# Effects of Chronic Administration of *P*-Cymene in an Animal Model of LPS-Induced Autism



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### ABSTRACT

p-Cymene is a monoterpene found in over 100 plant species. It shows a range of biological activity, including anti-inflammatory and antimicrobial effects. It is possibly a new therapeutic alternative for autism spectrum disorder characterized by deficits in interaction and behavioral abnormalities. These symptoms can occur in response to maternal immune activation through prenatal exposure to lipopolysaccharide. Thus, this study aimed to evaluate the behavioral, memory, and biochemical effects of chronic administration of p-cymene in an animal model of autism by prenatal maternal exposure to lipopolysaccharide. Twenty-four pregnant Wistar rats were used, who received 100 µg/kg of lipopolysaccharide or saline intraperitoneally (i.p.) on the 9.5 gestational day. After birth, the male offspring remained with the mothers until weaning and underwent model validation tests on postnatal day 30. From postnatal day 31 on, chronic administration, via i.p., of saline (1 mL/kg), risperidone (0.2 mg/ kg), or p-cymene (100 mg/kg) for 22 days was performed. The animals were submitted to behavioral (postnatal day 52) and memory tests (postnatal days 52-53) and subsequently sacrificed (postnatal day 54) when their brain structures were removed for quantification of proinflammatory cytokines (TNF- $\alpha$ , interleukin 1 $\beta$ , and interleukin 6). Prenatal exposure to lipopolysaccharide significantly increased episodes of stereotyped movement (p=0.0001) and decreased parameters of social interaction in offspring, including sniffing, following, mounting, and allowing mounting (p = 0.0043, p < 0.0001, p = 0.0009, and p = 0.0200, respectively). Chronic p-cymene treatment was not significant for behavioral, memory, and biochemical tests. However, due to their pharmacokinetic characteristics, p-cymene nanomaterials' formulation may be an exciting alternative to be tested for further results.

### ABBREVIATIONS

ASD	autism spectrum disorder
ATP	adenosine triphosphate
CNS	central nervous system
GD	gestational day
IFN-γ	interferon-gamma
IL-1β	interleukin 1 beta
IL-6	interleukin 6
KC	keratinocyte-derived chemokine
LPS	lipopolysaccharide
LTM	long-term memory
MAPK	mitogen-activated protein kinase
MCP-1	monocyte chemoattractant protein-
MIA	maternal immune activation
MMP	matrix metalloproteinases
PND	postnatal day
RISP	risperidone
SAL	Saline solution (0.9% NaCl)
STM	short-term memory
TIMP	tissue inhibitors of MMPs

## Introduction

*p*-Cymene belongs to the monoterpene class and is characterized as an aromatic organic compound [1, 2] precursor to carvacrol [3], naturally found in the oil of more than 100 species of gymnosperm and angiosperm plants [2, 4], also occurring in over 200 foods, including carrots, raspberries, tangerines, grapes, orange juice, butter, nutmeg, oregano, and in almost all spices [5, 6]. Studies show that *p*-cymene has anti-inflammatory [7], antimicrobial [8], and other activities [1].

The antimicrobial activities of *p*-cymene are one of its primary mechanisms of action since, due to its broad spectrum against gram-positive and gram-negative bacteria [8], it has been shown to block the release pathways of proinflammatory cytokine MAPK and I*k*B. In mice with pulmonary emphysema, Games et al. [2] suggested that monoterpenes, including *p*-cymene, reduce the inflammatory process and oxidative stress. This action is due to the significant decrease in proinflammatory cytokines, KC, transcription factor NF-*k*B, and isoprostane levels, avoiding pulmonary remodeling due to the imbalance between MMs and TIMPs, beyond collagen deposition, enhancing its anti-inflammatory and antioxidant potential [4].

The search for new therapeutic alternatives that are more effective, safer, and less toxic has been a constant among scientists studying disorders such as ASD. ASD is defined as a continuous complex of disorders of neurological development with early onset in childhood [9, 10], presenting a not fully elucidated etiological basis, which involves a complex interaction between genetic susceptibility and environmental exposures [11], as well as changes in the immune system [12].

Studies show that autistic individuals, in addition to presenting cognitive, behavioral, and memory deficits and their entire pathophysiological framework, may also have suffered, in the prenatal period, changes in the immune environment, which in turn represent the interface between the organism and the environment [13, 14]. Brain immune molecules play an essential role in the development and homeostasis of the organism where, in response to immune activation, overexpression of these molecules is detrimental to neural development [15]. Such immune molecules become key points, both in the normal development of neuronal activities and in the pathophysiological processes involving the imbalance of these factors [16].

The effects of prenatal maternal infection on fetal brain development can be studied from the animal model of autism by MIA [17, 18]. MIA may be experimentally induced by immunogens such as bacterial LPS on the 9.5 GD [19]. The induction will influence maternal cytokine signaling by crossing the placenta, affecting the fetal brain's development, which will result in a series of neural and behavioral abnormalities [20, 21] similar to autistic-type characteristics.

Therefore, considering neuroinflammation as an etiological hypothesis of ASD, it is assumed that chronic and controlled administration of *p*-cymene can reverse the behavioral and neurochemical conditions presented by autistic individuals. Thus, the present study's main objective was to evaluate the behavioral, cognitive, memory, and anti-inflammatory effects of chronic *p*-cymene administration in an experimental LPS-induced model of autism in rats.

## **Results and Discussion**

## Validation of the autism model

Stereotype test: episodes and time of grooming and reciprocal social interaction

Increased stereotyped (*grooming*) movements are associated with the repetitive behavior of individuals with ASD. When compared to the SAL group, it was observed that prenatal exposure to LPS significantly increased the number ( $\triangleright$  Fig. 1a) and time ( $\triangleright$  Fig. 1b) of grooming (p<0.001).

The animal's social behavior, on the other hand, can be assessed through the reciprocal social interaction test [22], since one of the main characteristics of ASD is decreased social interaction [23]. This test showed that prenatal exposure to LPS caused significant decreases in all parameters, which were sniffing (p = 0.0043) ( $\triangleright$  Fig. 2a), following (p < 0.0001) ( $\triangleright$  Fig. 2b), mounting (p = 0.0009) ( $\triangleright$  Fig. 2c), and allowing mounting (p = 0.0200) ( $\triangleright$  Fig. 2d), when compared to the SAL group. Thus, it can be said that a decrease in interaction between LPS animals was observed.

These results showed that the 100  $\mu$ g/kg dose of LPS administered in GD 9.5 promotes impairment to offspring, causing autistic-type symptoms. The unhealthy behavior statistically demonstrated in offspring due to exposure to LPS refers to a decrease in locomotion, *rearing*, and *grooming* activities, as well as the presence of reciprocal social interaction deficits. These data are similar to the results presented by Spencer et al. [59] and Kirsten and Bernardi [19], thus validating the present experimental model.









## Behavioral assessments

## Open field test

Twenty-four hours after the last administration of drugs and solutions, behavioral assessments were initiated by the open field test, which aimed to measure stereotyped behaviors and exploratory locomotor activity. Changes in these behaviors may be related to hyperactive and stereotypical conditions, which are signs and symptoms associated with ASD [25]. Firstly, in the open field test, the stereotyped behaviors (*grooming*) and locomotor activity (*crossing* and *rearing*) presented by the offspring after chronic treatment were evaluated.

The cleaning time (seconds) performed by the animal, considering cleaning of the paw, legs, and/or tail and cleaning of the head and/or body and the nose and/or face (grooming), corresponded to the stereotyped movements [26, 27], which are shown in  $\triangleright$  Fig. **3a**. For this parameter, the autistic-type behavior group showed a significantly increased difference (p < 0.001) compared to the saline group, thus suggesting the presence of unhealthy behavior, as related in the work of Fortunato et al. [28] in rats exposed prenatally to LPS.

The crossing parameter (▶ Fig. 3b) aims to quantify the animal's exploratory locomotor activity [29], and, in this parameter, no significant difference was observed.

*Rearing* refers to the amount of elevation of the front paws without touching the open field walls, being defined as an exploratory locomotor activity [30, 31]. Although there was no significant statistical difference between the control (SAL + SAL) and induced autistic behavior (LPS + SAL) groups, LPS + SAL tended to decrease this





parameter compared to the control group (p = 0.0521) ( $\blacktriangleright$  **Fig. 3c**). Furthermore, an increase in *rearing* episodes was observed in rats with autistic-type behavior treated with RISP (LPS + RISP) compared to the group of *p*-cymene-treated autistic rats (LPS + *p*-cymene; p = 0.0328).

## Object recognition test

Studies show that the brains of autistic individuals have decreased functional connectivity, thus causing memory impairment [32]. In order to measure non-spatial memory, assessing the natural tendency of animals to explore new items [33], after chronic administration of saline, RISP, and *p*-cymene, the amount of exploration of the object was measured ( $\triangleright$  Fig. 4a), as well as the time spent on each exploratory act ( $\triangleright$  Fig. 4b).

According to Czerniawski and Guzowski [34], immunogenic agents, such as LPS and cytokines, can disrupt the hippocampus' learning and memory processes. Our results showed that chronic treatment with *p*-cymene in animals exposed prenatally to LPS, as well as their controls (SAL and RISP), did not differ in the object recognition index in both STM and LTM.

## Analysis of TNF- $\alpha$ , interleukin 1 $\beta$ , and interleukin 6 proinflammatory cytokines

Based on the critical role of the immune system in brain function, development, health, and disease, prenatal experiences become crucial for fetal health. Proinflammatory cytokines include IL-1 $\beta$ , IL-6, TNF- $\alpha$ , and IFN- $\gamma$ , resulting in amplifying inflammatory responses [35]. It is suggested that gestational complications in the peri-, pre-, and neonatal periods have a strong correlation with the development of ASD [36, 37]. Such imbalance in the maternal-fetal immune system may be related to neuroinflammation caused by the release of proinflammatory cytokines, most prominently IL-1 $\beta$ , IL-6, and TNF- $\alpha$ , involved in the genesis of brain damage. According to the preclinical study in Wistar rats by Spencer et al. [24], prenatal LPS exposure can induce inflammation in the fetal CNS by exacerbating the release of proinflammatory cytokines, which modulate neuronal activity, leading to neurotransmission deficits and, consequently, the development of behaviors similar to ASD.

The expression of TNF- $\alpha$ , IL-1 $\beta$ , and IL-6 cytokines was quantified in the animals' prefrontal cortex and hippocampus. As can be seen in ▶ Fig. 5, prenatal LPS exposure (LPS + SAL) showed no significant statistical differences in cytokine levels (TNF- $\alpha$ , IL-1 $\beta$ , and IL-6) in the prefrontal cortex (> Fig. 5a) and the hippocampus (> Fig. 5b) when compared to the control group (SAL + SAL). Regarding treatment in the prefrontal cortex, when measuring IL-1 $\beta$ (**Fig. 5a**, IL-1 $\beta$ ), there is a significant increase in this proinflammatory cytokine in the LPS + RISP (p = 0.0223) and LPS + p-cymenetreated groups (p = 0.0339) when compared to their respective control groups (SAL + RISP and SAL + p-cymene). The same is true for the assessment of IL-6 levels (▶ Fig. 5a, IL-6), where the groups chronically treated with p-cymene (LPS + p-cymene) and RISP (LPS + RISP) showed a significant increase of this cytokine (p = 0.05and p=0.01, respectively) when compared to their respective control groups (SAL + RISP and SAL + p-cymene). In the TNF- $\alpha$  analysis, no significant results were found either in the prefrontal cortex or in the hippocampus ( $\triangleright$  Fig. 5b, TNF- $\alpha$ , IL-1 $\beta$ , and IL-6).





The proinflammatory cytokine analyses showed that in the prefrontal cortex, both RISP and *p*-cymene increased the levels of IL-1 $\beta$  and IL-6 in animals exposed to LPS prenatally, which suggest that both compounds are acting as proinflammatory agents. Whereas in the hippocampus, the same analyses showed no statistical significance. Our data support the hypothesis that RISP affects cytokine production, and these data are corroborated in several other *in vivo*[38–40] and *in vitro* [41,42] studies. *In vitro*, RISP has been observed to increase Th2 cytokines, such as anti-inflammatory cytokine IL-10 [41,42]. Already in the culture of the mononuclear cell of peripheral blood [41] and monocyte-derived dendritic cells [42], there is an increase in proinflammatory cytokines IL-6, IL-8, and TNF- $\alpha$ [38,39].

In vivo, Song et al. [40] evaluated the influence of RISP treatment on proinflammatory cytokines in 62 schizophrenic patients for 6 months. Their results demonstrated that there was an initial anti-inflammatory effect, which reduced over time, followed by an increase in serum levels of IL-1 $\beta$ , IL-6, and TNF- $\alpha$  in the second month of treatment, until reaching levels of cytokines close to baseline levels in the sixth month.

Regarding *p*-cymene, studies show that when administered acutely, it had significant anti-inflammatory effects [43], reducing the production of proinflammatory cytokine production and infiltration of inflammatory cells. Mechanistically, *p*-cymene blocks the NF- $\kappa$ B and MAPK signaling pathways, reducing TNF- $\alpha$ , IL-6, and IL- $\beta$  production in LPS-treated RAW 264.7 macrophages [44]. Our data, however, differ from them, possibly due to the chronic effect of using *p*-cymene. Contrary to what is known about acute adminis-

tration, *p*-cymene showed proinflammatory activity in the CNS when used for a prolonged period.

The results found in this work may be related to the dose-dependent vasorelaxation capacity of *p*-cymene, described in the aorta of Wistar rats with the  $K_{ATP}$  and  $K_{IR}$  participation channels [45]. The  $K_{ATP}$  (ATP-sensitive potassium channels) are members of the  $K_{IR}$  superfamily (internal rectifying K<sup>+</sup> channels), initially described in the myocardium [46], but which are also widely expressed in the CNS. In response to a metabolic challenge,  $K_{ATP}$  opens up, specifically to a decrease in ATP and/or an increase in adenosine diphosphate, leading to its hyperpolarization [47], playing an important role in regulating linking cellular metabolism to electrical activity of cell membranes [48]. These data suggest that chronic administration of *p*-cymene culminates in the hyperpolarization of neuronal cells through  $K_{ATP}$  channels, thus contributing to an increase in the recruitment of immune cells in the brain.

Finally, the differences regarding the anti-inflammatory effects of chronic administration of *p*-cymene found in the present study can be justified due to the limitation of monoterpenes concerning bioavailability and stability. *p*-Cymene has low water solubility and a brief half-life time of its pharmacological properties  $(0.44 \pm 0.07 \text{ h})$ . Added to this is its high volatility ( $T_{max} = 0.33 \pm 0.11 \text{ h}$ ), which, consequently, significantly reduces its stability [49, 50]. Also, according to Walde et al. [51], *p*-cymene (100 mg/kg) has almost complete urinary excretion of its metabolites (60-80% of the dose) within 48 h. However, of the 18 urinary metabolites already found and identified from *p*-cymene, two were not excreted by the studied rats. It is possible that due to the chronicity of the treatment,



Fig. 5 Effect of chronic administration of saline, risperidone, and *p*-cymene on proinflammatory cytokine levels TNF- $\alpha$ , IL-1 $\beta$ , and IL-6 in an animal model of autism. Concentrations of proinflammatory cytokines in the prefrontal cortex (**a**) and hippocampus (**b**) of chronically treated animals. Data are expressed as the mean ± standard deviation of n = 06 *06 correct*? for each group; \*p<0.05, \*\*p<0.01 (two-way ANOVA with Bonferroni posttest).

only the non-excreted secondary metabolites of *p*-cymene are reaching the brain and promoting proinflammatory effects. *p*-Cresol stands out among the different metabolites, deriving from *p*-cymene obtained by administering eucalyptus oil to *Trichosurus vulpecula*[52]. Such a compound, which easily crosses the bloodbrain barrier, appears to increase MCP-1 expression via NF-*κ*B p65 significantly [53]. MCP-1 is a crucial chemokine acting to regulate the migration and infiltration of monocytes and macrophages in tissues [54]. This data brings to light the hypothesis that possibly *p*-cresol from *p*-cymene is a neuroinflammatory agent. In turn, neuroinflammation will promote the recruitment of monocytes, T cells, and dendritic cells, thus increasing the release of proinflammatory cytokines TNF- $\alpha$ , IL-1 $\beta$ , and IL-6, which alter brain development by modifying the fate and function of maternal and fetal cells [55, 56].

To optimize the use of *p*-cymene, nanoformulations are considered a viable choice, as subcellular channels would decrease significantly concerning intracellular volume. Also, the formulation of *p*-cymene nanoparticles would increase its stability and solubility, and dissolution rate, decrease its volatility, and reduce possible local irritation, leading to more significant biological activity, be-

sides providing a longer duration of action and pharmacological properties [57]. Given this, further studies are needed to assess the availability of *p*-cymene in the CNS and its behavioral effects and proinflammatory cytokine modulation when administered chronically.

## Materials and Methods

## Drugs

*p*-Cymene was obtained from Sigma-Aldrich (purity 99%). RISP solution 1 mg/mL was purchased from Supera Pharmaceutical Laboratories SA (batch no. 01192/11, purity 99.9%).

## Animals

Twenty-four virgin adult female rats weighing 250-300 g were used and mated with 12 male rats from the Federal University of Santa Catarina (UFSC). The animals were kept under controlled sanitary standards, acclimatized at  $22 \pm 2$  °C, a light/dark cycle (12 h), and free access to feed and water *ad libitum*.



The use of animals in this study followed the rules of the Brazilian Directive for the Care and Use of Animals for Scientific and Didactic Purposes (2013) and approved by the Animal Use Ethics Commission of the University of Southern Santa Catarina – UNISUL on May 2, 2018 (protocol number 18.006.4.01.IV).

## Mating

Females were placed at the end of the light cycle (19:00 - the start of the dark cycle) in male-containing cages, in the ratio of two females to one male. At 07:00 the next day, vaginal washing was performed to test for pregnancy. The vaginal swab slide was verified under an optical microscope, and the presence of sperm was considered GD 0 or zero gestational day [58].

## Maternal immune activation

Matrices were randomly divided into two groups to characterize the animal model of autism by MIA. The LPS-treated group received a single intraperitoneal LPS administration obtained by phenolic extraction from *Escherichia coli* serotype 0127:B8 (Sigma) at a dose of 100 µg/kg on GD 9.5 diluted in 50 mg/kg aqueous solution of sterile NaCl 0.9% [16]. The control group received an equivalent volume of sterile NaCl solution (0.9%). The substances were administered in the morning (between 09:00 and 11:00).

## Brooding standardization and treatment

After birth, sexual differentiation was performed by analyzing the anogenital distance. All animals were kept with their mothers until weaning, on PND 21. Male offspring (n = 72) remained in the vivarium until the end of the experiment, while female offspring were submitted to painless assisted death. On PND 30, the male offspring

were subjected to social interaction and stereotype tests to validate the experimental model.

From PND 31, the animals were chronically treated for 22 days with substances previously defined for each group, which were divided into six:

- Negative control groups [SAL+SAL (n=12) and LPS+SAL (n=12)]: 0.9% NaCl solution at a dose of 1 mL/kg;
- Positive control groups [SAL+Risp (n=12) and LPS+Risp (n=12)]: RISP at a dose of 0.2 mg/kg;
- Test groups [SAL+p-cymene (n = 12) and LPS+p-cymene (n = 12)]: p-cymene at a dose of 100 mg/kg.

The application protocol is described in ► **Fig. 6**.

## Social interaction and stereotype tests

Social interaction and stereotypy tests were performed in rats on PND 30, following Schneider and Prezewlocki and Battisti et al., respectively [60, 61]. In the social interaction test, the parameters of sniffing, following, assembling, and allowing assembly were evaluated. The stereotyped movement was considered the animal's permanence in a stationary position was exhibiting rapid movements with the head and front paws.

## Open field habituation test

The open field test consists of a  $40 \times 60$  cm apparatus surrounded by 50 cm high walls with three wooden walls and a glass front wall. It was performed in PND 52 in a dark room with red light, in which the animals were kept individually for 5 min, thus allowing free exploration. The following parameters were evaluated: *Grooming:* the amount of cleaning performed by the animal in seconds, considering cleaning the paw, leg, and/or tail and cleaning the head and/or body and nose and/or face, corresponding to stereotyped movements [26, 27].

*Crossing:* the number of horizontal crossings performed by the animal, considering the four legs' entrance in the quadrant [29], suggesting exploratory locomotor activity [30].

*Rearing*: the amount of elevation of the front paws, without touching the walls of the open field, performed by the animal, referring to the exploratory locomotor activity [30, 31].

## **Object recognition test**

The object recognition test evaluated non-aversive memory, being performed in the open field apparatus, where 24 h before the experiment, to habituate animals, they were allowed to explore the apparatus for 5 min. According to Dere et al. [62], the following parameters were considered:

Training phase PND 52: two identical plastic or wooden objects (I and I) were attached to the arena with tape, allowing free exploration for 5 min.

STM PND 52: 180 min later, the animals explored the box for 5 min by placing a test session object (I) and a new object (II).

LTM: 24 h after the habituation test (PND 53), object II was replaced by a new object (III), allowing the free exploration of the animal for 5 min again.

The three moments (training, STM, and LTM) were evaluated through the animals' behavior involving the number of times the object had been explored (sniffing and touching) and each object's exploration time.

## Analysis of TNF- $\alpha$ , interleukin 1 $\beta$ , and interleukin 6 proinflammatory cytokines

On PND 54, the treated male pups and controls were euthanized, and the areas of interest (prefrontal cortex and hippocampus) were dissected on a frozen surface and stored in a -80 °C freezer. Tissue was homogenized with a PBS (pH 7.2) solution with Tween 20 (0.05%), phenylmethylsulphonyl fluoride (0.1 mM), EDTA (10 mM), aprotinin (2 ng/mL), and benzethonium chloride (0.1 mM) in an ice bath. The homogenates were centrifuged at  $3000 \times q$  (15 min, 4°C), and supernatants were collected and stored at -80 °C until further analyses. The amount of protein was measured from the supernatant using the Bradford method [63]; a volume of 100 µL was used to measure the TNF- $\alpha$ , IL-1 $\beta$ , and IL-6 cytokine levels with commercially available rat ELISA kits (R&D Systems) and estimated by interpolation from a standard curve. Measurements were done using an ELISA plate reader (Perlong DNM-9602; Nanjing Perlove Medical Equipment Co.) at 450 nM with a correction wavelength of 540 nM, and the results are expressed as pg/mg of protein.

## Statistical analysis

The results are presented as the mean  $\pm$  S.D. (SEM, 95%) in the behavioral tests and analysis of proinflammatory cytokines. Behavioral analyses were performed using non-parametric, two-tailed tests, and the groups were compared with Mann-Whitney. In contrast, the analysis of proinflammatory cytokines data between the groups was performed by two-way analysis of variance (ANOVA),

with Bonferroni post-test. Statistical analysis was performed using GraphPad Prism, version 8.1.

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## Conflicts of Interest

The authors declare that they have no conflict of interest.

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