observed in MMR and PCM combination treatment with repetitive/stereotype behavior. Similar activity of repetitive behavior was observed with DPT but decline locomotor activity while LPS showed diminished locomotor activity. The pups treated with LPS and PCM also had lower sensitivity to the spinal pain without adversely affecting the time for alignment on negative geotaxis paradigms, motor skills and cerebral integration. The motor skills were also scrutinized through swimming performance test and no significant adversity was accorded in any of the treatment groups, which is in line with the negative geotaxis paradigm. Limited environmental exploration is one of the core features in the clinical as well as preclinical cases of autism (Karuppusamy and Pullaiah 2009). Decreased exploratory behavior has been accredited to the reduced number of purkinje fibers or alteration in the neuronal structure of cortex and amygdale. A distinguished decline in the locomotor activity, suggesting decreased exploratory behavior was also observed through EPM paradigm. Slight decrease in NEOA was observed as a measure of anxiety like behavior in the pups. Comparing to the various communicational difficulties, the autistic subject are evident for the social deficits which has been argued to be associated with amygdala and the same was explored through olfactory discrimination test. The pups were ascertained with the decreased nest-seeking response subsequent of PCM treatment contributing the social deficit. The behavioral abnormalities (except motor coordination) was evident in the current experiment are inline with the various antecedents preclinical finding. It is of note that the use of PCM was observed to be too much more closely associated with behavioral abnormalities in comparison to the vaccine alone. Moreover, the LPS and PCM treatment was more profoundly associated with behavioral changes among all.

In addition to oxidative stress, changes in the immune and inflammatory response, abnormal mitochondrial functions are suggested in the pathogenesis of ASD. Marked increase was in the markers of lipid (TBARS) and protein (protein carbonyl) peroxidation. The triple regulated enzymatic defense of SOD/catalase/GSH represent a cellular mechanism to combat oxidative stress (Venkataswamy et al. 2010; Purohit et al. 2013). A significant decline in the enzymatic defense of SOD/catalase/GSH was observed in the present experiment irrespective of the treatment. The decline in the oxidative defense clearly reflects the participation of oxidative stress. It is to be noted that the LPS and PCM treatments were more markedly affected in comparison with other groups. The cholinergic abnormalities are the well-studied phenomenon in autistic subject. In fact the basal forebrain which is involved in the

<table>
<thead>
<tr>
<th>Groups</th>
<th>Treatment</th>
<th>COX-1 (% inhibition)</th>
<th>COX-2 (% inhibition)</th>
<th>15-LOX (% inhibition)</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>Control</td>
<td>0.029 ± 0.03</td>
<td>0.020 ± 0.001</td>
<td>0.022 ± 0.005</td>
</tr>
<tr>
<td>II</td>
<td>PCM</td>
<td>0.072 ± 0.01</td>
<td>0.026 ± 0.0003**</td>
<td>0.023 ± 0.004</td>
</tr>
<tr>
<td>III</td>
<td>MMR</td>
<td>0.020 ± 0.001</td>
<td>0.026 ± 0.0003**</td>
<td>0.014 ± 0.005</td>
</tr>
<tr>
<td>IV</td>
<td>MMR + PCM</td>
<td>0.12 ± 0.009***b</td>
<td>0.026 ± 0.000**</td>
<td>0.0035 ± 0.0003ac</td>
</tr>
<tr>
<td>V</td>
<td>LPS</td>
<td>0.026 ± 0.032</td>
<td>0.032 ± 0.0003***</td>
<td>0.020 ± 0.000</td>
</tr>
<tr>
<td>VI</td>
<td>LPS + PCM</td>
<td>0.13 ± 0.0042***b</td>
<td>0.027 ± 0.002**b</td>
<td>0.043 ± 0.004***</td>
</tr>
<tr>
<td>VII</td>
<td>DPT</td>
<td>0.038 ± 0.002</td>
<td>0.024 ± 0.002**</td>
<td>0.037 ± 0.0007***</td>
</tr>
<tr>
<td>VIII</td>
<td>DPT + PCM</td>
<td>0.006 ± 0.0004</td>
<td>0.021 ± 0.001*</td>
<td>0.0074 ± 0.001a</td>
</tr>
</tbody>
</table>

Values are expressed as Mean ± SD, each group contains 8 animals
Statistical Significance compared using one-way ANOVA followed by Student–Newman–Keuls test
All groups were compared to the control (**P < 0.001, *P < 0.01, *P < 0.05)
Group III, V, VII compared to Group IV, VI, VIII, respectively (*P < 0.001, bP < 0.01)
attention is majorly confined with cholinergic neurons is adversely regulated in autistic subjects. A recent study has demonstrated that the increase in the acetylcholine levels can improve the cognitive rigidity and social deficit in the mouse model of autism (Kumar et al. 2009), suggesting decreased cholinergic innervations in autism. Similar inline of finding were contemplated in the present study, with increased AchE activity. It would be pertinent to mention that the PCM treatment was associated with more profound rise in AchE activity than vaccines. Henceforth, an inflated level of neuronal acetylcholinesterase (AchE) enzyme inactivates the acetylcholine in brain, especially in the basal fore brain which interfere with learning and cognitive functions in an affected subject.

The cytokines play a pivotal role in governing the inflammatory and immunological responses in the neurological...
circuits. The underlying mechanism of the immune dysregulation and neuronal dysfunction is poorly understood. However, there are preclinical and clinical evidence suggesting that dysregulation in cytokine production/signaling/regulation is associated with neurodevelopmental impairment and behavioral changes (Amr and Maysa 2010; Nigam and Paarakh 2011). Considering these factors we determined the levels of proinflammatory (IL-1β and IL-6), anti-inflammatory (IL-4 and IL-10) and immune-regulatory (IL-2) cytokines. The IL-6 and IL-1β are reported to be associated with neuronal proliferation, survival, death along with regulation of synaptic plasticity (Gilmore et al. 2005; Vinothapooshan and Sundar 2010; Barker et al. 2001).

Previous clinical studies have argued for the over expressed levels of proinflammatory cytokines in the autistic children either with regression or without regression. A strong correlation was reported between the autism-associated behavioral abnormalities and the proinflammatory cytokines. A significant upsurge in the IL-6 was observed in all the treatment groups when compared to control, which is in corroborations with the previous preclinical and clinical findings (Thirunavukkarasu et al. 2009). However, treatment with the vaccines and LPS attributed maximum upsurge in the level of IL-6. Moreover, the PCM therapy afforded further increase in the IL-6 levels. When compared with the IL-1β, the vaccines and PCM treatment were able to downregulate the expression of IL-1β, which is not in line with previous finding. The present authors are in opinion that the IL-1β is proinflammatory cytokines expressed as an early immune response and in view of the long duration of the study one can expect a compensatory downregulation in the IL-1β levels to counteract the inflammation. The autistic subjects are reported to have an overload pro inflammatory cytokines. In fact a positive correlation has been shown between the social impairment and IL-6: IL-10/IL-1β: IL-10. The present study compared the effects of the DPT vaccine when given alone and MMR vaccine when given in combination with PCM could afford to have highest proinflammatory load in terms of IL-6: IL-10 and IL-1β: IL-10.

IL-2 is signaling molecule of immune system and is reported to be overexpressed 2–3-fold in autistic subjects. Similar pattern of overexpressed IL-2 was perceived after the MMR and DPT vaccines in the present study. The subsequent use of PCM afforded nonsignificant downregulation of IL-2 in the MMR and DPT treated groups. The upregulated immune response has been associated with increased impairment in social interaction, communication and stereotypical behavior in autistic subjects (Rafatullah et al. 1994; Minaiyan et al. 2012). The previous reports as well as the current study suggest that the immune dysfunction could affect the core features associated with ASD. The IL-4 and IL-10 are the anti-inflammatory cytokines, among which the IL-4 is reported to be poorly correlated with no significant variability between the control and autistic subjects. Similar line of observation was contemplated through present piece of work except for the MMR and DPT treated groups. The autistic subjects are very well documented for the decreased IL-10 expression and same was found present study. The correlation analysis revealed various levels of positive correlation was observed between behavioral domains (nociception, anxiety and motor coordination) and LPS and PCM combination therapy. Similarly, MMR and PCM treatment was also observed to be positively correlated with motor coordination and thermal nociception paradigms. The cytokines profiling as depicted in our study highlight a relation between the expressions of various cytokines and is complementing the previous clinical reports on this aspect (Howland et al. 2006; Tripathi 2008).

In a recent theory, poor sulfation has been accorded to be associated with autism and is hypothesized to be one of the possible mechanisms underlying the pathology. However, from the line of evidences derived from the current study, we are in opinion that it is not only PCM, rather the dysregulated immune/inflammatory circuits (due to immunization or any inflammatory disorder) and accumulation of unmetabolized catecholamine in the brain (due to poor sulfation of phenol and amines induced by PCM) (Sakat et al. 2012; Aggarwal et al. 2011) could render the highly susceptible brain of a new born for neurobehavioral and neurotoxic effects. The present hypothesis is also supported by one of the recent study from our laboratory, through which we inquested the effect of terbutaline siblings in precipitation of ASDs in albino rats (Sharma et al. 2015). The study showed that salbutamol, salmeterol and montelukast can precipitate variable symptoms associated with ASDs. We would also like to rule out that use of PCM to combat vaccine-induced fever can precipitate ASDs in newborn due to poor sulfation, first owing to the results derived from the present study and second to that fact, that the use of PCM in newborns is not restricted to vaccine-associated pyrexia only.

Altogether, we conclude that the use of PCM to counteract vaccine/s induced pyrexia can precipitate different symptoms pertaining to autism. It would be appropriate to mention that epidemiological studies in the newborns vaccinated with painless vaccines (e.g., DTaP vaccine) would be helpful to provide further into the issue. Authors would also like to add that considering the variable set of accompanying phenotypes, is the need of hour to bifurcate various subgroups of ASD on immunological criteria and develop a set diagnostic marker to provide a valuable implications for the diagnosis as well as monitoring of ASDs. Authors would also like to mention that the data presented in the present manuscript should be treated with caution until validated further.
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Compliance with ethical standards

Conflict of interest The authors declare that they have no competing interest.

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