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Inflammatory response and long-term behavioral assessment after neonatal CO_2-pneumothorax: study in a rodent model

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ABSTRACT

Background: Carbon-dioxide (CO_2)-pneumothorax during minimally invasive surgery induces well-known metabolic changes. However, little is known about its impact on the central nervous system. The aim of this work is to evaluate the acute impact of CO_2-pneumothorax over central cytokine response and its long-term effect on animal behavior.

Methods: This is an experimental study where neonatal Sprague–Dawley rats are submitted to CO_2-pneumothorax. Peripheral and central cytokine response was evaluated 24 h after insufflation, and peripheral immune cell phenotyping was evaluated 24 h and 4 weeks post-insufflation. Progenitor cell survival was evaluated in the hippocampal dentate gyrus, and the behavioral analysis was performed in adulthood to test cognition, anxious-like, and depressive-like behavior.

Results: Significantly increased IL-10 levels were observed in the cerebrospinal-fluid (CSF) of animals submitted to CO_2-pneumothorax, while no differences were found in serum. Regarding pro-inflammatory cytokines, no differences were observed in the periphery or centrally. CO_2-pneumothorax event did not alter the survival of newborn cells in the hippocampal dentate gyrus, and no impact on long-term behavior was observed.

Conclusions: Neonatal animals submitted to CO_2-pneumothorax present acutely increased CSF IL-10 levels. The CO_2-pneumothorax seems to result in no significant outcome over neurodevelopment as no functional behavioral alterations were observed in adulthood.

The thoracoscopic repair of congenital malformations in neonatal patients has been increasingly performed in the last decade. Esophageal atresia (EA) is one of the conditions that have been taking advantage of this minimally invasive approach since its first performance by Rothenberg and Lobe in 1999 [1]. In recent years, numerous reports on the successful outcomes of this minimally invasive approach have been published [2], however pertinent concerns about the safety of the CO_2-pneumothorax have been drawing the attention of health professionals. The effects on brain oxygenation and perfusion are the most investigated events [3–6]. It has become of utmost importance the investment in research studies to unravel the acute and long-term effects of CO_2-insufflation in neonatal patients [7]. Being a relatively recent surgical approach, the long-term human studies are far from being completed, and the use of altricial neonatal animal models may in the meanwhile contribute to clarify some of those concerning issues, as well as allow to explore the acute changes at the brain level during CO_2-pneumothorax.

Every surgical event promotes a stress response, that when excessive can lead to systemic inflammatory response and prolonged catabolism of body stores [8]. Considering that the function of the immune cells can be inferred by parameters such as cytokine *milieu*, and knowing the correlation between cytokine production in neonates and long-term morbidity [9], it is important to understand the interplay between several parameters of the immune system after CO_2-pneumothorax event, and their relationship with long-term behavior. To unravel those issues, we made use of a neonatal rodent model where the peripheral and central cytokine response after CO_2-pneumothorax was evaluated. The functional consequences of this early-life event were assessed by evaluating progenitor cell survival in the hippocampal dentate gyrus (DG), an important neurogenic area highly involved in cognition, and by long-term behavioral assessment of cognition, anxious-like and depressive-like behavior when adults.

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1. Materials and methods

This study was 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 (DGAV) (0421/000/000/2015). All the animal procedures were done in accordance with the EU Directive 2010/63/EU and all personnel involved in animal procedures are approved as competent for animal experimentation by DGAV.

1.1. Animals

Sprague Dawley rats (Charles River, Saint-Germain-sur-l'Arbresle, France) were maintained in an animal facility with controlled temperature (21 ± 1 °C), humidity (50–60%) and artificial 12-h light/dark cycle (from 8:00 a.m. to 8:00 p.m.). Irradiated food (4RF25-GLP, Mucedola, Settimo Milanese, Italy) and sterilized water were available *ad libitum*. Pregnant females were submitted to daily handling sessions during pregnancy and nest material was provided to each animal cage. No bedding changes were performed on the last days of pregnancy and the day of birth was designated as postnatal day (PND) 0. On PND 1 each litter was adjusted to 8 pups to normalize weight and animals were randomly assigned to the following experimental groups: SHAM, no pneumothorax (PT_0) and pneumothorax of 2 mmHg (PT_2). The experimental timeline is represented in Fig. 1.

1.2. Anesthesia, mechanical ventilation, and CO_2-insufflation

On PND 10, pups were anesthetized with ketamine 40 mg kg^{-1} (Imalgene, Merial, France) and xylazine 5 mg kg^{-1} (Rompum, Bayer, Germany), intraperitoneally. Endotracheal intubation was performed with the help of a videoendoscopic system as described by Miranda et al. [10] and animals were connected to a rodent ventilator (CW-SAR 1000, Small Animal Ventilator, CWE-Inc., USA). A 22-gauge catheter was inserted in the left hemithorax and connected to an electronic endoflator (Karl Storz GmbH & Co, Germany) for 30 min, at an insufflation pressure of 0 mmHg (PT_0) or 2 mmHg (PT_2). In both groups, ventilator settings were adjusted until achievement of physiological blood pH (7.35–7.45), $PaCO_2$ (35–45 mmHg) and oxygen saturation above 95%, evaluated in arterial blood from the carotid artery. After the insufflation period, animals were allowed to recover from anesthesia and were returned to the dam when breathing and capable of spontaneous movement. Body temperature was controlled with the help of a homoeothermic pad (ATC2000, World Precision Instruments, UK) during anesthesia. Animals from SHAM group were maternally separated and maintained in a warming chamber for the same period as experimental animals. No anesthesia or any additional procedure was performed to SHAM animals.

1.3. Blood sampling for corticosterone and arterial blood gas analysis

Blood sampling from the carotid artery was performed immediately before the end of the CO_2-insufflation time. Serum corticosterone measurements were determined by multiplex analysis on a Luminex MAGPIX instrument (Bio-Rad, Hercules, CA, USA) using a Milliplex MAP Rat Stress Hormone Magnetic Bead Panel. Blood gas analysis was performed using an i-Stat blood analyzer (CG4+ cartridge; i-Stat analyzer; Abbott, Chicago, IL, USA).

1.4. Blood and CSF sampling for cytokine measurements

Cytokine measurements (IL-10, IL-1β, TNFα, and IFNγ) were performed in blood and cerebrospinal fluid (CSF), 24 h after the surgical approach. Blood and CSF were collected from the carotid artery and cisterna magna, respectively, and cytokine levels were determined by multiplex analysis on a Luminex MAGPIX instrument (Bio-Rad, Hercules, CA, USA) using a Milliplex MAP Rat Cytokine/Chemokine Magnetic Bead Panel (Merck Millipore, Billerica, MA, USA).

1.5. Immune cells phenotyping

The impact of thoracic CO_2-insufflation over immune cell populations was evaluated. Immune cell phenotyping was performed on PND 11 (PSD 1) and PND 38 (PSD 28). For that, under anesthesia, blood was collected in heparinized tubes directly from the aorta. After erythrocytes lysis with ACK lysing buffer, cells from each individual rat were incubated with specific antibodies, according to Table 1. Fifty thousand events were acquired on a BD LSR II flow cytometer using the FACS Diva software. Cell enumeration was performed by using counting beads (AccuCheck counting beads, Invitrogen, USA). Analysis of the cell populations was performed using Flow Jo software (TreeStar, Ashland, OR, USA).

1.6. Immunostaining procedures

The effect of thoracic CO_2-insufflation on the hippocampal cells undergoing division prior to the insufflation event was investigated. Animals received a single intraperitoneal injection of BrdU (300 mg/kg) at PND 9 and were submitted to CO_2-insufflation on PND10. Twenty-eight days after BrdU injection, animals were deeply anesthetized with sodium pentobarbital (100 mg/kg i.p.) and transcardially perfused with cold 4% paraformaldehyde in 0.1 M phosphate buffer. Brains were collected and processed for immunohistochemistry to evaluate cell survival. Briefly, brain sections, 20 μm thick, were cut in a frozen section cryostat (Leica Instruments, Germany) and every 8th section throughout the hippocampus was processed for BrdU immunohistochemistry (1:50; Dako, Glostrup, Denmark). Cell survival was estimated in the

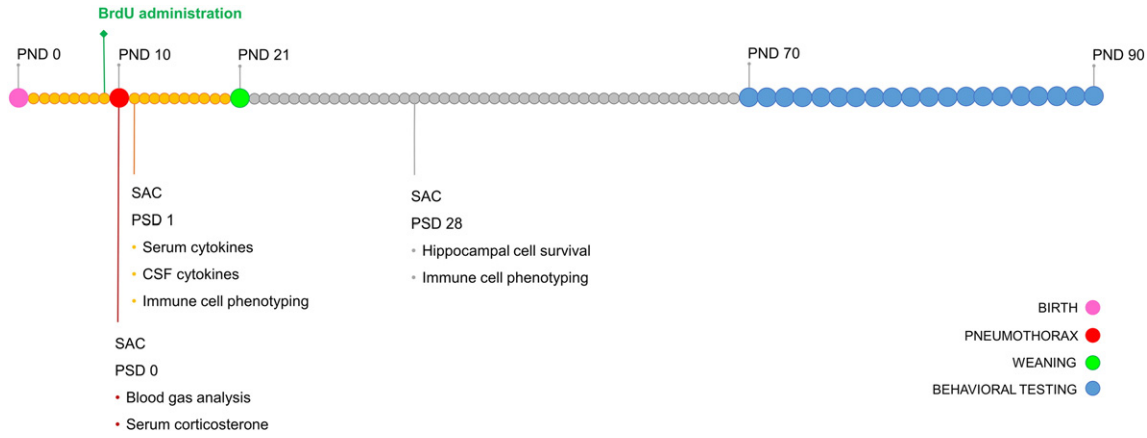

Fig. 1. Experimental timeline: Postnatal day (PND); post-surgical day (PSD); 5-bromo-2′-deoxyuridine (BrdU).

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Table 1
Antibodies combination for leucocyte identification.

Antibody	Fluorochrome	Clone	Supplier
PANEL 1			
CD3	Biot	G4.18	ebioscience
CD45	FITC	OX-1	BioLegend
CD45RA	APCCy7	OX-33	BD Pharmingen
CD4	Pecy7	W3/25	Biolegend
CD8	Pe	G28	Biolegend
Streptavidine	BV 421		Biolegend
PANEL 2			
CD161	Alexa 647	10/78	Biolegend
CD11b/c	PercpCy5.5	Ox-42	BioLegend
CD45	FITC	OX-1	BioLegend
CD4	Pecy7	W3/25	Biolegend
CD3	Biot	G4.18	ebioscience
Streptavidine	BV 421		Biolegend

subgranular zone (SGZ) and granular cell layer (GCL) of the dentate gyrus by estimating cell density of BrdU positive cells. Briefly, using an Olympus BX51 optical microscope and Newcast software (Visiopharm, Hoersholm, Denmark), every immune-stained cell following inside the contour of the SGZ and GCL was counted, and cell density was estimated as the ratio between the total number of immunostained cells and the area of the SGZ and GCL. Quantification was performed by one researcher blind to the experimental conditions.

1.7. Behavioral testing

Animals were submitted to behavioral testing in adulthood. Behavioral tests were performed on consecutive days, between 9 a.m. and 6 p.m., in the following order: sucrose preference test (SPT), elevated plus maze (EPM), open field test (OFT), novel object recognition (NOR), forced swimming test (FST) and Morris water maze (MWM). During behavioral testing, the researcher was blind to the experimental groups. All tests sessions requiring video recordings were scored by an investigator blind to the experimental groups.

Sucrose consumption test

Anhedonia was assessed by the sucrose consumption test as described by Bessa JM et al. [11]. Briefly, animals were habituated to the sucrose solution during 1 week, in which animals were presented with two pre-weighed drinking bottles, one with water and other with 1% (m/v) sucrose for 1 h. Before each recording, rats were food and water-deprived for 20 h. Percentage of sucrose preference was calculated according to the formula: % sucrose preference = [sucrose intake/ (sucrose intake + water intake)] X 100 [12].

Elevated-plus maze

Anxious-like behavior was assessed through the EPM test, in a 5 min session as previously described [13]. The percentage of time spent in the open arms was used as an index of anxious-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 exploratory activity [14] and as an additional measure of anxious-like behavior [15]. Animals were tested individually for 5 min in a transparent acrylic square arena (43.2 × 43.2 cm) illuminated by a bright white light. With the help of a 16-beam infrared system and a tracking software, the position of the animal was monitored (Activity Monitor software, MedAssociates, VT, USA) considering two previously defined areas: a central and an outer area. The following parameters were recorded: (i) % time spent in the centre of the arena (measure of anxious-like behavior), (ii) total distance traveled (measure of general locomotor activity) and (iii) number of rearings (measure of exploratory activity).

Novel object recognition test (NOR)

Cognitive function was assessed in the NOR test. Rats were habituated to the testing arena for 10 min. On the next day, each animal was allowed to explore two identical objects placed in the arena for 10 min. One hour later, rats explored the same arena for 5 min, 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) [16].

Forced swimming test

The FST was used as a measure of depressive-like behavior [17]. Each animal was placed in a transparent cylinder with 40 cm of diameter, filled with water (24 °C) to a depth of 50 cm. Assays were conducted 24 h after a pre-test session, by placing the rats in the cylinders for 5 min. Immobility time (time floating without evident efforts to escape) and latency to immobility (time from the beginning of the test until stop swimming for the first time) were assessed. Depressive-like behavior was defined as an increase in time of immobility and a decrease in latency to immobility.

Morris Water Maze

The MWM test was performed for four consecutive days in a black, circular (170 cm diameter) tank, filled with water (24 °C) to a depth of 31 cm. A video camera fixed on the ceiling captured the image to a video tracking system (Viewpoint, Champagne au Mont d'Or, France). Four virtual quadrants [north (N), east (E), south (S) and west (W)] were then assigned to the computer and a circular platform was placed within one of the quadrants, 1 cm below water surface (invisible to the rats) and kept in the same position throughout the days. Animals were given four trials each day to find the platform, each starting from a different quadrant [18,19]. Trials were automatically ended once the animals reached the platform or 120 s had elapsed. If an animal failed to find the platform, it was guided to it and allowed to remain there for 30 s before starting a new trial. Time to escape to the platform and distance swum during that period were automatically recorded.

1.8. Statistical analysis

Repeated measures analysis of variance (ANOVA) was used to analyze learning tasks in MWM. The remaining data were analyzed by one-way ANOVA, and when necessary, *post-hoc* Bonferroni's multiple comparisons was conducted. Statistical analysis was performed using IBM SPSS statistics 20.0 (IBM Corporation, Armonk, NY, USA). In all cases, statistical significance was set at $p \leqslant 0.05$. All data are presented as the mean ± S.E.M. The effect size (practical significance) was calculated as follows: for the one-way ANOVA the eta squared (η^2p) was calculated as the ratio of the between groups 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 of 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 [20].

2. Results

2.1. Serum corticosterone analysis

The analysis of corticosterone production, revealed statistically significant differences between the 3 groups studied ($F_{2,19} = 12.79$; $\boldsymbol{p < 0.001}$; $\eta^2 = 0.6007$). A significant increase in corticosterone levels was observed when comparing SHAM animals with PT_0 ($p < 0.05$) and PT_2 ($p < 0.001$), suggesting that even intubation/mechanical ventilation induce surgical stress, Fig. 2-A.

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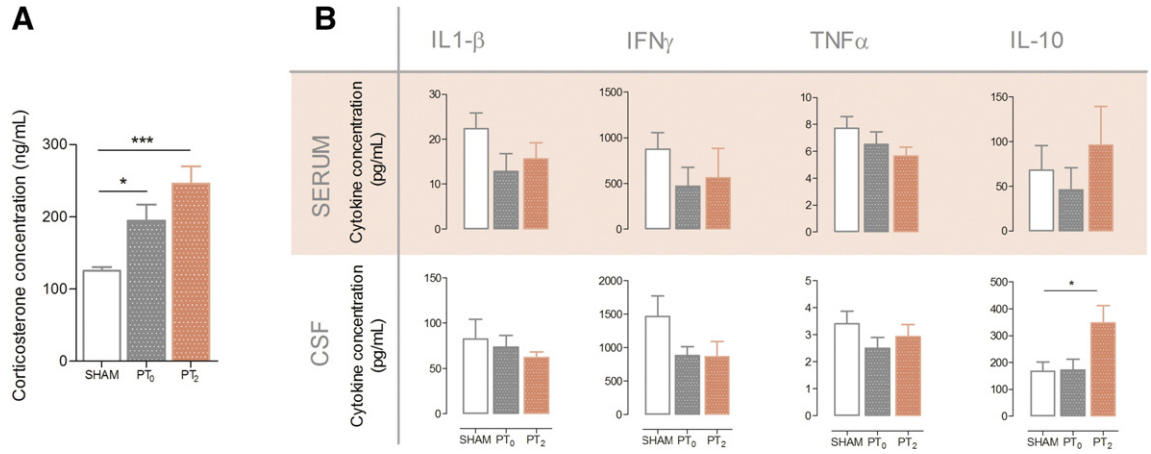

Fig. 2. Serum corticosterone **(A)** immediately after thoracic CO_2-insufflation in SHAM (white bars; n = 8), PT_0 (gray bars; n = 6) and PT_2 (pink bars; n = 6) groups. Peripheral and central IL-1β, IFN-γ, TNF-α and IL-10 **(B)** concentrations after thoracic CO_2-insufflation in SHAM (n = 11), PT_0 (n = 8) and PT_2 (n = 7) groups. Serum and cerebrospinal fluid (CSF) cytokine concentrations measured 24 h after CO_2-insufflation. Data analyzed using 1-way ANOVA followed by a Bonferroni's *post-hoc* multiple comparison test. Values represented as mean + SEM. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

2.2. Peripheral and central cytokine analysis

24 h after CO_2-insufflation, no significant changes were observed in the peripheral production of pro-inflammatory cytokines IL-1β ($F_{2,25} = 1.881$; $p = 0.1752$; $\eta^2 = 0.1405$), IFNγ ($F_{2,25} = 0.9719$; $p = 0.3934$; $\eta^2 = 0.07793$), TNFα ($F_{2,25} = 1.421$; $p = 0.2619$; $\eta^2 = 0.1100$). Regarding the anti-inflammatory cytokine, IL-10, no statistically significant differences were also observed ($F_{2,25} = 0.5442$; $p = 0.5876$; $\eta^2 = 0.04518$).

Regarding central inflammatory response, and similarly to what was observed in the periphery, no significant differences were observed in the pro-inflammatory CSF cytokine concentrations IL-1β ($F_{2,24} = 0.321$; $p = 0.7286$; $\eta^2 = 0.02837$), IFNγ ($F_{2,24} = 0.880$; $p = 0.1764$; $\eta^2 = 0.1459$), TNFα ($F_{2,24} = 0.986$; $p = 0.3889$; $\eta^2 = 0.08227$). However, regarding anti-inflammatory cytokines, the IL-10 concentration in CSF was significantly increased in PT_2 group when compared to both SHAM and PT_0 ($F_{2,24} = 4.619$; $\mathbf{p = 0.0211}$; $\eta^2 = 0.2957$), Fig. 2-B.

2.3. Immune cell phenotyping

Given the high sensitivity of the immune system to alterations in cytokine *milieu*, we analyzed the impact of CO_2-pneumothorax in the cell number of main leucocyte populations. No significant differences were observed in total leucocyte cell number at 24 h ($F_{2,21} = 0.7043$; $p = 0.5069$; $\eta^2 = 0.0690$) and at 4 weeks ($F_{2,10} = 0.1840$; $p = 0.8354$; $\eta^2 = 0.0440$) after insufflation, Fig. 3-B(i). When analyzing each leucocyte sub-population, no statistically significant differences were observed in the number of B-cells ($F_{2,21} = 0.3340$; $p = 0.7202$; $\eta^2 = 0.0340$), CD4 ($F_{2,21} = 0.8901$; $p = 0.4271$; $\eta^2 = 0.0857$) and CD8 T-cells ($F_{2,21} = 0.6378$; $p = 0.5394$; $\eta^2 = 0.0629$) at 24 h post-insufflation. The same profile was observed 4 weeks post-insufflation: B-cells ($F_{2,10} = 0.1595$; $p = 0.8552$; $\eta^2 = 0.0383$), CD4 ($F_{2,10} = 1.251$; $p = 0.3367$; $\eta^2 = 0.2382$) and CD8 T-cells ($F_{2,10} = 1.427$; $p = 0.2952$; $\eta^2 = 0.2629$), Fig. 3-B(ii). It was also observed that CD4/CD8 ($F_{(24h)\,2,21} = 1.041$; $p = 0.3724$; $\eta^2 = 0.0988$), ($F_{(4w)\,2,10} = 0.1749$; $p = 0.8427$; $\eta^2 = 0.0419$) and T/B-cell ($F_{(24h)\,2,21} = 0.0318$; $p = 0.9688$; $\eta^2 = 0.0033$), ($F_{(4w)\,2,10} = 4.528$; $p = 0.0484$; $\eta^2 = 0.5319$) ratios are well preserved in animal exposed to CO_2-insufflation in both time points, Fig. 3-B(iii). Regarding innate immune system, no differences were observed between groups regarding Monocytes ($F_{2,21} = 1.812$; $p = 0.1904$; $\eta^2 = 0.1602$), Granulocytes ($F_{2,21} = 1.641$; $p = 0.2201$; $\eta^2 = 0.1473$) and NK-cell number ($F_{2,21} = 0.2264$; $p = 0.7995$; $\eta^2 = 0.0233$) 24 h after insufflation. Four weeks later, no disturbances in innate immune system were observed: Monocytes ($F_{2,10} = 1.117$; $p = 0.3792$; $\eta^2 = 0.2420$), Granulocytes ($F_{2,10} = 0.4452$; $p = 0.6577$; $\eta^2 = 0.1128$) and NK-cells ($F_{2,10} = 0.1294$; $p = 0.8807$; $\eta^2 = 0.0357$), Fig. 3B-iv.

2.4. Cell survival

In order to determine the influence of CO_2-pneumothorax on the survival of newborn cells in the hippocampal dentate gyrus, BrdU was administered according to a temporal design, Fig. 1. Briefly, BrdU was injected 24 h before the thoracic CO_2-insufflation, and BrdU positive cells were detected 4 weeks after the injection, Fig. 4A. BrdU-positive cells in SHAM animals revealed to be non-significantly different than animals exposed to mechanical ventilation (PT_0) or thoracic CO_2-insufflation (PT_2) ($F_{2,12} = 0.07603$; $p = 0.9273$; $\eta^2 = 0.0150$), Fig. 4B.

2.5. Behavioral testing

No effect of CO_2-pneumothorax exposure was observed in any of the behavioral tests in which the animals were submitted in adulthood, Fig. 5. In anxious-like behavior, evaluated by EPM test, no differences were observed between groups ($F_{2,30} = 0.6725$; $p = 0.5185$; $\eta^2 = 0.0458$). In OF test, also used as measure of anxious-like behavior by quantifying the distance traveled in the central area of the arena, no significant differences were also observed between groups ($F_{2,31} = 0.4220$; $p = 0.6597$; $\eta^2 = 0.0283$). In the same test, and by evaluating the total distance traveled in the open field arena, we observed that the locomotor behavior was also not altered ($F_{2,31} = 0.2289$; $p = 0.7968$; $\eta^2 = 0.0155$) as well as the exploratory behavior evaluated by the number of rearings ($F_{2,31} = 0.5600$; $p = 0.5773$; $\eta^2 = 0.0372$). Regarding depressive-like behavior, evaluated by the SPT and by the latency to immobility and the total immobility time in FST, no significant differences were observed between groups: SPT ($F_{2,31} = 0.4138$; $p = 0.6650$; $\eta^2 = 0.0278$), $FST_{latency}$ ($F_{2,31} = 0.2939$; $p = 0.7475$; $\eta^2 = 0.0199$) and $FST_{immobility}$ ($F_{2,30} = 2.352$; $p = 0.1137$; $\eta^2 = 0.1438$), respectively. In the cognitive assessment, again, no significant impact of CO_2-pneumothorax exposure was detected in the spatial reference memory in MWM ($F_{2,29} = 1.348$; $p = 0.2757$; $\eta^2_p = 0.0545$) and in NOR ($F_{2,31} = 1.198$; $p = 0.3164$; $\eta^2 = 0.0763$) tests.

3. Discussion

Rodents at postnatal day 7–10 are traditionally used as animal models of developmental brain injury as they are considered equivalent to a term human infant based on the measurement of brain growth

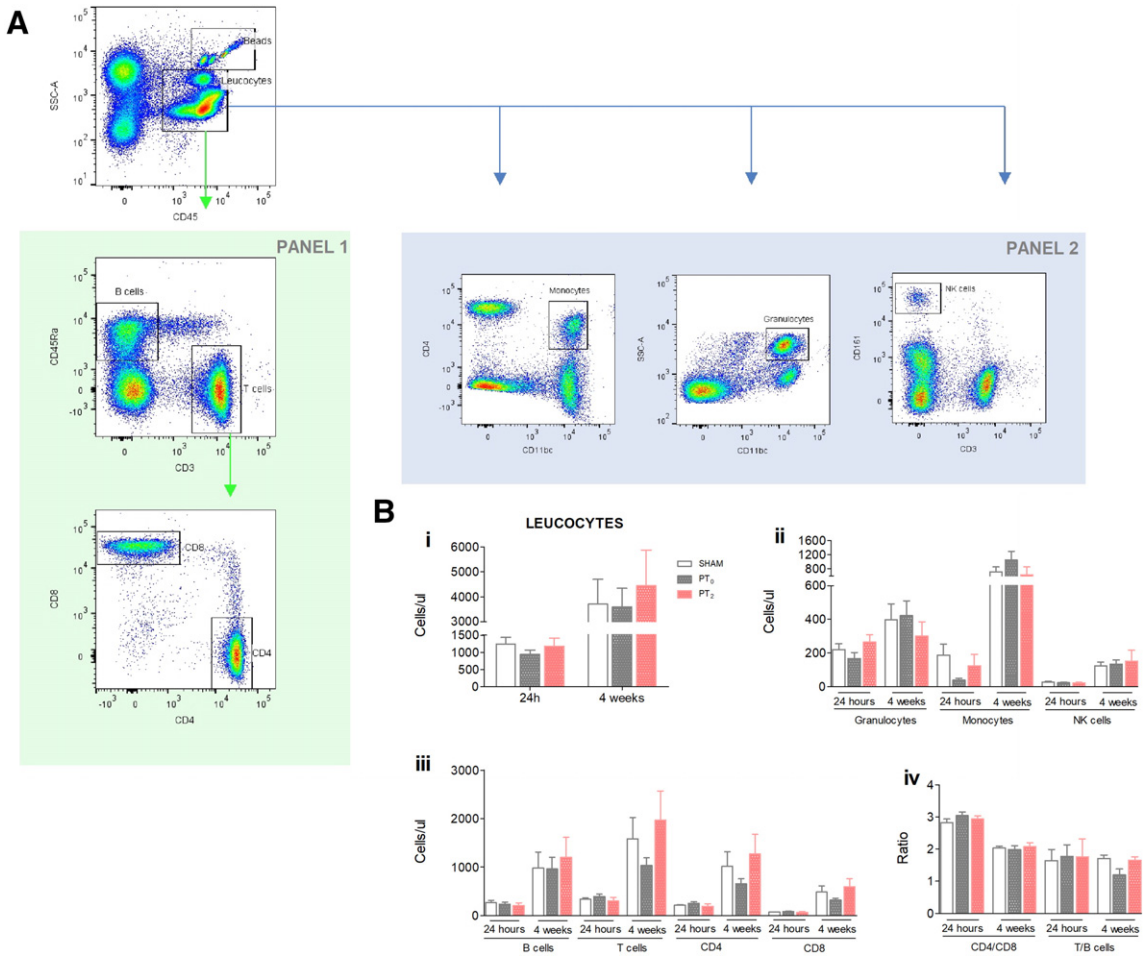

Fig. 3. Blood was processed for flow cytometry and labeled with specific antibodies for general leukocyte identification. Singlets were selected by plotting FCS-H to FSC-A, and leucocyte subset discrimination was obtained by applying the gating strategy represented in (**A**). Groups were compared by one-way ANOVA (**B**): adaptive immune system (**B-ii**); CD4/CD8 and T/B cells ratios (**B-iii**); and innate cells (**B-iv**) of the immune system were analyzed 24 h [SHAM (n = 8), PT_0 (n = 7) and PT_2 (n = 7)] and 4 weeks [SHAM (n = 4), PT_0 (n = 4) and PT_2 (n = 3)] after CO_2-insufflation. Each bar represents the mean + SEM.

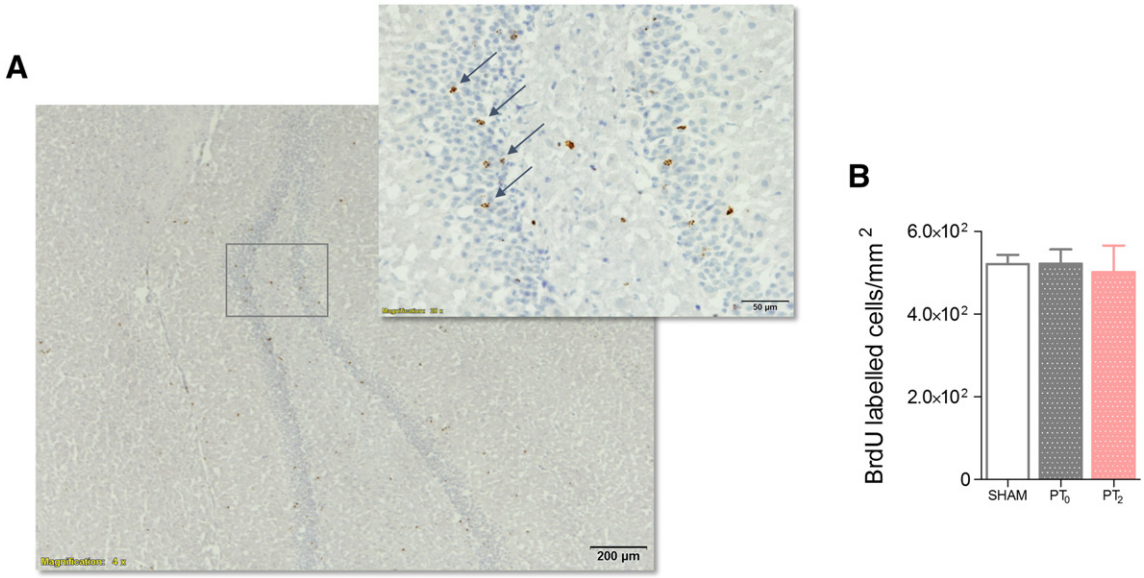

Fig. 4. Coronal sections were taken at the level of the dorsal hippocampus, and BrdU-labeled cells (gray arrows as some examples) were identified in the subgranular zone (SGZ) and granular cell layer (GCL) of the dentate gyrus with optical microscopy (**A**). The total number of BrdU + cells per mm^2 was determined 28 days after BrdU administration to examine cell survival (**B**). SHAM (n = 4), PT_0 (n = 4) and PT_2 (n = 4). Results represented as mean + SEM.

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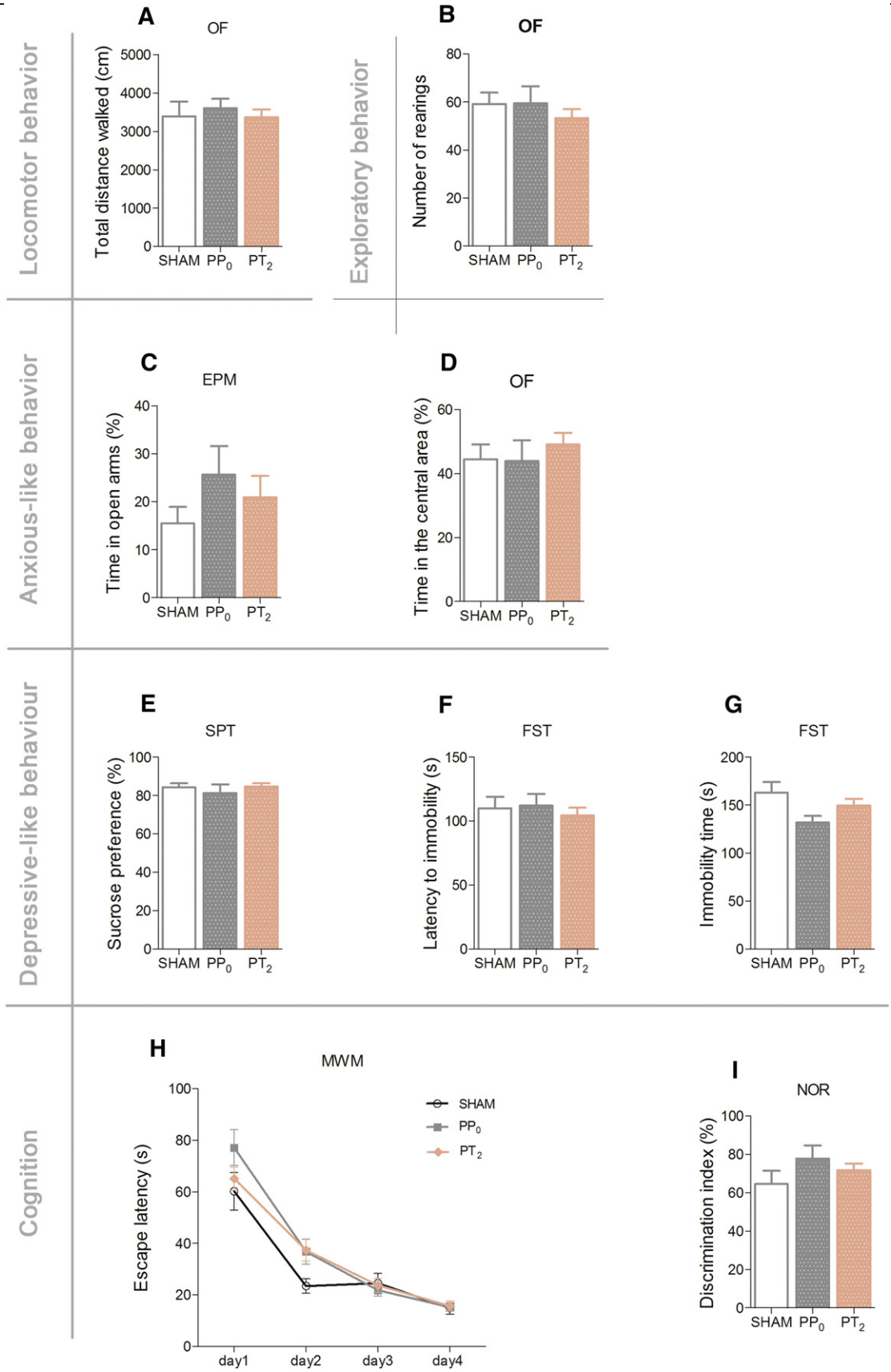

Fig. 5. Adult behavior of rats submitted to CO_2 pneumothorax in neonatal period of life: SHAM animals (white bars); PT_0 (gray bars); PT_2 (pink bars). **Locomotor activity**: (A) Total distance traveled in the open field (OF) arena; **Exploratory behavior**: (B) Number of rearings in the OF arena; **Anxious-like behavior**: (C) Time in open arms in elevated plus maze (EPM) test and (D) percentage of distance traveled in the centre of the OF arena; **Depressive-like behavior**: (E) Percentage of sucrose consumption in SPT and (F) latency to immobility and (G) immobility time in forced swimming test (FST); **Cognitive assessment**: (I) Discrimination index in novel object recognition test (NOR) and (H) escape latency time in Morris water maze (MWM) test. SHAM (n = 7), PT_0 (n = 8) and PT_2 (n = 17). Results represented as mean + SEM.

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spurt [21]. More recently, studies comparing time course of postnatal brain development across species considered that, while the time scale is considerably different, the sequence of key events is largely consistent between humans and rodents [22]. The rodent brain development proceeds on a timeline of days *versus* weeks to months in humans [23]. Therefore, the insufflation period of 30 min applied in our study, although less than the average surgical time for EA repair in humans, will cover relatively larger sequence of brain maturation events when compared to humans. Regarding the insufflation pressure applied in this study, the pressure of 2 mmHg was chosen based on rodent and human CO_2-pneumoperitoneum correlation studies that highlight that the application of human standard working pressures in the rat model will correspond to abnormally high insufflation pressures [24], which may lead to peritoneal capillaries occlusion and microcirculatory failure [25], not simulating routine working pressures employed in humans.

Several studies focused on the early neurodevelopment and long-term psychological outcome of EA surgical repair have been published since many years, associating this high-risk group with psychomotor delay and lower scores in expressive language subscale [26–28] which are associated with either general anesthesia, associated malformations or the surgical approach. However, and since the beginning of the application of minimally invasive surgery in neonatal patients worldwide, no similar longitudinal studies were performed. Recent reports have suggested a potentially deleterious impact of CO_2-insufflation during thoracoscopic approaches over neonatal brain owing to perioperative reduction of brain oxygenation [3–5], however no longitudinal studies were performed confirming whether this minimally invasive approach aggravates the developmental alterations previously described, or if those alterations are a consequence of the postoperative morbidity associated with the open-surgical approaches, namely the thoracotomy.

Every surgical insult triggers a stress response, and the presumed role of this response is to prevent secondary damage and increase the availability of substrates required by organs and healing tissues, contributing to animal survival. However, and contrary to the presumption of survival promotion, earlier studies in pediatric patients demonstrated that the attenuation of surgical stress response was associated with improved outcomes and reduced complications. Therefore, efforts are made to minimize the stress response with minimally invasive techniques [29,30]. The increased corticosterone levels observed in our anesthetized and mechanically ventilated control animals suggest that even the anesthetic perioperative management contributes to some degree of surgical stress but, interestingly, the pneumothorax event did not significantly impact corticosterone production when compared to anesthetized control animals, which may indicate no additional significant surgical stress induced by CO_2-pneumothorax. These findings are in good accordance with the literature since video-assisted thoracic surgery is associated with less tissue injury and consequently reduced acute-phase response and early postoperative stress [31,32]. Nevertheless, most studies do not isolate the surgical approach *per se*, associating the surgical approach (thoracotomy *vs.* thoracoscopy) with the surgical procedure and associated organ trauma, which can overlap the stress effect of CO_2-insufflation.

The surgical stress response reflects a combination of endocrinological, immunological and metabolic changes. Depending on the extent of surgical trauma and neuroendocrine stress response, the systemic glucocorticoid release is many times accompanied by alterations in cytokine production, which modulate the activity of both innate and adaptive immune system. In addition, a surgical insult also triggers a local response, including cytokine production linked to tissue trauma [30]. In our study, we observed that CO_2-pneumothorax did not induce any disturbances in the peripheral pro and anti-inflammatory cytokines when compared to sham or anesthetized control animals. Additionally, no impact on cellular immune system balance was found, since there was no impact on the main leucocyte populations in the blood. Although most human studies compare open thoracotomy with thoracoscopic approaches, studies demonstrate that thoracoscopic approaches result in significantly lower levels of inflammatory factors [33,34] and better preserves postoperative immune function [35]. After every surgical event, there is a delicate balance between the production of pro and anti-inflammatory cytokines since an exaggerated pro-inflammatory response may lead to hemodynamic decompensation and multi-organ failure while a compensatory anti-inflammatory response can cause immunosuppression. In this work, we observed that animals submitted to thoracic CO_2-insufflation present a very well preserved immune function, equivalent to SHAM and anesthetized mechanically ventilated animals.

Interestingly, and regarding CSF cytokine concentrations, the observed increase in CSF IL-10 levels of animals submitted to CO_2-pneumothorax suggests a central anti-inflammatory profile not exactly anticipated by the observed serum levels. This finding might suggest that a cytokine response in CNS could be elicited independently of the systemic one, a phenomenon already described in human patients submitted to non-neurological surgery [36,37]. The upstream mechanisms of this central anti-inflammatory profile need further investigation to clarify whether these findings are related with the gas itself or with the mechanical effects of the thoracic insufflation on blood pressure and circulation. The increased intrathoracic pressure caused by the capnothorax may directly affect the venous drainage from the head and neck owing to venous compression. This phenomenon may consequently lead to increased intracranial pressure (ICP) and significant changes in cerebral perfusion and oxygenation. In the literature, there are contradictory findings relating CSF IL-10 levels and variations in ICP and brain oxygenation/perfusion. Studies focused on the interplay between CSF concentrations of anti-inflammatory mediators in patients with severe traumatic brain injury (TBI) have found that CSF IL-10 levels were significantly increased in patients with high ICP [38,39]. Interestingly, a correlation between IL-10 levels and ICP was emphasized by another study, where an experimental increase in ICP was able to induce a systemic release of IL-10 [40] however, the CSF IL-10 levels were not evaluated. On the other hand, additional studies could not find a correlation between IL-10 and ICP [41]. A study focused on the evolution of cytokine patterns in patients with TBI has explored the relationship between CSF cytokines, ICP, and brain tissue oxygenation. The study concluded that there is no clear association between the temporal pattern of CSF IL-10 levels and ICP, brain tissue oxygenation and the presence of swelling in the computed tomography scan. These findings might suggest that IL-10 does not play a role in the pathogenesis of ICP and brain tissue oxygenation and that IL-10 has an independent evolution than these two clinical variables [42]. Studies in patients with TBI may not be ideal to evaluate the impact of ICP on CSF cytokine levels since these patients usually have severe intracranial injuries, many times associated with additional extracranial injuries, which makes difficult the extrapolation to our study. Since brain oxygenation and perfusion was not monitored in our work, future clinical studies addressing CSF cytokine concentrations after MIS must be correlated with these intraoperative parameters.

A single developmental insult can initiate a cascade of alterations that may not be detected structurally or functionally until much later in life. Thus, these effects may be manifested at a time much removed from the critical developmental window. However, we observed that the CO_2-insufflation had no impact on the survival of the newborn hippocampal cells. Additionally, no functional impact was observed in the long-term behavior in any of the behavioral domains analyzed. However, it is important to recall that animals were submitted to a single insufflation event. Repeated and prolonged insufflations may have different long-term outcomes, and further studies must evaluate those experimental conditions.

4. Conclusion

An anti-inflammatory response takes place in the cerebral compartment after MIS. This peculiar distribution of IL-10 needs further

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investigation and interpretation of its significance in the postoperative period. Most importantly, the CO_2-insufflation seems to result in no significant outcome over neurodevelopment. This absence of structural and functional alterations might contribute to clarify, step-by-step, some of the ambiguities about the impact of CO_2-insufflation over the immature brain of the neonates.

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