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Neurodevelopment impact of CO_2-pneumoperitoneum in neonates: experimental study in a rat model

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ABSTRACT

Background: Laparoscopy is becoming more common in neonates. However, concerns remain about the impact of the carbon-dioxide (CO_2)-insufflation over the neonatal brain. We aim to evaluate the peripheral (serum) and central (cerebrospinal fluid [CSF]) cytokine response after neonatal CO_2-pneumoperitoneum and its impact over neurodevelopmental milestones acquisition and long-term behavioral outcomes.
Materials and methods: Rats were subjected to a systematic assessment of neurodevelopmental milestones between postnatal day 1 (PND 1) and PND 21. At PND 10, neonatal rats were anesthetized, mechanically ventilated, and exposed to different pressures and times of abdominal CO_2-insufflation. Immediately after pneumoperitoneum, corticosterone was analyzed in serum. Twenty-four hours after intervention, serum and CSF were collected to assess inflammatory response (interleukin [IL]-10, IL-1β, tumor necrosis factor [TNF]-α, and interferon [IFN]-γ). In adulthood, animals from each group were submitted to several tests to assess different behavioral domains (locomotion, anxiety, mood, and cognition).
Results: The antiinflammatory cytokine IL-10 was significantly increased in CSF in CO_2-insufflated groups, with no other significant changes in the other biomarkers. Acquisition of neurodevelopmental milestones was maintained in all studied groups. No significant differences were observed in adult behavior in the different CO_2-insufflation conditions.
Conclusions: Neonatal CO_2-pneumoperitoneum does not seem to have any negative impact on neurodevelopment or induce behavioral alterations in adulthood. Minimally invasive surgery results in a central antiinflammatory profile, and further studies on the functional consequences of these phenomena are needed.

Introduction

Minimally invasive surgery (MIS) has been widely used in adult patients, and the natural translation to infants and neonates was easily anticipated.[1,2] The following factors have contributed to the success of its application in neonatal patients: the continuous development of technology and instruments and the increasing number of MIS training courses.[3]

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As in adults, laparoscopic surgery brings to neonates numerous advantages over the traditional open surgery, such as less time until full feeding, less postoperative emesis and less postoperative pain.[2,4,5] However, little is known about the impact of the metabolic, hemodynamic, and inflammatory changes induced by the carbon-dioxide (CO_2) pneumoperitoneum over the developing organs of neonates, namely the brain. Recently, several communications focused on the neurodevelopmental impact of general anesthetic and sedation drugs in children aged <3 years have been suggesting a putative mechanism of neurotoxicity during this vulnerable period of life, where the brain is still under development.[6,7] But concerns have also been extended to the type of surgical approach applied in this population,[8] with the minimally invasive approaches and the neurodevelopmental impact of peritoneal CO_2-insufflation, focus of numerous recent publications.[9-12] Since many questions are still without clear answers, any finding on the isolated effects of these surgical-related events will result in critical changes in neonatal medical practice. Therefore, additional preclinical and clinical research on anesthesia, mechanical ventilation, postoperative care, and the surgery itself are needed.[8]

Neonatal-brain injury, induced by a variety of insults, is mediated by cytokine production, inflammatory cell recruitment, and neuroinflammation.[8] It is known that any surgical event promotes an inflammatory response; however, in non-neurological surgeries, the central inflammatory response and its deleterious impact are described as being different according to the type of surgery, being the cardiac surgery associated with the most concerning outcomes.[13-16] In addition, the inflammatory response in central nervous system (CNS), as a consequence of peripheral surgery, has been associated with postoperative delirium, cognitive decline, and depression, especially in elderly patients.[17-19] To our knowledge, the effect of abdominal CO_2-insufflation in the central inflammatory response is completely unknown. In this work, we evaluated the impact of abdominal CO_2-insufflation on peripheral and central inflammatory response and its impact on neurodevelopment milestones acquisition and in the long-term adult behavior.

Methods

This study was performed following the EU Directive 2010/63/EU, approved by the Animal Ethics Committee of the institution where the study was performed (SECVS 093/2013) and by the competent national authority for animal protection Direção Geral de Alimentação e Veterinária (0421/000/000/2015). All personnel involved in the procedures are approved as competent for animal experimentation by the Direção Geral de Alimentação e Veterinária.

Animals

Experimental design is represented in Figure 1. Pregnant Sprague Dawley rats (Charles River, Barcelona, Spain) were maintained in an animal facility with controlled temperature (22°C), humidity, and artificial 12-h light/dark cycle (from 8:00 a.m. to 8:00 p.m.). Irradiated food and sterilized water were available *ad libitum*. Nest material was provided to each dam, and no bedding changes were performed on the last days of pregnancy. The day of delivery was designated as postnatal day (PND) 0 and, on PND 1, each litter was adjusted to eight male pups. Males were chosen because it is suggested that slight differences may not reach statistical significance in studies performed in females due to higher resistance to brain senescence.[20]

Anesthesia, mechanical ventilation, and CO_2-insufflation

On PND10, the pups were anesthetized with an intraperitoneal injection of a combination of ketamine 40 mg kg^{-1} (Imalgene, Merial, France) and xylazine 5 mg kg^{-1} (Rompum, Bayer, Germany) and endotracheally intubated with the help of a videoendoscopic system as described by Miranda *et al.*[21] Animals were connected to a rodent ventilator (SAR-1000 Small Animal Ventilator, CWE Inc, PA) and ventilator settings were adjusted until achievement of physiological blood pH (7.35-7.45), $PaCO_2$ (35-45 mmHg) and oxygen saturation (sO_2)

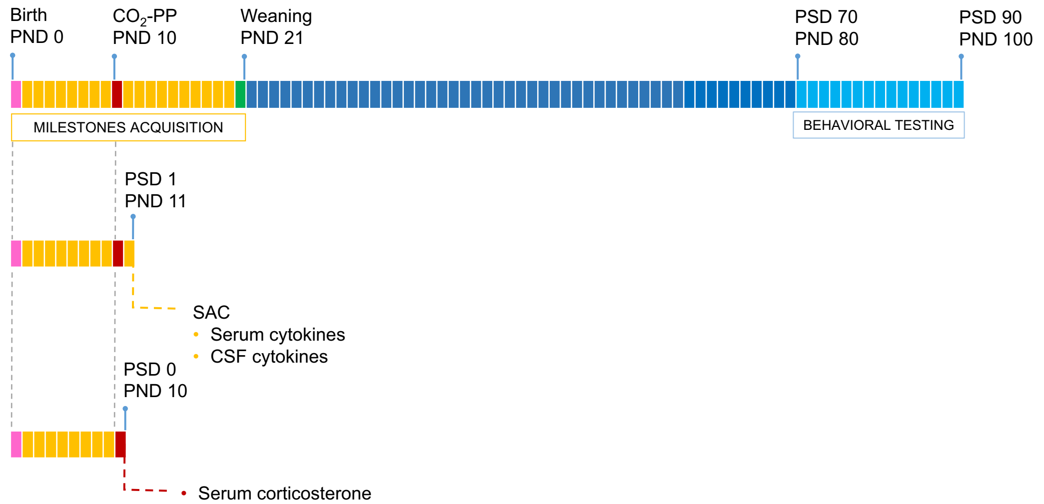

Fig. 1 – Experimental timeline: birth (pink); insufflation day (red); weaning (green). PSD = postsurgical day; PND = postnatal day. (Color version of figure is available online.)

above 95% (i-Stat analyzer, Abbott, Chicago, IL). Body temperature was maintained at 37°C during the procedures with a homeothermic pad (ATC2000, World Precision Instruments, UK).

For the CO_2-insufflation groups, a 20-gauge catheter was inserted in the right lower quadrant of the abdomen and connected to an electronic endoflator (Karl Storz GmbH & Co, Germany) at a flow rate 0.1 L/min. Insufflation pressures of 8 mmHg (PP_8) or 12 mmHg (PP_{12}) were applied, and animals were insufflated for 30 (minor procedure). The same groups were constituted for 60-minute insufflation (major procedure) but were only submitted to corticosterone measurements and long-term behavioral analysis. Since CO_2-insufflation groups required artificial ventilation and the respiration method could contribute to the observed differences, an additional control group (PP_0) was included to assess the influence of mechanical ventilation. SHAM animals were not submitted to anesthesia or CO_2-insufflation. For cytokine analysis, an additional open surgery (OPEN) group was included, in which animals were anesthetized, mechanically ventilated, and submitted to an exploratory laparotomy. Every experimental group included animals from at least two different litters.

Blood and cerebrospinal fluid sampling for multiplex analysis

Blood samples from the carotid artery were collected immediately after CO_2-insufflation for serum corticosterone determinations. Additional blood sampling was performed 24h after the CO_2-insufflation for cytokine measurements (IL-10, IL-1β, TNFα, and IFNγ). Cerebrospinal fluid (CSF) sampling for cytokine measurement was performed in the cisterna magna, 24h after the surgical approach. Corticosterone and cytokine levels were determined by multiplex analysis on a Luminex MAGPIX instrument (Bio-Rad Laboratories, Hercules, CA) using a Milliplex MAP Rat Stress Hormone Magnetic Bead Panel and a Milliplex MAP Rat Cytokine/Chemokine Magnetic Bead Panel (Merck Millipore, Billerica, MA), respectively.

Neurodevelopment milestones

Neurodevelopmental assessment included analysis of physical growth and maturation and acquisition of neurological reflexes.[22] Evaluator was blind to experimental treatment.

Somatic parameters

Animals were weighed daily from PND 2 to PND 21. Day of eye and ear opening were recorded as indicative of physical maturation.

Neurological reflexes

Neurological reflexes were evaluated daily at 9:00 a.m. from PND 2 to PND 21. The following reflexes were evaluated: i) walking: newborns were observed for 1 minute, and mature walking was considered when the animal could move with the body completely supported by the four limbs, without dragging the belly over the surface; ii) air-righting reflex: each newborn was held on its back 30 cm above a soft surface. The animal was released, and the position in which the animal reached the soft pad was recorded. The reflex was achieved when the neonate landed on the surface with all four paws.

Behavioral tests

At 3 month of age, animals were submitted to behavioral testing for 10 consecutive days, between 9 a.m. and 6 p.m. Behavioral tests were performed in the following order: sucrose preference test, elevated plus maze, open field (OF) test, novel object recognition (NOR), forced swimming test (FST) and Morris water maze (MWM).

Sucrose consumption test

Anhedonia was assessed after two habituation trials. Animals were presented with two preweighed drinking bottles, one with water and the other with 1% (m/v) sucrose. Before testing, rats were food- and water-deprived for 18 h and exposed to the drinking solutions for 1 h. Sucrose preference was calculated according to the formula: sucrose preference = [sucrose intake/(sucrose intake + water intake)] × 100.

Elevated plus maze

Anxiety-like behavior was evaluated in a 5-minute session in a plus maze containing two open arms and two closed arms (MED-NIRPMNR; Med Associates Inc, St Albans, VT), as previously described.[23] Animals were placed in the central junction facing an open arm, and an entry in the arm was defined when all four paws were positioned within one arm. The percentage of time spent in the open arm was used as an index of anxiety-like behavior (time spent in the open arms/total time spent in all arms). The degree of anxiety was indirectly related to the time spent in the open arms.

Open field

This test was performed to assess locomotor and anxious-like behavior. Animals were tested individually for 5 minutes in a transparent acrylic square arena with a white floor (43.2 × 43.2 cm) illuminated by a bright white light. The session started with the animal placed in the center of the arena and, with a 16-beam infrared system and a tracking software, the position of the animal was monitored using the Activity Monitor software (Med Associates Inc, VT), considering two previously defined areas as follows: a central (10.8 × 10.8 cm) and an outer area. The following parameters were recorded: (i) ratio between the distance traveled in the central area/total distance traveled (a measure of anxious-like behavior) and (ii) total distance traveled (a measure of general locomotor activity).

Novel object recognition test

Cognitive function was assessed in the NOR test. Rats were habituated to the testing arena for 10 minutes. On the next day, for 10 minutes, each animal was allowed to explore two identical objects placed in the arena. An hour later, the rats explored the same arena for 5 minutes but this time with one familiar object and one novel object. Recognition memory was expressed by the percentage of time spent exploring the novel object (time of exploration novel object/total time of exploration).

Forced swimming test
The FST was used to evaluate the ability of rats to cope with an inescapable situation (behavioral despair), as a measure of depressive-like behavior. Each animal was placed in a transparent cylinder (27 cm diameter), filled with water (25°C) to a depth of 50 cm, so the rat had no solid support for the rear paws or tail. Assays were conducted 24 h after a 10-minute pretest session, by placing the rats in the cylinders for 5 min. A video camera was used to record test sessions and was later scored by an investigator kept blind to the experimental groups. Latency to immobility (time that each animal takes from the beginning of the test to stop for the first time) and time of immobility (time that the animal stayed floating without evident efforts to escape) were assessed. Depressive-like behavior was defined as an increase in time of immobility and a decrease in latency to immobility.[24]

Morris water maze
The MWM test was designed to study the animal's capacity to learn the platform location for 4 consecutive days—spatial reference memory [65]. MWM was made in a black, circular tank (170 cm diameter), filled with water (24°C) to a depth of 31 cm in a dimly illuminated room with spatial clues on the walls. A video camera fixed on the ceiling, above the center of the tank, captured the image to a video tracking system (View Point, Champagne au Mont d'Or, France). Four virtual quadrants were then assigned, and a circular platform, 12 cm diameter, 1 cm below the water surface (invisible to the rats) was placed within one of the quadrants. The platform was kept in the same position throughout the test, and the animals were given four trials to find the platform, each trial starting from a different quadrant. The test was performed during 4 days and, in each day, the starting quadrant was different. Trials were automatically completed once the animals reached the platform or 120 s had elapsed, whichever occurred first. If an animal failed to find the platform in 120 s, it was gently guided to it and allowed to remain there for 30 s before starting a new trial. The time to escape to the platform was automatically recorded.

Statistical analysis

All data are presented as the mean ± standard error of mean (SEM). Normality and homogeneity of variances were checked with Shapiro–Wilk and Lavene's tests, respectively. Whenever appropriate, data were analyzed by one-way or two-way analysis of variance (ANOVA), and Bonferroni's multiple comparisons test was applied for *post hoc* analysis. Statistical analysis was conducted using statistical software (IBM SPSS Statistics, version 22 for Windows.). Overall, tests were considered significant when $P < 0.05$. As a measure of the magnitude of a difference, the effect size (practical significance) was calculated as follows[25]: for the one-way ANOVA, the eta-square (η^2) was calculated as the ratio of the between-group's sum of squares (SS_{btw}) and the total SS (SS_{tot}; $\eta^2 = SS_{btw}/SS_{tot}$); for the two-way ANOVA the partial η^2 (η^2_p) was calculated as the ratio between the SS_{btw} and the sum of the SS_{btw} and the residual SS [SS_{res}; $\eta^2_p = SS_{btw}/(SS_{btw} + SS_{res})$]. For η^2 and η^2_p, a small effect size was considered for values between at least 0.01 and less than 0.06, medium between at least 0.06 and less than 0.14, and a large effect size at least 0.14.

Results

Peripheral and central cytokine profile

Twenty-four hours after pneumoperitoneum, serum IL-10 concentrations did not significantly differ between the groups ($F_{(4;40)} = 0.4693$, $P = 0.7579$, $\eta^2 = 0.04956$); however, a tendency in increased concentration is observed in CO_2-pneumoperitoneum groups, Figure 2A1. But interestingly, statistically significant differences were found in CSF IL-10 levels ($F_{(4;29)} = 4.783$, $P = 0.0053$, $\eta^2 = 0.4335$). Bonferroni's multiple comparison showed that the animals submitted to very high pneumoperitoneum insufflation pressures (PP_{12}) presented statistically significant higher IL-10 concentrations when compared to SHAM ($P < 0.05$) and OPEN groups ($P < 0.05$), Figure 2A2. Regarding the proinflammatory cytokines analyzed, no main effect was observed in the surgical approach over IL-1β ($F_{(4;40)} = 1.260$, $P = 0.3037$, $\eta^2 = 0.1228$) and IFNγ ($F_{(4;40)} = 1.534$, $P = 0.2136$, $\eta^2 = 0.1492$) concentrations, respectively Figure 2B1, C1. However, regarding serum TNFα concentrations, it was observed that statistically significant differences exist between the groups ($F_{(4;40)} = 4.776$, $P = 0.0034$, $\eta^2 = 0.3467$). Bonferroni's *post hoc* test showed that the animals submitted to open surgery presented significant higher serum levels of TNFα when compared with animals submitted to pneumoperitoneum Figure 2D1. In respect to proinflammatory cytokines, no significant differences were observed between groups in CSF IL-1β ($P = 0.9352$), IFNγ ($P = 0.4447$), and TNFα ($P = 0.2631$), Figure 2B2-D2.

Stress response

Statistically significant differences were observed regarding corticosterone concentrations ($F_{(4;40)} = 10.06$, $P < 0.0001$, $\eta^2 = 0.5897$). Bonferroni's multiple comparisons showed that SHAM animal presented statistically significant lower corticosterone levels when compared to all other groups ($P_{\text{SHAM versus PP0}} < 0.05$), ($P_{\text{SHAM versus PP8}} < 0.05$), ($P_{\text{SHAM versus PP12}} < 0.001$), and ($P_{\text{SHAM versus OPEN}} < 0.001$), Figure 3A. When evaluating the effect of CO_2-insufflation time (30 min and 60 min) and CO_2-insufflation pressure (8 mmHg and 12 mmHg) by 2-way ANOVA, no effect of insufflation pressure, insufflation time or interaction effect was observed over corticosterone production ($F_{(2;30)} = 2.109$, $P = 0.1390$, $\eta^2_P = 0.0309$), ($F_{(1;30)} = 3.855$, $P = 0.0589$, $\eta^2_P = 0.1233$), and ($F_{(2;30)} = 0.4780$, $P = 0.6247$, $\eta^2_P = 0.1139$), respectively Figure 3B.

Developmental milestones acquisition

Regarding body weight, no statistically significant differences were observed between groups from birth until weaning, Figure 4A. In respect to physical maturation, no statistically significant differences were observed between groups because animals in all groups presented eye opening between PND13 and PND15 and ear opening from PND12 to PND14. Furthermore, no differences were observed in the acquisition of

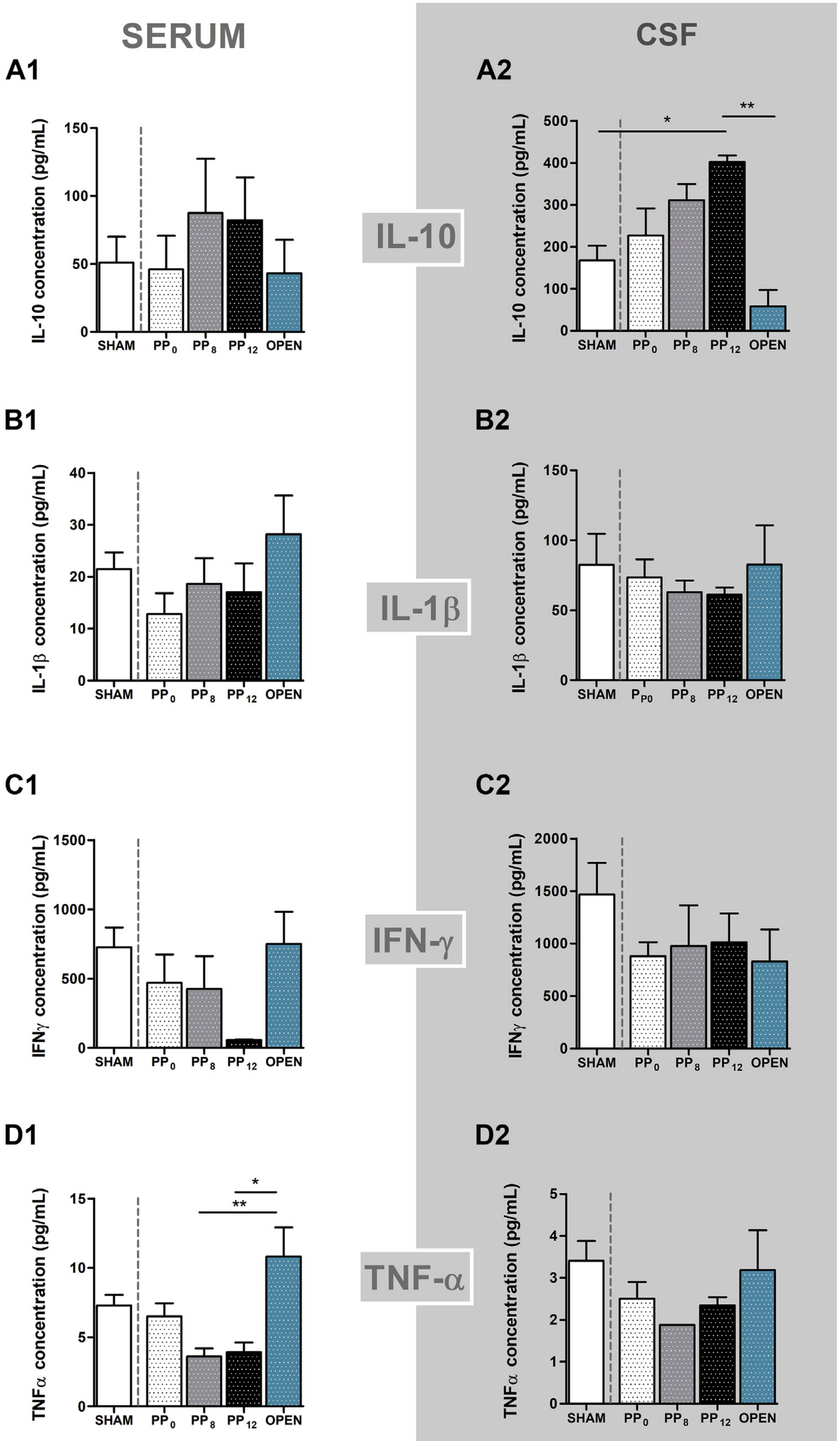

Fig. 2 – IL-10 (A), IL-1β (B), IFN-γ (C), and TNF-α (D) concentrations 24h after CO_2-insufflation (P30). Serum concentrations (A1, B1, C1, D1) in SHAM (white bars; $n = 17$), PP_0 (light gray bars; $n = 8$), PP_8 (dark gray bars; $n = 5$), PP_{12} (black bars; $n = 5$), and OPEN (blue bars $n = 6$) groups. CSF concentrations (A2, B2, C2, D2) in SHAM (white bars; $n = 11$), PP_0 (light gray bars; $n = 8$), PP_8 (dark gray bars; $n = 4$), PP_{12} (black bars; $n = 4$) and OPEN (blue bars $n = 4$) groups. Data analyzed in a one-way ANOVA. Values represented as mean +SEM. $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$. (Color version of figure is available online.)

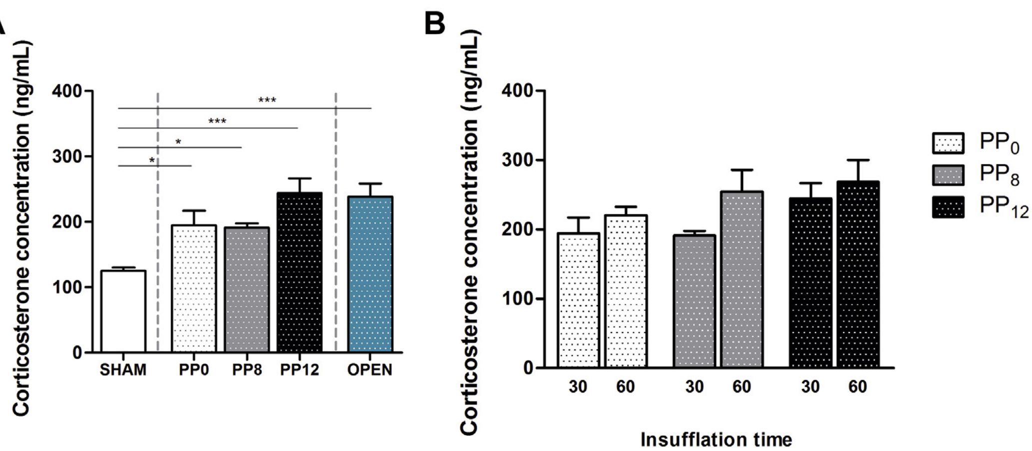

Fig. 3 – (A) Serum corticosterone levels after 30-minute CO_2-insufflation (P30) in SHAM (white bars; $n = 8$), PP_0 (light gray bars; $n = 6$), PP_8 (dark gray bars; $n = 7$), PP_{12} (black bars; $n = 6$), and OPEN (blue bars; $n = 6$) groups. Group means were compared by one-way ANOVA followed by Bonferroni's *post hoc* multiple comparison test. (B) Serum corticosterone levels in animals submitted to 30- or 60-minute CO_2-insufflation (P30 versus P60). Differences between PP_0, PP_8, and PP_{12} groups were analyzed using a two-way ANOVA. Results represented as mean +SEM. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$. (Color version of figure is available online.)

air-righting reflex, with animals presenting the innate response between PND14 and PND16. Animals displayed similar neurodevelopmental profile regarding walking ability, acquired between PND11 and PND14, Figure 4B.

Long-term behavior

In anxious-like behavior, evaluated using the ratio of time spent in the open arms versus total time of the elevated plus maze test, no effect of CO_2-insufflation pressure, insufflation time, or interaction effect was observed, ($F_{(2;92)} = 2.183$, $P = 0.1185$, $\eta^2_P = 0.045$), ($F_{(1;92)} = 0.534$, $P = 0.467$, $\eta^2_P = 0.006$), and ($F_{(2;92)} = 0.610$, $P = 0.545$, $\eta^2_P = 0.013$), respectively. In addition, all groups were compared with SHAM animals, and no differences were observed ($F_{(6;132)} = 1.215$, $P = 0.3026$, $\eta^2 = 0.05233$), Figure 5A. The ratio of distance walked in the central area versus total distance traveled in the OF test was also assessed as measure of anxious-like behavior, and again no effect of CO_2-insufflation pressure, insufflation time, or interaction effect was observed, ($F_{(2;97)} = 1.603$, $P = 0.207$, $\eta^2_P = 0.032$), ($F_{(1;97)} = 0.167$, $P = 0.684$, $\eta^2_P = 0.002$), and ($F_{(2;97)} = 0.627$, $P = 0.536$, $\eta^2_P = 0.013$), respectively. When comparing all groups with SHAM animals, no statistically significant differences were observed ($F_{(6;139)} = 0.6202$, $P = 0.7138$, $\eta^2_P = 0.026$), Figure 5B. The total distance traveled in the OF arena was used as a measure of locomotor activity, and no effect of the CO_2-insufflation pressure ($F_{(2;96)} = 0.4159$, $P = 0.6609$, $\eta^2_P = 0.009$), duration of insufflation ($F_{(2;96)} = 0.043$, $P = 0.835$, $\eta^2_P = 0.000$), or any interaction effect was observed ($F_{(2;96)} = 0.188$, $P = 0.829$, $\eta^2_P = 0.004$). Furthermore, no

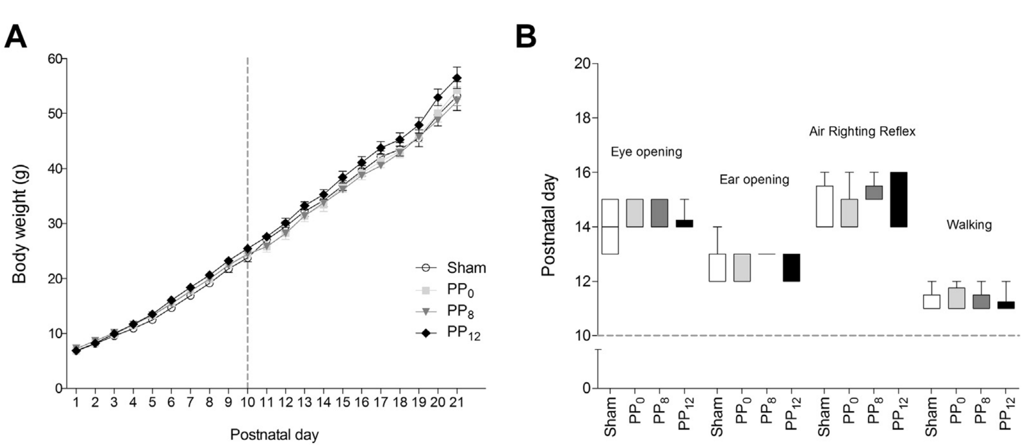

Fig. 4 – Developmental milestones after 30-minute CO_2-insufflation (P30) in SHAM (white symbols); PP_0 (light gray symbols); PP_8 (dark gray symbols); and PP_{12} (black symbols). Dashed line corresponds to insufflation day. (A) Body weight gain from birth until weaning. (B) Somatic development and neurological reflexes acquisition. Results presented as median PND at which animals showed a mature response: eye opening, ear opening, air-righting reflex, and mature walking.

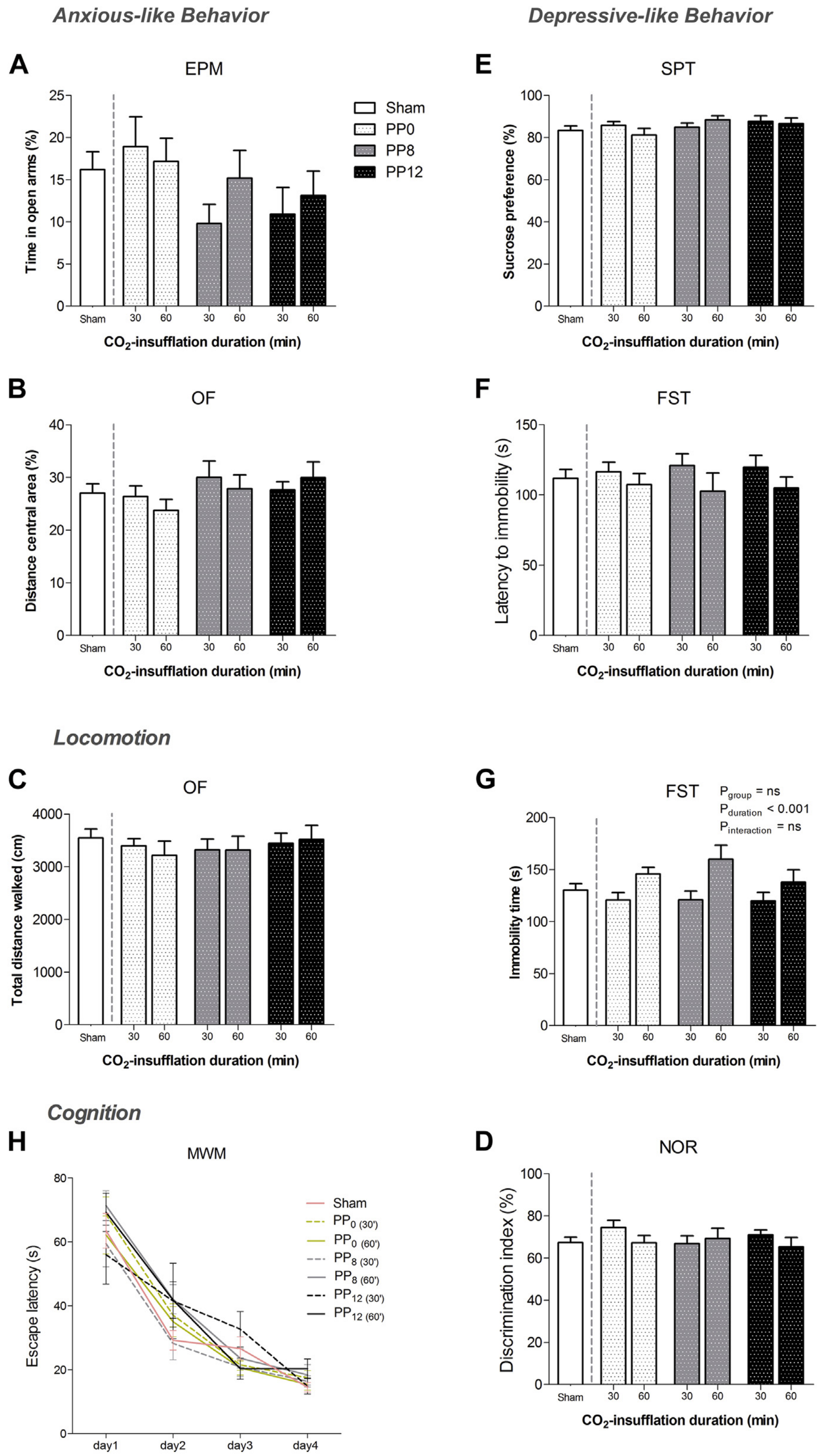

Fig. 5 – Behavioral tests performed in adult animals, 3 months after CO_2-insufflation: SHAM animals (white bars; $n = 43$;); PP_0 (light gray bars; $n_{30min} = 23$; $n_{60min} = 13$); PP_8 (dark gray bar; $n_{30min} = 16$; $n_{60min} = 16$); and PP_{12} (black bars; $n_{30min} = 19$; $n_{60min} = 15$). (A) Time in open arms in elevated plus maze (EPM) test and (B) percentage of distance traveled in the center of the OF arena as measure of anxious-like behavior; (C) Total distance traveled in the OF arena as measure of locomotor activity; (E) Percentage of sucrose consumption in sucrose preference test (SPT) and (F) Latency to immobility and (G) Immobility time in FST as measure of depressive-like behavior; (D) Discrimination index in the NOR test and (H) Escape latency time in MWM test for cognitive assessment. Results represented as mean + SEM from two independent experiments. (Color version of figure is available online.)

differences were observed between groups when compared with SHAM animals ($F_{(6;138)} = 0.3147$, $P = 0.9285$, $\eta^2_P = 0.0135$), Figure 5C.

Depressive-like behavior was addressed using the sucrose preference test and FST. No effect of the CO_2-insufflation pressure ($F_{(2;97)} = 1.491$, $P = 0.2302$, $\eta^2_P = 0.03$), insufflation duration ($F_{(1;97)} = 0.1443$, $P = 0.7049$, $\eta^2_P = 0.001$) or any interaction effect ($F_{(2;96)} = 1.443$, $P = 0.2413$, $\eta^2_P = 0.029$) was observed in the sucrose consumption. When comparing to SHAM animals, by one-way ANOVA, no differences were observed between groups ($F_{(6;139)} = 0.9122$, $P = 0.4881$, $\eta^2 = 0.039$], Figure 5E. The same profile was observed in the FST, where no effect of CO_2-insufflation pressure ($F_{(2;97)} = 0.0025$, $P = 0.9975$, $\eta^2_P = 0.00$), insufflation duration ($F_{(1;97)} = 3.607$, $P = 0.0605$, $\eta^2_P = 0.036$), or interaction effect ($F_{(2;97)} = 0.131$, $P = 0.878$, $\eta^2_P = 0.003$) was observed over latency to immobility Figure 5F. In the same test, no effect of CO_2-insufflation pressure ($F_{(2;97)} = 0.7405$, $P = 0.4795$, $\eta^2_P = 0.015$) or any interaction effect ($F_{(2;97)} = 0.6150$, $P = 0.5428$, $\eta^2_P = 0.013$) was observed, but interestingly an effect of insufflation duration ($F_{(2;97)} = 12.336$, $P = 0.001$, $\eta^2_P = 0.113$) was observed in total immobility time, with the animals submitted to 60-minute procedure, presenting increased immobility time, regardless the application of CO_2-insufflation Figure 5G. When comparing all groups with SHAM animals, no statistical significant differences were observed in latency to immobility time ($F_{(6;139)} = 0.6046$, $P = 0.7263$, $\eta^2 = 0.025$) and in total immobility time ($F_{(2;97)} = 2.582$, $P = 0.0211$, $\eta^2 = 0.1003$).

Spatial reference memory was assessed in MWM, and it was observed that no statistical significant differences exist between the groups in test performance ($F_{(6;273)} = 0.8061$, $P = 0.5677$, $\eta^2_P = 0.044$), Figure 5H. Cognitive function was also assessed by NOR test, and none of the factors, insufflation pressure ($F_{(2;87)} = 0.3441$, $P = 0.7098$, $\eta^2_P = 0.008$), insufflation duration ($F_{(1;87)} = 1.090$, $P = 0.299$, $\eta^2_P = 0.012$), or any interaction effect ($F_{(2;87)} = 0.754$, $P = 0.473$, $\eta^2_P = 0.017$) influenced the test performance. No differences were observed between groups when compared with SHAM group ($F_{(6;130)} = 0.8358$, $P = 0.5444$, $\eta^2_P = 0.037$), Figure 5D.

Discussion

Every surgical event promotes an inflammatory response, but changes in the CNS are less known, especially during nonneurological surgery. The local and systemic inflammatory mediators produced after a surgical trauma have been shown to influence inflammatory process in the brain, leading to the activation of the microglia and concurrent endogenous production of cytokines.[26] The aim of the present study was to investigate the acute postoperative changes in peripheral and central inflammatory cytokines after CO_2-pneumoperitoneum in a neonatal rodent model and evaluate the long-term effects of CO_2-insufflation on adult behavior.

In our study, open surgery was the condition that leads to more disturbances in peripheral proinflammatory cytokines, showing significantly increased serum levels of TNFα, whereas in CO_2-insufflation groups this proinflammatory profile was not observed. During MIS, macrophages have been pointed as the primer CO_2-cellular target via inhibition of proinflammatory cytokine production. *In vitro* experiments have shown an inhibition of proinflammatory cytokine production by lipopolysaccharide (LPS)-stimulated peritoneal macrophages obtained from animals previously submitted to capnoperitoneum.[27] The same inhibition was obtained in LPS-stimulated macrophages incubated with CO_2, whereas no inhibition was obtained when incubated with helium or air.[28] Moreover, *in vivo* studies reported that a CO_2-insufflation pretreatment significantly reduces IL-6 plasma levels after LPS-contaminated laparotomy[29] and decrease ascites volume, inflammatory cell number and serum TNFα and IL-6 levels in acute pancreatitis model.[30] All these studies were performed in models of sepsis, which in this context leads to an exacerbation of the modifying effects of CO_2-insufflation on the inflammatory response. Although our study was performed in aseptic conditions, because our aim was to evaluate the isolated effect of pneumoperitoneum, we were able to identify significant differences in acutely measured proinflammatory marker TNFα when comparing open surgery with pneumoperitoneum groups, which confirms the significantly different inflammatory profile of the two surgical approaches. Interestingly, the observed changes in the serum TNFα levels in the OPEN group were not found in the CSF, suggesting that this peripheral inflammation did not impact on the CNS homeostasis. However, it is important to highlight that our OPEN group was only submitted to abdominal wall opening, which does not exactly mimic an open surgery, in which organs and organ systems are disturbed.

Regarding the serum levels of the antiinflammatory cytokine IL-10, although the differences were not statistically significant between groups, the pneumoperitoneum groups presented a tendency in increased concentrations of this antiinflammatory cytokine. This tendency is in good accordance with the literature because an increase in IL-10 levels was observed in the LPS-sepsis models submitted to CO_2-pneumoperitoneum, having contributed to increased survival rates and suggesting a “rescuing” capability of the capnoperitoneum.[31] The upstream mechanisms of this antiinflammatory profile are yet to be identified, but interestingly, the same study described increased peripheral IL-10 production in animals submitted to acidification of the peritoneal cavity, whether induced by CO_2-pneumoeritoneum or by peritoneal acidic buffered lavage.[32] Interestingly, in our CO_2-pneumoperitoneum groups, it was observed an increase in CSF IL-10 levels, with significantly increased levels in the group exposed to high CO_2-insufflation pressures (PP_{12}). Since peripheral IL-10 does not seem to cross the intact blood–brain barrier,[33] it is possible that circulating IL-10 acts directly in the brain through regions devoided of the blood–brain barrier, such as the circumventricular organs and the choroid plexus.[34] Furthermore, it is also suggested that peripherally produced cytokines can elicit CNS inflammation by binding to receptors associated with peripheral afferent nerves, such as part of the vagus nerve, which relay signals to the brain that set off cytokine synthesis.[35-38] However, in our work, we observed a much greater increase in CSF IL-10 levels than in serum, and similar phenomena were observed in human patients submitted to nonneurological surgery, suggesting that an inflammatory reaction in CNS could be elicited independently of the systemic one.[13,39] Although this study does not include a follow-up on the peripheral and central inflammatory response, human studies have shown that after surgery, and without continuous stimuli, peripheral cytokine levels return to baseline levels.[40,41] For this reason, this study aimed to evaluate the acute central inflammatory response in the first 24h, which is the postoperative period where the most disturbing inflammatory peripheral changes occur. Future follow-up studies will help to clarify whether this central antiinflammatory profile lasts over time, even without continuous external stimuli (the CO_2-insufflation).

Since corticosterone is also an antiinflammatory molecule produced upon a stress response, we also have evaluated its production in our model. We observed that anesthesia and mechanical ventilation itself result in increased corticosterone levels when comparing to SHAM, which is not aggravated by CO_2-insufflation. Another important aspect is that OPEN group presented corticosterone levels similarly high. Moreover, in CO_2-insufflation groups, the surgical stress does not seem to be aggravated by duplicating the insufflation time from 30 minutes to 60 minutes. In our study, since corticosterone levels in pneumoperitoneum groups did not exceed the values from OPEN group, we considered that the stress response elicited by CO_2-insufflation is, therefore, lower or equivalent (when applying very high insufflation pressures) to the stress response elicited by open surgical approaches. Therefore, if this early-life event has contributed to a long-lasting disruption in the hypothalamic–pituitary–adrenal axis function and to increased basal levels of corticosterone in the adult progeny, this would occur regardless the CO_2-insufflation.

The interplay between corticosterone during the first days of life and several parameters of the immune system has been shown to impact adult behavior.[42] In fact, studies have demonstrated that chronic stress during early postnatal life has two important neurodevelopmental outcomes: accelerate some somatic milestones, while delaying the acquisition of neurological reflexes.[43] In our study, CO_2-pneumoperitoneum had no impact on physical growth and maturation, as well as over sensorimotor development. It is important to recall that, although corticosterone levels significantly increased when compared to SHAM animals, this was secondary to an acute and single stressful event in neonatal life which, in our study, seems to result in no significant outcome over neurodevelopment. Moreover, the CO_2-insufflation during the postnatal period did not induce any long-term alterations in the following several behavioral domains analyzed: locomotion, anxiety, depression, and cognition. One aspect that needs further study is the observed trend to the depressive-like behavior of groups submitted to 60-minute intervention, including PP_0 group. This finding suggests that the duration of neonatal anesthesia and mechanical ventilation might contribute to long-term outcomes in adult behavior, rather than capnoperitoneum itself. This finding might suggest that whenever MIS approaches contribute to the reduction of the length of surgical procedures, this might be beneficial to avoid any long-term behavioral outcomes.

The absence of peripheral and central proinflammatory response, combined with an antiinflammatory profile, in animals submitted to CO_2-insufflation, could have contributed to the absence of alterations both on the development milestones and adult behavior. Recent studies have found that synaptic function and neurodevelopment are regulated through the interaction of the IL-10 released from the microglia with the IL-10 receptors expressed on the hippocampal neurons in the early neurodevelopmental stage. Moreover, IL-10 was shown to increase the number of dendritic spines and excitatory and inhibitory synapses, even without external stimuli.[44] The role of IL-10 was also demonstrated in other works, where activated regulatory T-cells promoted neuronal stem cell proliferation via IL-10, suggesting a new therapeutic approach for ischemic stroke.[45]

In summary, MIS results in a peripheral and central antiinflammatory profile, and further studies on the quantification of IL-10 in different brain regions after pneumoperitoneum may help to clarify wheather this antiinflammatory cytokine has a protective role in the neonatal brain after the surgically related harms. The findings of this work may also be a forerunner for the study of the incidence of postoperative cognitive dysfunction after MIS versus open surgery, especially in elderly patients. Postsurgical neuroinflammation has been associated with impaired cognitive functioning,[17,46,47] which together with the age-related alterations in immune regulation may account for the increased incidence of these surgical-related behavioral changes in the elderly.[48,49] Moreover, inhibition of central proinflammatory cytokine signaling was shown to attenuate postoperative memory impairment in rodents.[26,50-52] Therefore, if MIS approaches lead to less inflammation and even contribute to an antiinflammatory profile, the incidence of postoperative cognitive dysfunction may be different between patients submitted to MIS versus open surgery. The replacement of open surgery by MIS may later result in less surgery-induced changes in cognition and mood in elderly patients. Further preclinical and clinical studies may help to clarify this issue.

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Disclosure

The authors report no proprietary or commercial interest in any product mentioned or concept discussed in this article.

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