Universidade do Minho
Escola de Medicina

Alice Augusta Macedo Miranda

Studies on thoracic and peritoneal CO_2-insufflation in neonatal rats: implications for minimally invasive neonatal surgery

dezembro de 2017

Studies on thoracic and peritoneal CO_2-insufflation in neonatal rats: implications for minimally invasive neonatal surgery
Alice Augusta Macedo Miranda
UMinho | 2017

Universidade do Minho
Escola de Medicina

Alice Augusta Macedo Miranda

Studies on thoracic and peritoneal CO_2-insufflation in neonatal rats: implications for minimally invasive neonatal surgery

Tese de Doutoramento em Ciências da Saúde

Trabalho efetuado sob a orientação do
Professor Doutor Jorge Correia-Pinto
e do
Professor Doutor José Miguel Pêgo

dezembro de 2017

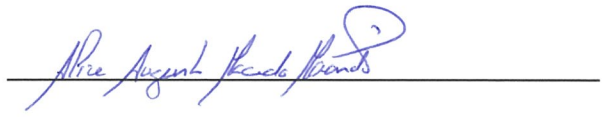
Statement of integrity

I hereby declare having conducted my thesis with integrity. I confirm that I have not used plagiarism or any form of falsification of results in the process of the thesis elaboration.
I further declare that I have fully acknowledged the Code of Ethical Conduct of the University of Minho.

University of Minho, December 22nd of 2017.

Full name: Alice Augusta Macedo Miranda

Signature:



Acknowledgements

À Escola de Medicina e ao Instituto de Investigação em Ciências da Vida e Saúde agradeço a oportunidade de realizar o doutoramento num ambiente estimulante e de rigor científico.

Ao Professor Doutor Jorge Correia-Pinto, Coordenador do Domínio de Investigação em Ciências Cirúrgicas do ICVS, que me acolheu com entusiasmo no seu grupo de investigação, agradeço a orientação deste trabalho, a confiança depositada, a partilha de ideias e o estímulo contínuo.

Ao Prof. Doutor José Miguel Pêgo pela orientação desta tese, disponibilidade e palavras de incentivo.

À Prof. Doutora Margarida Correia-Neves por me ter acolhido e confiado. Pela exigência e seriedade, compreensão e empatia, pelas palavras de encorajamento e motivação.

À Cláudia Nóbrega e Susana Roque pelo acolhimento, ensinamento e partilha diários.

Ao Bruno Rodrigues, Cláudia Miranda, Daniela Calçada, Nuno Santos, Palmira Silva, e todos os MCN's, sem os quais este caminho teria sido muito difícil de percorrer.

A todos os amigos SSRD com quem tive o privilégio de colaborar e que tanto me ensinaram. Todos os MIRD pelo excelente acolhimento, desde a minha chegada ao ICVS até hoje. A todos os NERD pelo ambiente estimulante e de companheirismo.

Special thanks to Vincent Beysang for all the concern and support. For the kind words and excellent work environment, making everything easier.

À Manuela Carneiro pela sua generosidade e positivismo. Obrigada pela amizade.

À equipa do biotério Magda Carlos, Agostinha Costa, Celina Barros, Cláudia Pereira, Daniela Gonçalves, Denise Silva, Filipe Malheiro e Susana Santos. Pela boa disposição e espírito de equipa.

A todos os meus amigos povoenses. Aos meus grandes amigos da UTAD. Amigos que contribuíram para aquilo que hoje sou.

À minha família, Pai, Mãe e Gina, sempre presentes com o seu amor incondicional.

À Licinha por ser a minha maior inspiração!

"The important thing in science is not so much to obtain new facts as to discover new ways of thinking about them"

Sir William Lawrence Brag

This work was performed in the Life and Health Sciences Research Institute (ICVS), School of Medicine, University of Minho, Braga, Portugal (ICVS/3B's – PT Government Associate Laboratory, Braga/Guimarães, Portugal) and supported by Foundation for Science and Technology (FCT) by means of a PhD grant (SFRH/BD/52059/2012) and by FEDER funds through the Operational Programme Competitiveness Factors - COMPETE and National Funds through FCT under the project POCI-01-0145-FEDER-007038; and by the project NORTE-01-0145-FEDER-000013, supported by Norte Portugal Regional Operational Programme (NORTE 2020), under the PORTUGAL 2020 Partnership Agreement, through the European Regional Development Fund (ERDF).

Studies on thoracic and peritoneal CO_2-insufflation in neonatal rats: implications for minimally invasive neonatal surgery

Abstract

Minimally invasive surgery (MIS) has been increasingly used and the continuous development of technology and instruments allowed the application of this surgical approach, not only in adults, but also in paediatric and neonatal patients to correct several birth defects. Since the beginning of its application in adult patients, several studies on the hemodynamic, metabolic and inflammatory changes during MIS are being extensively performed, with a special focus on the effects of CO_2-insufflation. More recently, concerns about the impact of the CO_2-insufflation over the developing organs and organ systems of neonates have been published, with brain oxygenation and perfusion the most investigated events. In this thesis, we aimed to unravel some of these issues and contribute to the understanding of the impact of the thoracic and peritoneal CO_2-insufflation in this sensitive phase of life, that is the neonatal period.

Surgery leads to specific endocrine, immunologic and metabolic changes but, during MIS, the isolated impact of CO_2-insufflation on these parameters is limited. Studies on animal models arose as a methodical way to evaluate the impact of CO_2-insufflation, by avoiding confounding factors associated with human studies. Nevertheless, most studies are performed in adult animals, with only a few studying these impacts on neonatal models. Additionally, many studies are performed without ventilatory support which is the key player for the maintenance of normal blood gas partial pressures and pH during CO_2-insufflation. In this work we developed an intubation technique that could be applied in neonatal rats, allowing respiratory support during CO_2-pneumoperitoneum and -pneumothorax. The adjustments in ventilatory parameters allowed the development of a neonatal animal model

of CO_2-pneumothorax and CO_2-pneumoperitoneum, which was then used to evaluate acute and long-term impact of CO_2-insufflation over neonatal organ systems.

In our anesthetized, endotracheal intubated, mechanically ventilated neonatal rat model, the impact of the CO_2-insufflation over the immune system was evaluated at different levels. The impact of CO_2-insufflation over immune cell populations was evaluated immediately after CO_2-pneumothorax and 4 weeks after exposure, and no major alterations were detected when compared to control animals, suggesting that CO_2-insufflation do not exert a significant impact on cellular immune system balance. But when evaluating peripheral (serum) and central (cerebrospinal fluid [CSF]) cytokine response, our findings support that the old paradigm of an immunologically privileged CNS may not be sustained perioperatively during MIS surgery, since increased anti-inflammatory cytokine IL-10 levels in CSF were found in animals submitted either to CO_2-pneumothorax or CO_2-pneumoperitoneum. The impact of this finding in the developing brain is still unclear, and further study is needed.

It is known that a single developmental insult in the neonatal period can initiate a cascade of structural alterations, manifested much later in life or under specific pathological/stress conditions. Since little is known about the functional consequences of the associated hemodynamic, respiratory and inflammatory consequences of CO_2-insufflation event over brain development, neurodevelopmental milestones acquisition and long-term behavioral outcomes were evaluated in our neonatal animal models of CO_2-pneumothorax and CO_2-pneumoperitoneum. Different insufflation pressures and times were evaluated during CO_2-pneumoperitoneum. This work supports that this early-life event does not seem to induce any negative impact on neurodevelopment or induce behavioral alterations in adulthood.

The findings presented in this thesis provide new insights of the central inflammatory response after MIS and suggests possible implications in other brain related perioperative phenomena. The finding presented in thesis contribute to clarify, step-by-step, some of the ambiguities about the impact of CO_2-insufflation over the immature brain of neonates.

Insuflação torácica e abdominal com CO_2 em ratos recém-nascidos: implicações na cirurgia minimamente invasiva

Resumo

A Cirurgia Minimamente Invasiva (MIS) é cada vez mais utilizada em todo o mundo e o contínuo desenvolvimento da tecnologia e instrumentos cirúrgicos têm permitido a sua aplicação, não só em adultos, mas também em crianças e recém-nascidos para corrigir os mais diversos defeitos congénitos. Desde o início da sua aplicação, vários estudos sobre as alterações hemodinâmicas, metabólicas e inflamatórias têm sido publicados, com especial ênfase sobre os efeitos da insuflação com dióxido de carbono (CO_2) nas cavidades corporais. Recentes publicações sobre o impacto da insuflação com CO_2 nos órgãos em desenvolvimento dos recém-nascidos têm sido publicados, em que os fenómenos de oxigenação e perfusão do cérebro têm sido foco de especial atenção. Nesta tese, temos como objetivo esclarecer algumas dessas questões e contribuir para a melhor compreensão sobre o impacto da insuflação torácica e peritoneal com CO_2 nesta fase de especial vulnerabilidade que é o período neonatal.

A cirurgia induz mudanças específicas a nível endócrino, imunológico e metabólico, mas estudos sobre o impacto isolado da insuflação de CO_2 nestes parâmetros são limitados. Os estudos em modelos animais são muito frequentemente usados para avaliar o impacto da insuflação com CO_2 durante procedimentos minimamente invasivos, contudo, a maioria dos estudos são realizados em animais adultos, com apenas alguns deles recorrendo a animais recém-nascidos. Adicionalmente, os estudos com animais recém-nascidos são realizados sem ventilação mecânica, fator essencial para a eliminação do CO_2 absorvido durante a insuflação, e consequente manutenção de valores fisiológicos de pressão parcial de gases e do pH. Nesta tese otimizámos uma técnica de entubação endotraqueal que pudesse ser

aplicada num modelo roedor neonatal e que permitisse a posterior ventilação mecânica durante a insuflação torácica e abdominal com CO_2. Ajustes nos parâmetros ventilatórios permitiram o desenvolvimento de um modelo animal neonatal de CO_2-pneumotórax e CO_2-pneumoperitoneu.

No nosso modelo neonatal ventilado mecanicamente e exposto a insuflação com CO_2, o impacto da insuflação com CO_2 foi avaliado a diferentes níveis. O impacto da insuflação com CO_2 sobre as principais células do sistema imunitário foi avaliado imediatamente após a insuflação torácica com CO_2 e 4 semanas após esta exposição e não foram encontradas alterações significativas quando comparadas com animais controlo. Estes dados sugerem que a insuflação com CO_2 não constitui um fator perturbador no equilíbrio do sistema imunitário. Mas quando foram avaliados os níveis de citocinas periférica (séricas) e central (líquido cefalorraquidiano), os nossos achados sustentam que o antigo paradigma de um sistema nervoso central (SNC) imunologicamente privilegiado pode não ser mantido no período peri-operatório durante a cirurgia minimamente invasiva. Níveis elevados da citocina anti-inflamatória IL-10 foram detetados no líquido cefalorraquidiano de animais submetidos a insuflação torácica e abdominal com CO_2. O impacto deste achado no cérebro em desenvolvimento é, contudo, desconhecido e novos estudos são necessários.

Um único insulto no período de desenvolvimento neonatal pode iniciar uma cascata de alterações estruturais, manifestadas muito mais tarde na vida ou sob condições patológicas ou de stress. As consequências funcionais dos efeitos mecânicos e químicos da insuflação com CO_2 sobre o desenvolvimento cerebral é desconhecido, e por esse motivo este trabalho teve como objetivo avaliar as possíveis alterações no neurodesenvolvimento assim como avaliar o impacto a longo prazo no nosso modelo animal neonatal. Diferentes pressões e tempos de insuflação foram avaliados. Este evento neonatal não parece resultar em qualquer impacto negativo no desenvolvimento neurológico ou induzir alterações comportamentais na idade adulta. O trabalho aqui apresentado destaca a ausência de consequências funcionais a nível comportamental, mas abre novas portas para futura investigação noutros fenómenos peri-operatórios.

Table of contents

Abbreviations

BSA	Body surface area
CDH	Congenital diaphragmatic hernia
Cdyn	Dynamic compliance
CI	Cardiac index
CNS	Central nervous system
CO_2	Carbon dioxide
CPP	Cerebral perfusion pressure
EA	Esophageal atresia
$EtCO_2$	End-tidal CO_2
FiO_2	Fraction of inspired oxygen
HCA	Hypercapnic acidosis
HR	Heart rate
IAP	Intra-abdominal pressure
ICP	Intracranial pressure
IFN	Interferon
IL	Interleukin
ISP	Intraspinal pressure
LPS	Lipopolysaccharide
MAC	Metabolic acidosis
MAP	Mean arteria blood pressure
MAS	Minimal access surgery
MIPS	Minimally Invasive Pediatric Surgery
MIS	Minimally Invasive Surgery
MV	Minute volume
MW	Molecular weight
$PaCO_2$	Partial pressure of arterial CO_2
PIP	Peak airway pressure
PMN	Polymorphonuclear cells
$rScO_2$	Regional cerebral oxygen saturation
SaO_2	Arterial oxygen saturation
SpO_2	Peripheral oxygen saturation
SPP	Spinal perfusion pressure
TEF	Tracheoesophageal fistula
TNF	Tumor necrosis factor
V_ECO2	Volume of exhaled CO_2
Vgas	Gas volume
$\dot{V}O_2$	Oxygen consumption
V_T	Tidal volume

PART I | Introduction

CHAPTER 1.
Minimally Invasive Pediatric Surgery

1.1. Brief history of Minimally Invasive Surgery in Pediatrics (MIPS)

Until the mid-19th century, the best surgeon was the fastest one, who thereby caused less pain to his restrained and non-anesthetized patient. However, with the first general inhalational anesthesia in the Massachusetts General Hospital (1846), the field of surgery truly expanded, and increasingly sophisticated techniques were developed. At that time, larger incisions were absolutely necessary for an adequate surgical exposure, however these large operative wounds were responsible for significant peri-operative stress and postoperative pain and morbidity. The idea of performing invasive surgeries without large incisions was timeless appealing (Georgeson *et al.*, 2000). This appeal was responded with progressively less aggressive surgical techniques and has culminated in the modern surgical procedures ranging from small incisions to endoscopic procedures using natural orifices as ports (NOTES) as our group has explored in research environment.

The German, Dr. Philipp Bozzini (1773 – 1809) is considered the father of modern endoscopy being the first to hypothesize that adequate organ exposure could be performed without skin incision, being the pioneer in Minimal Access Surgery (MAS) (Figure 1). Dr. Bozzini developed an apparatus called *Lichtleiter*, which used a wax candle as light source and an urethral cannula to direct light into the internal organs through external orifices (1804) (Bush *et al.*, 1974). Almost 50 years later, the word endoscopy was invented by the French physician Desormeaux (1853), who described an endoscope for cystoscopy using a *gazogene* lamp (fueled by alcohol and turpentine) as external light source, which occasionally resulted in burns to the face of the physician and to the legs of patients (Harrell *et al.*, 2005; Bax, 2008). Internal visualization remained poor and only with the invention of the incandescent lamp by Thomas Edison in 1879, a cystoscope with a built-in light bulb was constructed by the German Maximilian Nitze and the Austrian Josef Leiter (1887). The first

examination of the abdomen using a Nitze cystoscope was performed in a dog in 1901 by the German surgeon George Kelling (Ellis, 2007) who termed this procedure *celioscopy*. Despite those significant hallmarks, the introduction of adequate light into the body and the acquisition of clear and undistorted picture was still far from ideal. It was the British physicist, Hopkins, who invented the now classic rod-shaped lenses, a system that has revolutionized image transmission and dramatically improved the resolution and contrast of endoscope image. A system still much in use today (Hopkins *et al.*, 1953). Until the mid-1970s the surgeon had to look with one eye through the telescope, thus close to the patient, which was unappropiate from the point of view of sterility and surgeon ergonomics (Bax, 2004). When video cameras allowed real-time video transmission onto a TV screen, huge advantages to endoscopic surgery arose: binocular view from a convenient distance; a significantly enlarged image; easy observation of minute changes; observation of the procedure by the entire team; and the ability to obtain permanent records (Berci *et al.*, 1986).

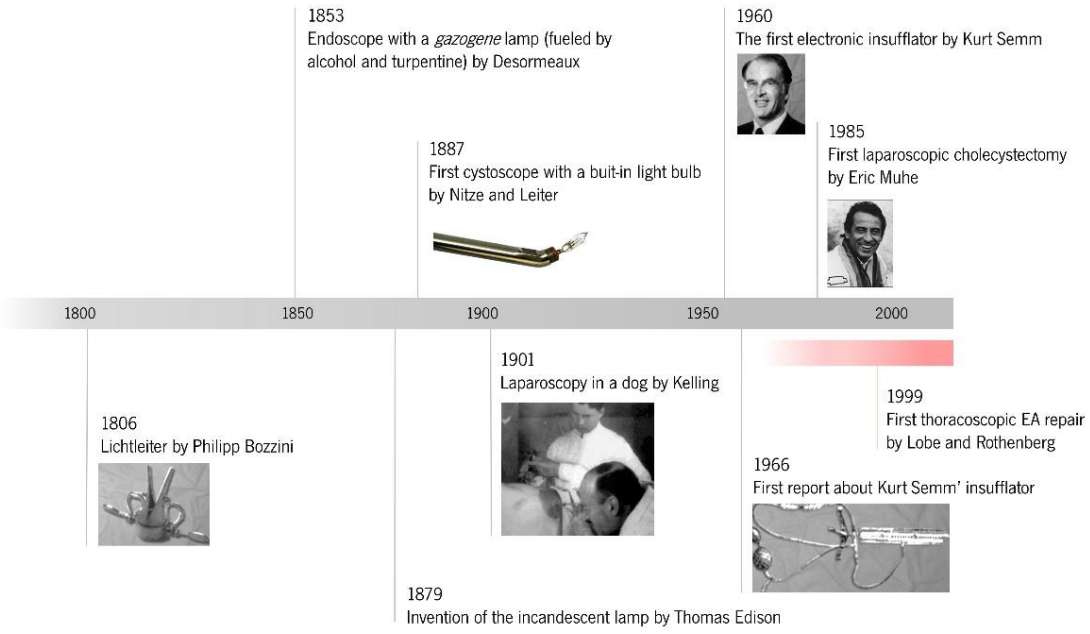


FIGURE 1 MILESTONES IN THE HISTORY OF MIS (GRAY) AND BEGINNING OF MIPS (PINK)

The jump from diagnostic MAS to general surgery began when Eric Muhe, in Germany, performed the first laparoscopic cholecystectomy in 1985 (Mühe, 1992). In 1987, Philippe Mouret and F. Dubois helped popularize the laparoscopic cholecystectomy, and this technique soon became a standard surgical procedure (Georgeson *et al.*, 2000). Since that time, MAS has been applied to numerous other procedures with good results in adult patients, propelling the evolution of MIS.

MIS has been widely performed in adult patients for more than 30 years, however, its use in pediatric and neonatal patients was slowly introduced in the daily practice (Table 1). In 1973, Stephan Gans and George Berci were the pioneers in pediatric laparoscopy but mainly for diagnostic purposes (Gans *et al.*, 1973). A long lag period in MIPS had occurred since its first breakthrough by Drs Gans and Berci, and many epistemological factors have contributed to this delay: (i) many surgeons believed that laparoscopic cases did not really apply to children - the need for cholecystectomy was relatively uncommon in children; (ii) the efficacy of laparoscopy in children was not proven and potential risks and benefits for both patient care and total hospital cost *per* procedure were uncertain; (iii) there was a widely held belief that young children did not experience postoperative pain and surgical stress - as they cannot adequately articulate distress (Schechter, 1989); and (iv) the advantages of smaller incisions were underestimated by pediatric surgeons, already prided on the ability to work with small incisions (Georgeson *et al.*, 2000). In addition to these factors, two technical aspects have also contributed to this delayed development in the pediatric endoscopic surgery: (i) the lack of specialized equipment suitable for pediatric patients, and (ii) the limited number of skilled surgeons performing these procedures. Pediatric surgery is a specialty dedicated to rare conditions and, due to this fact, pediatric endoscopic surgeons were often dependent on the developments of devices used in adult surgery (van der Zee, 2017). The medical industry started running MIS training courses for general surgeons, but very few were prepared towards pediatric surgeons. Thom Lobe, and then Raleigh Thompson were the first to organize pediatric surgery training courses however, surgical training was performed in large animals and with large instruments. Nevertheless, these courses gave

visibility to MIPS, and the industry began to understand its needs. The first set of miniaturized laparoscopic instruments had not been designed for pediatric surgery, but soon they were tested and refined by pediatric surgeons and engineers. In the last decade, companies were encouraged to produce 3 mm instruments, helping the pediatric endoscopic surgical field to evolve (Rothenberg, 2015). Another hallmark in MIPS occurred in 1999 when Drs. Lobe and Rothenberg performed the first thoracoscopic esophageal atresia (EA) repair during a live surgery demonstration at the International Pediatric Endoscopy Group (IPEG) meeting in Berlin (Lobe *et al.*, 1999). Since then, similar achievements were reported by the medical community in peer reviewed literature, documenting its safety and efficacy. In 2001 the first European report of a laparoscopic repair of duodenal atresia was published (Bax *et al.*, 2001), and shortly after, the first case series was released (Steven Rothenberg, 2002). Few years after, the first multi-institutional study of tracheoesophageal fistula (TEF) repair was published by Holcomb (Holcomb Iii *et al.*, 2005). All these publications gave credibility to neonatal and pediatric MIS, and convinced resistant or sceptical surgeons of its advantages (Figure 2). The documentation on the safety of endoscopic procedures and the reports of successful interventions using sophisticated new techniques also propelled pediatric surgeons to develop new and bold thoracoscopic and laparoscopic surgical approaches (Lacher *et al.*, 2014).

TABLE 1 MAIN ACHIEVEMENTS IN HISTORY OF LAPAROSCOPIC AND THORACOSCOPIC SURGERY

SURGICAL PROCEDURE	FIRST DESCRIBED IN PEDIATRIC PATIENTS
Pyloromyotomy	(Alain *et al.*, 1991)
Fundoplication	(Dallemagne *et al.*, 1991)
Thoracoscopic repair of EA	(Lobe *et al.*, 1999)
Duodenoduodenostomy	(Bax *et al.*, 2001)
Thoracoscopic repair of EA with TEF	(Steven S Rothenberg, 2002)

The evolution of MIPS is undoubtedly supported by its benefits to the pediatric patient. Besides better cosmetic outcomes and less wound-related complications (Nwokoma *et al.*, 2009), several studies reported other relevant advantages of MIPS over open surgical

approaches, namely less time to return to full feedings, lower incidence of postoperative emesis, shorter hospital stays (T Fujimoto *et al.*, 1999; Takao Fujimoto *et al.*, 1999; Peter *et al.*, 2006; Adams *et al.*, 2016; Chiarenza *et al.*, 2017), less postoperative requirement for narcotic analgesia (Peter *et al.*, 2006; Gourlay *et al.*, 2009; Bishay *et al.*, 2013a) and shorter duration of additional postoperative ventilation (Gourlay *et al.*, 2009). The possibility of bringing such benefits to all pediatric patients is indeed appealing to any pediatric surgeon.

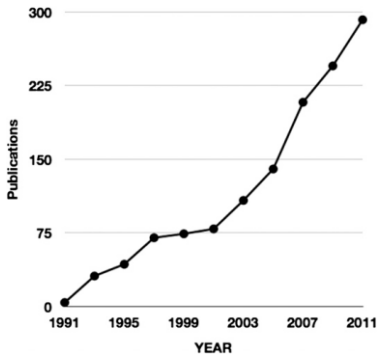


FIGURE 2 NUMBER OF PUBLICATIONS IN PUBMED CONTAINING THE TITLE WORD "LAPAROSCOPIC" AND THE KEYWORD "PEDIATRIC" BY YEAR OF PUBLICATION (Blinman *et al.*, 2012).

1.2. Evolution of pneumoperitoneum and pneumothorax in MIS

The first reference to pneumoperitoneum occurred before the 20th century when a German surgeon, George Kelling, realized that hemostasis could be achieved by increasing the intra-abdominal pressure. Kelling hypothesized that pneumoperitoneum might had a therapeutic use, and hoped that it could arrest the gastrointestinal and intraabdominal bleeding in conditions such as ectopic pregnancy, bleeding ulcers and hemorrhagic pancreatitis (Bax, 2008). He introduced the concept of *lufttamponade*, which consisted in introducing high-pressure air insufflation into the abdominal cavity to stop bleeding. Kelling performed his experiments in anesthetized dogs and fortunately observed that "*After an examination, a dog is as cheerful as it was before*". During his experiments Kelling was driven to evaluate the intra-abdominal effects of *lufttamponade*, namely in organ ischemia, and thus *celioscopy (Koelioskopie)* was born. It was in 1901 that Kelling described for the first time that intra-

abdominal organs could be observed by a cystoscope introduced in the abdomen through a trocar, after the induction of pneumoperitoneum by injecting air through a sterile cotton filter. Despite his reports on successful animal experiments, he was unable to encourage patients to be submitted to this technique (Harrell *et al.*, 2005).

In late 1910 and early 1911, Hans Jacobaeus, a Swedish internist, used the term "*laparothorakoskopie*" for the first time, and described the filtered air pneumoperitoneum as the first step for performing laparoscopy and thoracoscopy for diagnostic purposes in humans. Jacobaeus made clinical use of this technique in I7 cases of ascites (by drawing off the fluid through the trocar and then pumping air before inserting the cystoscope); in the diagnosis of one case of metastatic nodule in the liver; in stomach carcinoma; and in carcinosis of the intestines. He also used the method in the pleural cavity of human patients (Jacobeus, 1911). A response by Kelling appeared months later disputing Jacobeus' claim, stating that he was the first to successfully use "*celioscopy*" in humans between 1901-1910. Since then, some reports were published describing the examination of the abdominal cavity via pneumoperitoneum. In 1911, Bertram Bernheim, an assistant surgeon at John Hopkins University Hospital in the United Sates, described the "organoscopy" by inserting a 12 mm proctoscope through the abdominal wall, referring that "*By inserting the tube further in then and sweeping it around, always keeping the parietal peritoneum in view, the abdominal cavity can be inspected with surprising freedom*". He was the first to introduce the laparoscopy in the United States (Bernheim, 1911).

At this time pneumoperitoneum was still maintained through syringe injections, until Otto Goetze has described an automatic pneumoperitoneum needle (1918) characterized by its safe introduction in the peritoneal cavity and operated with a foot pump (Harrell *et al.*, 2005). In 1924, Otto Steiner at Grady Hospital in Atlanta, unaware of the experiences performed by other researchers, also used a cystoscope and trocar to perform "abdominoscopy" but applied oxygen instead of air to insufflate the abdomen. Steiner claimed then that "abdominoscopy" was a method of immense diagnostic value, describing it as "*the fulfillment of a dream*" (Steiner, 1924). By the same year, started the debate on the

insufflating gas to be used, as until then, the potential dangers associated with insufflation were apparently not understood. Karl Fervers was one of the first to perform laparoscopy for therapeutic purposes and described the use of oxygen to insufflate the abdomen before performing lysis of adhesions using cold cautery electrosurgery. Fervers described an explosion inside of the peritoneal cavity with multiple audible "detonations" and "flames", visible through the abdominal wall. Although the patient fully recovered after several days, Fervers thereafter wisely argued against the use of oxygen to induce pneumoperitoneum (Fervers, 1933). Richard Zollikofer, a gynecologist from Switzerland, was one of the first to prefer carbon dioxide (CO_2) to create pneumoperitoneum, due to its fast absorption and noncombustible properties (Zollikofer, 1924).

In 1938, the introduction of a spring-loaded blunt stylet type of needle with a side hole, so called Veress needle, contributed to a safe penetration of the abdominal wall. Janos Veress, a thoracic surgeon from Hungary was the responsible for the invention of this instrument (Veress, 1938). Although originally designed to treat pulmonary tuberculosis, it was quickly recognized as an effective device to safely induce pneumoperitoneum and minimize intra-abdominal organ injury (Harrell *et al.*, 2005). Until this time, internists were still creating pneumoperitoneum by pumping in air but soon those manual insufflators were no longer adequate for longer operating times and insufflating flow requirements. In early 1960, the gynecologist Kurt Semm realized that, from a technical standpoint, tubal insufflation was similar to creating pneumoperitoneum, as in both procedures CO_2 was injected in the human body. Semm knew that intra-abdominal pressure had to be controlled continuously, the insufflation flow should be limited, and the volume of insufflated gas had to be registered. Therefore he developed the first automatic carbon dioxide insufflator (Litynski, 1998). Afterwards other insufflators were developed with sophisticated features as pre-warmed CO_2, electronic displays and alarm systems.

6.3. Carbon dioxide (CO_2)-insufflation during MIPS

Carbon dioxide is the gas of choice for cavity insufflation during MIS as it is highly soluble in blood, thereby reducing the risk of gas embolism, is rapidly excreted by respiration when absorbed, and does not support combustion when using electrocautery (Tobias, 2002). Other gases have been considered, like air, oxygen, nitrous oxide, helium and argon, however the CO_2 gathers most of the characteristics considered optimal for an insufflation gas (Table 2).

TABLE 2 CHARACTERISTICS OF GASES CONSIDERED FOR CAVITIES INSUFFLATION IN MIS

GAS	SOLUBILITY IN BLOOD	RISK OF EMBOLIZATION	METABOLIC CONSEQUENCES WHEN ABSORBED	SUPPORT COMBUSTION
Carbon dioxide	High	Low	Major	No
Air	Low	High	Minor	Yes
Oxygen	Low	High	Minor	Yes
Nitrous oxide	High	Low	Minor	Yes
Helium	Low	High	Minor	No
Argon	Low	High	Minor	No

The safe application of MIS in pediatric patients requires a thorough understanding of the physiological effects of CO_2-insufflation in this population. Besides the physiological effects induced by tissue trauma associated to every surgical intervention, two main factors associated with the CO_2-insufflation influence physiological responses during MIS: (i) mechanical effect of the increased intra-abdominal or intrathoracic pressure and (ii) chemical effect induced by CO_2 absorption through the visceral and parietal peritoneum and pleura (Figure 3). The most described and studied physiological phenomena associated with MIS is the development of severe hypercapnia and acidosis. Some authors claim that the acidotic profile during MIS results from the mechanical effect of the increased intracavitary pressure over the cardiovascular system, resulting in tissue hypoperfusion, anaerobic metabolism and lactic acidosis (Taura *et al.*, 1998). While others claim that the acidosis is predominantly respiratory due to CO_2 absorption during insufflation (Leighton *et al.*, 1993).

Furthermore, others report acidosis to be 44% metabolic, 21% mixed and 8% respiratory in patients submitted to laparoscopic cholecystectomy (Gándara *et al.*, 1997), suggesting that more than one factor is taking part in the development of acidosis during MIS. This mechanical, chemical and, consequently, metabolic impact of CO_2-insufflation is however manifested in other organ systems as cardiovascular, respiratory and immune systems (discussed in the next sections).

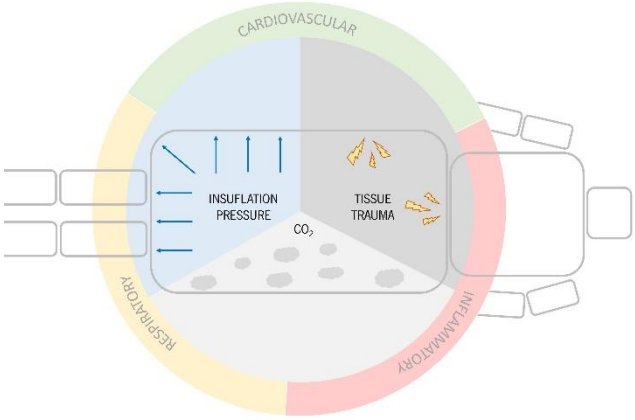


FIGURE 3 MECHANICAL AND CHEMICAL FACTORS INDUCED BY CO_2-INSUFFLATION INFLUENCING PHYSIOLOGICAL RESPONSES DURING MIS

6.3.1. The pediatric patient

The CO_2 absorption is different between adult and pediatric patients (McHoney *et al.*, 2003). These differences may be explained by the Fick's Law of diffusion [$Vgas = D \cdot A \cdot (P_1-P_2)/d$] that states that the diffusion of a given volume of gas through a membrane (Vgas) is inversely proportional to the membrane thickness (d), and proportional to the: (i) surface area of the membrane in contact with the gas (A), (ii) difference in gas partial pressure between the two compartments (P_1-P_2), and (iii) diffusion constant (D). The diffusion constant is proportional to the solubility of the gas (α^{sol}) divided by the square root of its molecular weight (MW) ($D = \alpha^{sol}/\sqrt{MW}$). Therefore, it is expected that children absorb a higher proportion of CO_2 than adults, as their ratio [surface area : body mass] is increased and their peritoneal and pleural

thickness is lower than in adults (Lasersohn, 2011). These anatomical features may not fully explain the differences between adult and pediatric patients regarding the CO_2-absorption. McHoney *et al.* has studied the CO_2-absorption during laparoscopy in children and concluded that younger, low weight children absorb more CO_2 than older individuals, despite the positive correlation between the total volume of CO_2 insufflated and the patient age. These conclusions were obtained by evaluating the CO_2 elimination through respiration (V_ECO_2). However V_ECO_2 includes both metabolically produced and absorbed CO_2 and, therefore, the observed increase in V_ECO_2 may either correspond to the higher CO_2 absorption and/or higher CO_2 metabolic production in younger children (McHoney *et al.*, 2003). Studies on the metabolic response of pediatric patients during laparoscopic surgery reported a hypermetabolic response characterized by an increase in oxygen consumption ($\dot{V}O_2$) and core temperature when compared to open surgery. Studies reported that oxygen consumption rose faster in the laparoscopic group (0.017 ml/kg/min more than in the open surgery group) and the core temperature rose 0.005 °C faster per minute of operation in the laparoscopic group. A positive correlation between $\dot{V}O_2$ and core temperature was found, and these observations highlight a higher metabolic response of children under laparoscopic surgery when compared to open surgery. Additionally, the same study reported that, in the laparoscopic group, younger and lighter children had a more pronounced rise in $\dot{V}O_2$, suggesting that age is modulating the metabolic response to laparoscopy (McHoney *et al.*, 2006). These studies highlight that the combination of both factors (i) laparoscopic approaches and (ii) young and low weight patients, lead to the requirement of scrupulous anesthetic management, as both factors are associated with increased metabolic response. However, no pediatric studies have evaluated whether this response is independent of the insufflated gas or if it is related with the CO_2.

6.3.2. CO_2-pneumothorax *versus* -pneumoperitoneum

Although it is not known what is the exact amount of the exhaled CO_2 that derives from cavities absorption (through peritoneum or pleura), it is certain that small children eliminate relatively more CO_2 during MIS than adults and for that reason, a more scrupulous anesthetic management is required. Within the pediatric patient, differences regarding the cavity accessed (thoracic and abdominal) also exist and the percentage of exhaled CO_2 originating from CO_2-pneumothorax was evaluated by Bishay *et al.*, who have compared the $^{13}CO_2/^{12}CO_2$ ratio from the medical CO_2 used for insufflation with the baseline breath of pediatric patients before CO_2-insufflation. This study concluded that 29% of exhaled CO_2 derived from the CO_2-pneumothorax rather than being of metabolic origin (Bishay *et al.*, 2011). By using the same isotope-ratio mass spectrometry technique, Pacilli *et al.* determined the absorption of exogenous CO_2 during laparoscopy *versus* open surgery in children, and estimated that 10 to 20% of the eliminated CO_2 is derived from peritoneum absorption (Pacilli *et al.*, 2006).

When comparing peritoneal with thoracic insufflation, studies in children have shown a significant increase on end-tidal CO_2 ($EtCO_2$) during thoracoscopy when compared to laparoscopy, despite the lower insufflation pressures usually applied in the first approach (Pacilli *et al.*, 2006; McHoney *et al.*, 2008). The differences observed between these two approaches are suggested to be related with the lung collapse during thoracic insufflation, which contributes to an impaired respiratory capacity. The same study observed a negative correlation between patient weight and maximum increase in $EtCO_2$ in children undergoing thoracoscopic surgery with one-lung ventilation, but this correlation was not observed in children undergoing thoracoscopic surgery with two-lung ventilation. These two findings suggest that thoracoscopic surgery in small age and low-weight patients, combined with single-lung ventilation may be particularly hazardous for the pediatric patients in terms of acid base imbalance (McHoney *et al.*, 2008).

6.3.2.1. Respiratory effects

Laparoscopic and thoracoscopic surgery introduce important challenges to anesthetic a and ventilatory management due to the (i) elevated intra-cavity pressure and (ii) CO_2-absorption. In laparoscopic surgery, pneumoperitoneum causes a cephalic displacement of the diaphragm, resulting in: reduction of functional residual capacity (FCR), that may ultimately lead to atelectasis; increase in peak airway pressure (PIP); reduction in pulmonary compliance; uneven distribution of ventilation and consequent ventilation perfusion (V/Q) mismatch, which can result in hypoxemia (Truchon, 2004). These mechanical effects were studied by Bannister *et al.* who have found a significant correlation between insufflation pressure, during laparoscopic surgery in infants, and $EtCO_2$, PIP, tidal volume (V_T) and dynamic compliance (C_{dyn}). No changes were detected at insufflation pressures of 5 mmHg, but became evident at insufflation pressures of 10 mmHg. The same study measured pulmonary changes in infants undergoing laparoscopic procedures with insufflation pressures between 12 and 15 mmHg and observed an 18% increase in PIP, and decreases of 33% in V_T and 48% in C_{dyn}. It was observed a 13 % increase in $EtCO_2$ and 2 to 11 % decrease in peripheral oxygen saturation (SpO_2), with approximately one-fourth of the patients presenting decreases above 5%. All changes in SpO_2 were corrected by ventilatory adjustments and required increases in PIP, in order to restore V_T, and only few patients required additional changes in respiratory rate (Bannister *et al.*, 2003). Bergesio *et al.* have also detected 26,6% increase in PIP and 38,9% decrease in C_{dyn} after the induction of pneumoperitoneum (Bergesio *et al.*, 1999). Similar findings were obtained by Manner *et al.*, who observed that the same trends were aggravated by the Trendelenburg position adopted during laparoscopic procedures in pediatric patients. The author observed a 17% decrease in lung compliance induced by the Trendelenburg position, which was further decreased by 27% during CO_2-insufflation at an intrabdominal pressure (IAP) of 12 mmHg. Furthermore, a 19% increase in PIP was detected when patients were in Trendelenburg position and aggravated by 32% during CO_2-insufflation (Manner *et al.*, 1998).

This derangement in ventilation due to the mechanical impact of the increased intracavity-pressure requires appropriate monitoring of exhaled CO_2 in order to prevent severe hypercapnia. Increases in minute volume (MV) of 30 to 40% are described as being required to obtain physiological $PaCO_2$ levels during 8 mmHg pneumoperitoneum (T Fujimoto *et al.*, 1999). Other studies, including thoracoscopic and laparoscopic procedures, have shown increases in MV of 22,6%, mostly associated with increases in tidal volumes rather than in respiratory frequency (Kalfa *et al.*, 2005). These adjustments in MV are essential for counterbalancing the reduced pulmonary compliance and increased respiratory resistance. However, increases in MV might be responsible for excessive increases in PIP which carries increased risk of barotrauma and hemodynamic changes (Kalfa *et al.*, 2005).

During thoracoscopic procedures, the mechanical effects of CO_2-insufflation are applied direct over lungs affecting its compliance due to the presence of the CO_2 in the pleural space. In neonates under minimally invasive surgery, significant changes and alterations in SpO_2 are more pronounced in thoracoscopic procedures when compared to laparoscopy (Bishay *et al.*, 2013a) and the requirement of an initial 100% in the fraction of inspired oxygen (FiO_2) was verified in pediatric patients submitted to thoracoscopic procedures, while in laparoscopy only a 58% FiO_2 was required (Kalfa *et al.*, 2005). These studies highlight that the ventilatory management during MIS must account for the mechanical impact of increased intra-cavity pressure over the respiratory system. CO_2 retention (hypercapnia) and hypoxia can occur if moderate hyperventilation is not adopted.

Studies have evaluated the association between blood gas derangement in neonates and children and possible long-term neurocognitive dysfunctions, most of them performed in preterm, low-weight children requiring mechanical ventilation. Some studies relate hypoxia and hypercapnia/acidosis with damage of the immature brain (Lavrijsen *et al.*, 2005; Hagen *et al.*, 2008; Volpe, 2009; Salmaso *et al.*, 2014) associated with dysfunctions years later (Leviton *et al.*, 2010), while others associate these events to neuroprotective benefits such as increased neurogenesis in the hippocampus, counteracting the adverse effects of brain lesions (Ladilov, 2012; Tregub *et al.*, 2016). A possible balance between injury and

neuroprotection determines the long-term functional consequences of transient hypercapnia and hypoxia in neonatal patients and the susceptibility of the newborn brain to damage may be determined by various factors, including severity and duration of the hypoxia and hypercapnia/acidosis events. However, no studies were yet performed focusing on the long-term impact of blood gas derangements induced by CO_2-insufflation during MIS.

6.3.2.2. Cardiovascular effects

The hemodynamic alterations induced by CO_2-insufflation during laparoscopic and thoracoscopic procedures may result in changes in end-organ perfusion and oxygen delivery. During MIS, both mechanical and chemical factors influence the cardiovascular system, the first one due to the elevation in intracavity pressure, and the second one due to the hypercapnia development after CO_2 absorption.

Animal studies on the isolated mechanical effects of the increased IAP, by applying peritoneal saline instillation in a rabbit model, have shown significant increases in cardiac output [Cardiac output = stoke volume x heart rate (HR)/body surface area (BSA)] when applying IAP under 20 mmHg. This initial increase in cardiac output may be attributed to a blood volume shift from the intra-abdominal to the intrathoracic cavity, caused by a compression of intra-abdominal blood vessels. The continuous increase in IAP above 20 mmHg led to a decrease in cardiac output, possibly as a result of excessive venous compression and, consequent decreased cardiac venous return (Robert Sümpelmann *et al.*, 2006). This initial increase in cardiac output was also verified in another study using CO_2-insufflation pressures of 8 mmHg. However, this increase was only observed in the first 30 minutes of pneumoperitoneum. From 30 to 210 min of CO_2-insufflation, a progressive decrease in Cardiac Index (CI) was observed [CI = CO/BSA], suggesting that prolonged pneumoperitoneum contributes to a decreased CI, even when applying constant and relatively low insufflation pressures (R Sümpelmann *et al.*, 2006).

Studies on the chemical effects of acidosis over the cardiovascular system, were performed in animal models. Hypercapnic acidosis (HCA) was induced by increasing the inspired fraction of CO_2 until reaching a pH of 7.1, with no CO_2-insufflation over body cavities. The HCA led to an increase in mean pulmonary artery pressure and pulmonary vascular resistance (PVR). However, these increases were not limited to the HCA group, being also verified during metabolic acidosis (MAC) induced by intravenous infusion of hydrochloric acid. Both, HCA and MAC, had the same effect on the pulmonary circulation indicating that the main stimulus in pulmonary circulation is the hydrogen ion concentration [H^+]. Regarding systemic circulation, only HCA resulted in increased mean arterial pressure (MAP) despite the reduction in systemic vascular resistance (SVR). Both types of acidosis lead to a decrease in stroke volume and increased HR suggesting that cardiac function is affected by [H^+]. These findings were supported by *in vitro* experiments, where contraction force of isolated cardiac tissues (right ventricular trabeculae) was decreased in acidic solution. Despite the observed decrease in stroke volume, the cardiac output was maintained in MAC and even increased in HCA as a result of the increase in HR. The increase in cardiac output observed in HCA group may be due to the stimulation of the central chemoreceptors in the *medulla* that are highly sensitive to variations in blood $PaCO_2$. Regarding the regional circulation, no alterations on renal perfusion were observed in HCA or MAC groups. However, HCA increased carotid, portal and total liver blood flow, suggesting a specific vasodilatory effect of CO_2 on the hepato-splanchnic circulation, which revealed to be independent of systemic pH changes (Stengl *et al.*, 2013). These animal studies support that cardiovascular alterations during MIS results not only from the acid-base changes after CO_2-absorption but also from the effect of the gas itself.

Human pediatric studies on the hemodynamic effects of CO_2-insufflation are influenced by several confounding factors namely the intravascular volume status, patient positioning, anesthetic technique and underlying cardiovascular status, which may explain inconsistencies between studies. In neonatal patients submitted to laparoscopic surgery, no significant changes in systolic blood pressure was observed when patients are submitted to

a limited insufflation pressure of 8 mmHg (T Fujimoto *et al.*, 1999). A prospective study with patients undergoing laparoscopic fundoplication in 33 children, found no significant changes in HR or blood pressure when insufflation pressures did not exceed 10 mmHg (Mattioli *et al.*, 2003). The same observations were obtained even when including laparoscopic and thoracoscopic procedures, presenting null variations in systolic blood pressure or, when present, were smaller than 5 mmHg. Only ten neonates (20%) included in this study have shown decreases in blood pressure above 10 mmHg, with nine patients requiring vascular expansion with 4% albumin due to systolic blood pressure < 45 mmHg (Kalfa *et al.*, 2005). Other studies have shown significant increases in MAP after pneumoperitoneum in infants (Tytgat *et al.*, 2015) and neonates (Li *et al.*, 2013). Nevertheless, in the first study, the initial MAP was low in comparison to non-anesthetized term infants with the same age (Tytgat *et al.*, 2015) and in the second one, CO_2 insufflation pressure ranged from 8 to 14 mmHg which may be considered a high upper limit (Li *et al.*, 2013). A study on the hemodynamic changes during laparoscopic herniorrhaphy in children between 2 and 6 years old had found significant hemodynamic changes when patients were submitted to an IAP of 12 mmHg. These changes include an increase in MAP and a decrease in HR and CI, returning to baseline levels when IAP was reduced to 6 mmHg. The reduction of 13% in CI, measured by transesophageal echocardiography, was considered by the authors as a non-substantial risk to a healthy child. Nevertheless, lower IAP should be applied whenever possible, especially in children with cardiocirculatory problems (Sakka *et al.*, 2000). These findings were shared by other studies, namely during laparoscopic fundoplication in children under 3 years old with insufflation pressures of 5 mmHg which resulted in increased CI, HR and MAP (De Waal *et al.*, 2003), where a decrease in cardiac output was observed during laparoscopic procedures with insufflation pressures of 12–13 mmHg (Kardos *et al.*, 2001). Findings suggest that there is a threshold in insufflation pressure at which significant changes in cardiac output and CI occur. Moderate increases in IAP influences venous vasculature promoting venous return, most likely by reducing the blood volume sequestrated in the splanchnic vasculature. This increase in venous return will consequently increase

right- and left- cardiac filling pressures, with a resultant increase in cardiac output and arterial pressure. Following this transient rise in venous return with the initiation of pneumoperitoneum, a steady state of decreased blood circulation comes after, due to the continued compressive effects. The compressive effects on the arterial vasculature and capillaries lead to an increased afterload, SVR, and arterial pressure, although stroke volume and cardiac output decrease. HR may rise transiently in response to increases in SVR and arterial blood pressure to maintain cardiac output, but most studies have reported no significant long-term changes during laparoscopy. When applying a very-high insufflation pressure, the inferior vena cava is compressed and venous return and preload decreases. Left ventricular function is therefore impaired and cardiac output decreases (Baroncini *et al.*, 2002).

Few studies have evaluated the cardiovascular impact of thoracic CO_2 insufflation in pediatric patients however, the findings are similar to pneumoperitoneum studies. Cardiac output was described as being constant until reaching an intrathoracic insufflation pressure of 2 mmHg, increasing significantly from 4 mmHg until reaching a peak at insufflation pressures of 6 mmHg (Mukhtar *et al.*, 2008).

The derangements of cardiac function during MIS may also be related with patient specificities, as neonates have a relatively fixed cardiac contractility because of their immature myocardial fibers. The relationship between the lengthening of the myocardial fibers during the ventricular filling and the contractility is known as the Frank-Starling Law, and the greater the lengthening of the fibers, the greater the ventricular contraction and hence, increased stoke volume and cardiac output (Blackburn, 2013). The contractility and ventricular compliance are effectively reduced in the neonates, and the myocardium is near the functional limit at baseline levels. Therefore, the efficiency of the cardiac contractions is influenced by underfilling or overfilling during the preload period, and adequate cardiac output is maintained primarily through an increase in HR (Vijayalakshmi *et al.*, 2013). All the described changes in cardiac function have a direct effect on tissue perfusion. To understand the influence of elevated IAP on the quality of tissue perfusion, Tytgat *et al.* have

investigated the alterations in buccal and sublingual microcirculation in neonates undergoing laparoscopic pyloromyotomy, in order to understand the influence of the increased IAP on the quality of tissue perfusion. This study has shown significant changes in microvascular diameter and a reversible vasodilation after cessation of the 8 mmHg pneumoperitoneum, but no changes were observed in perfused vessel density (recruitment or closure of microvasculature). These observations indicate no substantial perfusion changes in buccal and sublingual microcirculation, which is known to maintain a common vascular pathway that provides blood to the brain. This may suggest no cerebral microcirculatory instability (Tytgat *et al.*, 2013).

Blood pressure measurements during anesthesia are often used as an indicator for organ perfusion and, although all organs are at risk when poorly perfused, cerebral perfusion is the most critical for a neurocognitive outcome. Cerebral perfusion pressures vary directly with arterial blood pressures and maintaining blood pressure within the limits of cerebral autoregulation is essential for cerebral protection. However, the limits of cerebral autoregulation in neonates are not precisely known (McCann *et al.*, 2014). There is evidence of direct ischemic cerebral damage following a general anaesthetic in young infants submitted to major neurosurgery or cardiac surgery (Walker *et al.*, 2006), but no long-term studies have valuated the neurodevelopmental impact of circulatory instability during MIS in these fragile patients. During MIPS, the case-by-case identification and correction of risk factors such as intraoperative hypo and hypertention may be more important in terms of improving the long-term neurocognitive outcomes in these patients, rather than establishing general CO_2-insufflation pressure limits.

6.3.2.3. Inflammatory response

It is increasingly evident that the advantages of MIS are not only attributed to the reduced tissue injury secondary to smaller incisions. With the increasing complexity of surgeries performed by minimally invasive approaches, the magnitude of operations begins to

outweigh the magnitude of incisions, and the specific immunological differences between laparoscopic and thoracoscopic *versus* open surgery may result much from the unique properties of the gas used for cavity insufflation. Experimental studies have shown that acute immunological effects can be found locally, in the surfaces of the insufflated cavity, or can be followed by a systemic acute-phase reaction interfering with the function of several cell populations (Neuhaus *et al.*, 2004; Ure *et al.*, 2007). Experimental and clinical data suggest that the intensity of the acute-phase response after surgery is proportional to the extent of tissue damage (Ruzic *et al.*, 2005) and the postoperative levels of proteins and cytokines related to acute-phase response, e.g. tumor necrosis factor (TNF)-α, interferon (IFN)-γ, IL-1, IL-6, and IL-10, C-reactive protein, have been used as criteria to evaluate the magnitude of tissue trauma during surgery and the development of associated complications (Miyake *et al.*, 2002; Mutoh *et al.*, 2004; Fracalanza *et al.*, 2008).

Peritoneum contains approximately 300 cells/mm^3, mostly macrophages and some desquamated mesothelial cells and lymphocytes. However, the ability of the peritoneal cavity to generate polymorphonuclear cells (PMN) and macrophages is huge after abdominal injury (Heel *et al.*, 1996). During abdominal surgery, degranulation of cells releases vasoactive substances, complement, opsins and cytokines that regulate macrophage phagocytic function (Neuhaus *et al.*, 2004). A study focused on the pattern of peritoneal cell recruitment after CO_2- *versus* air-pneumoperitoneum have shown that air exposure triggered a higher rate of PMN migration from the intravascular compartment into the peritoneal cavity, associated with a decreased programed cell death. These two events suggest a higher peritoneal inflammatory status and longer resolution of inflammation process in air-insufflation groups. Interestingly, there was a significantly lower percentage of fluorescent labeled macrophages in the peritoneum after air-insufflation, which may indicate that there is a higher inflammatory stimulation which is causing higher clearance and increased migration of macrophages to draining lymph nodes (Moehrlen *et al.*, 2006). Similar findings were obtained by Ure et al. when comparing air with CO_2-insufflation during laparoscopy in

a pig model. Air laparoscopy resulted in increased peritoneal PMN and a decrease in the percentage of peritoneal macrophages (Ure *et al.*, 2002).

In vitro studies have found that lipopolysaccharide (LPS) stimulated macrophages derived from rats exposed to CO_2-pneumoperitomeum, presented a reduced TNFα production when compared to macrophages from animals submitted to gasless laparoscopy or laparotomy (Mathew *et al.*, 1999). Furthermore, intraperitoneal macrophages harvested from rats 3 days after undergoing laparoscopy with CO_2-insufflation presented the same response, which was not observed in macrophages derived from animals submitted to gasless or helium laparoscopy (Neuhaus *et al.*, 2000). *In vitro* incubation of murine macrophages with CO_2, but not in those incubated with air or helium-rich environment, also present a reduction in TNFα and IL-1 production. A marked inhibition of IL-1 was seen after 15 minutes of CO_2 exposure, while it was needed a minimum of 30 minutes to inhibit TNFα. This depression in cytokine production was reverted after incubation in a controlled atmosphere (West *et al.*, 1996, 1997). These *in vitro* immunomodulatory effects of CO_2 have also been shown in human primary peritoneal macrophages, with IL-1β and TNFα secretions increasing to normal levels only after a recovery period of 24 hours. Helium exposure had no suppressive effect on cytokine levels (Kopernik *et al.*, 1998).

Abdominal CO_2-insufflation, before experimental induction of acute pancreatitis, resulted in a significant decrease in ascites volume, peritoneal cell number and TNF α levels in serum and peritoneal lavage (Machado et al., 2010). Jacobi *et al.* have also demonstrated decreased TNF α and increased IL-10 levels in plasma of rats undergoing laparoscopy with CO_2-insufflation, as compared with controls or rats undergoing helium laparoscopy (Jacobi et al., 1998). Furthermore Hanly *et al.* observed that exposure to CO_2-pneumoperitoneum was associated with significantly lower serum TNF α levels when compared with helium or anesthesia control groups, and presented significant increased serum IL-10 levels and reduced mortality among animals with LPS-induced sepsis (Hanly et al., 2006).

West *et al.* attributed the impaired function of peritoneal macrophages to the cytosolic acidification caused by CO_2-insufflation. Both *in vitro* and *in vivo* experiments have shown

that a transient exposure to CO_2 leads to a reduction in intracellular and tissue pH, respectively. Additionally, *in vitro* assays have shown that pharmacological intracellular acidification reproduced the same inhibitory effect on macrophage TNFα production. These two findings support that resident peritoneal macrophage acidification induced by peritoneal CO_2-insufflation might contribute to blunting of the local inflammatory response during laparoscopic surgery (West *et al.*, 1997). Similar findings were shared by Rotstein *et al.* who observed that *in vitro* CO_2-exposure causes intracellular acidification of peritoneal macrophages, which in turn suppresses production of TNFα in response to LPS challenge (Rotstein, 2001). In accordance with these *in vitro* observations, *in vivo* acidification of the peritoneum by acidic lavage also leads to an increase in serum IL-10 levels and decrease in TNFα levels in response to LPS challenge. Furthermore, the degree of peritoneal acidification correlated directly with the degree of IL-10 production and inversely with the degree of TNFα production (Hanly *et al.*, 2007).

Other research groups have focused in understanding whether this attenuation of acute phase inflammatory response was a consequence of the systemic acidosis induced by the peritoneal CO_2 absorption or by the local acidosis induced by the presence of CO_2 in the abdominal cavity. Hanly *et al.* concluded that systemic acidosis is not responsible for the reduction in the acute phase inflammatory response, as no differences were observed between normocarbic and hypercarbic CO_2-insufflated groups. Both groups presented attenuation of the acute phase inflammatory response and these findings might suggest that the attenuated inflammatory response after MIS is attributed to the presence of CO_2 in the abdominal cavity and not from changes in systemic pH. Hanly *et al.* have also confirmed that peritoneum is acidotic during abdominal CO_2-insufflation, even when systemic pH is corrected by mechanical ventilation adjustments (Hanly *et al.*, 2005).

To understand if this local acidification, that seems to be independent of the systemic acid-base status, has impact in distant organs Ure *et al.,* by using a pediatric porcine model, compared air- with CO_2- laparoscopy and analyzed the distant pulmonary response. The author observed that macrophage numbers in alveolar lavage were significantly increased

after air- *versus* CO_2 -laparoscopy. The same author compared air- with CO_2- laparotomy groups, performed in a pressurized balloon filled with the corresponding gas and the author found no differences between laparotomy groups, which might suggest that laparotomy overruled the effects of CO_2 on distant organ injury (Ure *et al.*, 2002).

Pleural and peritoneal lavages of children and adolescents undergoing non-contaminated laparoscopic and thoracoscopic surgical procedures have been evaluated. A significantly higher percentage of macrophages was observed in the pleural compared to the peritoneal lavages, with fewer lymphocytes and a small amount of PMNs. The spontaneous and LPS-triggered cytokine release of pleural *versus* peritoneal macrophages was measured *in vitro* and the pattern of cytokine release was similar in both. This finding suggests that macrophages in the peritoneal and pleural cavity show a similar inflammatory response (Shimotakahara *et al.*, 2007). The same research group examined the effects of CO_2 on human adolescent pleural macrophages, harvested from patients undergoing elective surgery for pectus bar correction. After *in vitro* LPS-stimulation, macrophages from CO_2-exposure and not helium hypoxic animals, presented a significant reduction in TNFα production, as well as a significant increase in the release of IL-10 and IL-1β within the first 4h after incubation (Shimotakahara *et al.*, 2010). These studies show that both cavities, thoracic and abdominal, respond similarly to CO_2-insufflation, with reduction of TNFα and increase in IL-10 levels.

Several authors have suggested that the modulation of local macrophages by CO_2-insufflation, contribute to reducing the systemic stress response following laparoscopic surgery. (Novitsky *et al.*, 2004). The acute-phase response and cytokines are important components of immunological function and, although cytokine levels do not indicate immune status directly, they are good indicators of the activation of the systemic immune system. Higher serum levels of TNFα and other proinflammatory cytokines have been shown to correlate with mortality among patients with sepsis (Casey *et al.*, 1993; Gogos *et al.*, 2000) and the anti-inflammatory cytokine IL-10 has been shown to be protective in this context (Howard *et al.*, 1993; Rongione *et al.*, 2000). Furthermore, IL-10 has been shown to

downregulate TNFα in surgical models of sepsis (Rongione *et al.*, 1997; Chang *et al.*, 2002). However, studies have not shown how CO_2 stimulates IL-10 production (and/or TNFα inhibition, if through a different mechanism). The fact that IL-10 levels are increased in animals subjected to the biological activity of CO_2 suggests that increased IL-10 may be fundamental to the mechanism of CO_2-insufflation-specific immune protection during MIS (Hanly *et al.*, 2006).

In MIS, systemic immune responses undeniably are still activated. It is apparent, however, that laparoscopic and thoracoscopic surgery appears to induce a smaller injury, resulting in proportionally decreased immunologic changes when compared to conventional open surgery (Jacobi *et al.*, 2002). In addition to improved cosmetic and faster functional recovery, a patient undergoing laparoscopic and thoracoscopic surgery may benefit from a net immunologic advantage and the CO_2-insufflation is pointed as a key event to explain why laparoscopic surgery is so well tolerated (West *et al.*, 1997).

The understanding the unique pediatric inflammatory response and the exploration of the developmental biology of the innate immune system will improve strategies and anticipate responses to injury and interventions of this specific population. Studies have established a relationship between patients age and cytokine production by circulating cells of the immune system. A proportional relationship between age and monocyte production of IFN-γ, TNF-α, IL-12 and IL-2 was observed, whereas IL-10 production showed no age pattern. Studies have also demonstrated that stimulated macrophages from children show a greater anti-inflammatory-to-pro-inflammatory cytokine ratio (IL-10-to-TNFα ratio) than macrophages from adults. It is suggested that this attenuated systemic inflammatory response may confer protection to the pediatric patient, with less collateral and systemic damage, and that the pro-inflammatory-to-anti-inflammatory ratio is more important than the absolute cytokine levels to the restoration of homeostasis after injury (Wood *et al.*, 2010).

Many studies have compared the release of inflammatory cytokines following laparoscopic *versus* open surgery in children and most of the findings are in accordance with adult human studies, showing a faster restored or even preserved immune function (T Fujimoto *et al.*,

1999; Takao Fujimoto *et al.*, 1999; Li *et al.*, 2005; Wang *et al.*, 2009). However, no human pediatric studies were focused on different type of gas insufflation or on the isolated systemic effects of CO_2-insufflation by comparing these patients with the pediatric non-operated population.

CO_2-insufflation during MIS may impact the immune system at different levels. Besides the acute production of inflammatory cytokines, an activation of cellular immune mechanisms occurs in response to surgical injury. The stress response to injury is associated with an early rise in stress hormone levels combined with a decrease in cellular immune response. Regarding the cell-mediated immune function, many studies have compared laparoscopic with open surgical approaches, but few studies have evaluated the isolated peri- and post-operative disturbances induced by CO_2 during MIS. A study from Evrard *et al.* evaluated quantitatively the T-lymphocyte subsets following laparoscopic cholecystectomy in adult patients, in which each patient was his own control. The author evaluated the effects of CO_2-pneumoperitoneum on intraperitoneal lymphocytes, and no difference in lymphocyte viability was found before or after CO_2-pneumoperitoneum and the peritoneal CD4/CD8 ratio was similar before and after pneumoperitoneum. Regarding the circulating lymphocyte subpopulations, a significant reduction in the absolute lymphocyte count (CD3) and in CD4 cells occurred on postoperative day 1 and returned to preoperative values on postoperative day 2, which suggest brief, moderate immunodepression (Evrard et al., 1997). Although the author found a moderate immunosuppression, it is known that laparoscopic surgery is associated with less immunosuppression than open surgery (Novitsky et al., 2004). However, it is important to understand if this relatively low immunosuppression is associated with the gas used for insufflation. An in vitro study from Lee *et al.* evaluated the lymphocyte proliferation derived from mice submitted to a full laparotomy, whether performed in a sealed CO_2-chamber or in room air. The author found no differences between groups however, lymphocyte proliferation from these two laparotomy groups was significantly lower than both anesthetized controls and CO_2-pneumoperitoneum groups. These finding hypothesize that the suppression of lymphocyte proliferation may be more related with incision length than

with air- or CO_2-exposure. Additionally, no differences were found between anesthetized controls and CO_2-pneumoperitoneum groups (Lee et al., 2000).

It is general accepted that MIS is associated with reduced inflammatory response and minimal immunosuppression however (Novitsky et al., 2004), the specific effects of the CO_2 on the immune cell-mediated responses during this surgical approach is not well understood. No pediatric studies have evaluated the effects of different insufflation gases (CO_2, helium) on cell-mediated immunity, being the studies limited to comparing MIS with open approaches.

6.3.2.4. Brain events affected by CO_2-insuflation

Several studies on regional cerebral oxygenation ($rScO_2$) during laparoscopic and thoracoscopic procedures were performed in neonates and children by using near-infrared spectroscopy (Bishay *et al.*, 2011; Tytgat *et al.*, 2015, 2016; Stolwijk *et al.*, 2017). $rScO_2$ reflects a mixture of venous, arterial and capillary hemoglobin oxygen saturation in a proportion of 75:20:5 and is used to detect substantial changes in cerebral oxygenation, as an estimator of cerebral perfusion. Although it cannot be used as a robust quantitative method, it can detect changes in cerebral oxygenation within the same patient (Van Bel *et al.*, 2008). Research groups have evaluated the $rScO_2$ during pediatric laparoscopy and observed no significant changes between pre- and intra-operative levels (Tytgat *et al.*, 2015; Tuna *et al.*, 2016). Interestingly, one study has investigated the balance between oxygen delivery and oxygen consumption by calculating the cerebral fractional tissue oxygen extraction that can be obtained as the ratio $(SaO_2 – rScO_2)/SaO_2$. This study shows a gradual nonsignificant decrease in the cerebral fractional tissue oxygen extraction during CO_2–pneumoperitoneum, becoming statistically significant during the phase prior extubation, which indicates a gradual increased delivery of oxygen to the brain during laparoscopic procedures (Tytgat *et al.*, 2015). This increased oxygen delivery can be caused by (i) intracerebral microvascular dilatation, possibly due to the vasodilatory effect of the elevated

$PaCO_2$; (ii) increased blood pressure; or (iii) increased FiO_2 especially during anesthesia induction and prior to the extubating event. Interestingly, one study has found a significant reduction in $rScO_2$ during laparoscopic procedures and no reduction on peripheral oxygenation (SpO_2). This finding suggests that $rScO_2$ is strongly correlated with the hemodynamic consequences of IAP rather than with the CO_2 absorption. In the same report, the reduction in $rScO_2$ during laparoscopic inguinal hernia repair was about 3,5% (Pelizzo *et al.*, 2017) and similar findings were reported by Tsypin *et al.* where a 3% average reduction in $rScO_2$ in pediatric gynecological laparoscopic interventions was observed (Tsypin *et al.*, 2007).

Regarding thoracoscopic surgery, Bishay *et al.* have described a decrease in $rScO_2$ from 87% preoperatively to 75% during thoracoscopic repair of congenital diaphragmatic hernia (CDH) and esophageal atresia (EA) in neonates and infants. This decrease remained at least 24 hours postoperatively. The CO_2-insufflation pressure adopted in this study ranged from 5 to 10 mmHg, which may be considered a very high upper limit for CO_2-pneumothorax. Additionally, no information regarding ventilatory management of patients after induction of CO_2-pneumothotrax was described (Bishay *et al.*, 2011). Although it was observed a decrease in $rScO_2$, the values remained within the suggested reference range ($55\% < rScO_2 < 85\%$) (Pellicer *et al.*, 2013) intra- and postoperatively. A second study by Tytgat *et al.* in neonates undergoing thoracoscopic EA repair reported that, although observing a significant decrease in $rScO_2$ levels from 77% at anesthesia induction to 73% at 30 minutes of CO_2-insufflation, these values remained within reference range during and after the surgical procedure, with no suggested hampering of cerebral oxygenation. In this study, CO_2-insufflation pressure was limited to 5 mmHg and anesthetic maneuvers during CO_2 pneumothorax included adjustments in respiratory frequency, inspiratory pressure and FiO_2, in order to obtain stable anesthetic conditions based on $rScO_2$, SaO_2, end-tidal CO_2 and blood gas analysis (Tytgat *et al.*, 2016). Although these two studies report significant differences in $rScO_2$ between anesthesia induction and intraoperative periods, the high value of $rScO_2$ recorded at anesthesia induction may justify the statistically significant differences, possible

due to initial hyper-oxygenation. This factor was considered in Tytgat *et al.* work, and no significant differences were found when comparing intraoperative $rScO_2$ values with the baseline values in the awake patient in neonatal intensive care unit.

A study from Neunhoeffer *et al.* have found a significant correlation between CO_2-pneumothorax and a transient decrease in $rScO_2$, while SpO_2 remained normal. This finding, and similarly to Pelizzo findings in pediatric laparoscopic studies, suggests that brain oxygenation might be affected by the hemodynamic disturbances induced by CO_2-insufflation. In the same study, periods of $rScO_2$ 20% below from baseline were significantly more frequent during thoracoscopy than in abdominal open surgery. Interestingly, the authors concluded that those periods of decreased $rScO_2$ were completely inapparent when conventional monitoring was applied (HR, blood pressure, arterial oxygen saturation and end-tidal CO_2). The authors analyzed other parameters of cerebral microenvironment, which included cerebral fractional oxygen extraction and cerebral microcirculation and observed that regional cerebral blood flow remained unchanged during episodes of increased intrathoracic pressure but increased significantly throughout the surgery, which authors have associated with the tissue damage stimulation during surgery. Additionally, it was observed an increase in regional cerebral hemoglobin, that authors attributed to the impaired cerebral venous return, which is another factor that may contribute to the reduced $rScO_2$ (Neunhoeffer *et al.*, 2017). The hemodynamic impact of IAP over the central nervous system (CNS) was evaluated in the pig model by applying an insufflation pressure of 20 mmHg, which resulted in a statistically significant increase in intracranial (ICP) and intraspinal (ISP) pressures, associated with decreases in cerebral perfusion (CPP) and spinal perfusion (SPP) pressures. Decreases of CPP and SPP below 60 mmHg were accompanied by increases in IL-6, TNFα and lactate levels in cerebrospinal fluid suggesting CNS ischemia. Nevertheless, the abdominal desufflation was followed by restoration of ICP and ISP to baseline levels (Marinis *et al.*, 2010). A case series of 16 procedures in five patients submitted to thoracoscopic repair of Long Gap EA found three transient outliers in respect to $rScO_2$. Findings were related with low hemoglobin levels, decreased MAP or high FiO_2. This study draws attention to

intraoperative values of $rScO_2$ bellow and above the safety range of 55 – 85%. The lower rScO2 (<55%) observed in this study were attributed to low hemoglobin levels or hypotension while the higher levels (>85%) were mostly due to pre-oxygenation, particularly during the induction phase of anesthesia due to an increased supply of oxygen. In all cases, $rScO_2$ was normalized after the intervention of anesthesiologist and, in the case of low hemoglobin levels, erythrocytes transfusion and vasopressor support were the corrective measures adopted. Nevertheless, and similarly to other studies, the remaining patients included in this prospective study presented $rScO_2$ values within the safety range during the entire thoracoscopic procedure. This study recognizes the importance of oxygen delivery by hemoglobin and its importance in cerebral oxygenation, especially in patients with "physiological" lower hemoglobin levels (Stolwijk *et al.*, 2017).

The functional consequences of brain hypoxia, hypercapnia and hypoperfusion induced by CO_2-insufflation during MIS are yet unknown. Several animal and human studies have been exploring these events in ischemia-reperfusion and traumatic brain injury studies however, extrapolation to the context of MIS may not be possible. No studies on the long-term effects of CO_2-insufflation during MIS over CNS have been performed in neonates. Being a relatively recent surgical approach in this age range, longitudinal studies are far from being completed, whether comparing open approaches with MIS or MIS patients with non-operated ones. Neurodevelopmental follow-up studies are currently under way, focusing on the effects of hypercapnia and acidosis (Bishay *et al.*, 2013b; Tytgat *et al.*, 2016) but while no neurodevelopmental human studies come out, some Ethic Committee's reports have been advising against thoracoscopic repair of CDH (Pierro, 2015), at least while no enlightening studies on the neurodevelopmental impact of hypercapnia and acidosis were published. Systematic research on neurodevelopmental outcomes after neonatal surgery for major noncardiac anomalies have concluded that 23% of the patients develop cognitive and motor developmental delays, reporting that low birth weight, higher number of congenital anomalies, duration of hospital admission and repeated surgery are risk factors for the observed psychomotor delays (Stolwijk *et al.*, 2016). Other studies in children with

anatomical congenital abnormalities have shown that CDH and EA patients show impaired growth, lower psychomotor scores (Gischler *et al.*, 2009) and lower developmental scores in the expressive language (Walker *et al.*, 2013), compared with the healthy control children. However, no studies have evaluated whether the surgical approach constitutes a risk factor for the observed developmental delays. Interdisciplinary follow-up of children after surgical correction for congenital abnormalities is still lacking in many centers and future follow-up neurodevelopmental studies accounting for the surgical technique may help to understand if MIPS constitutes a risk factor for the observed neurodevelopmental delays or if it represents a long-term advantage over open approaches.

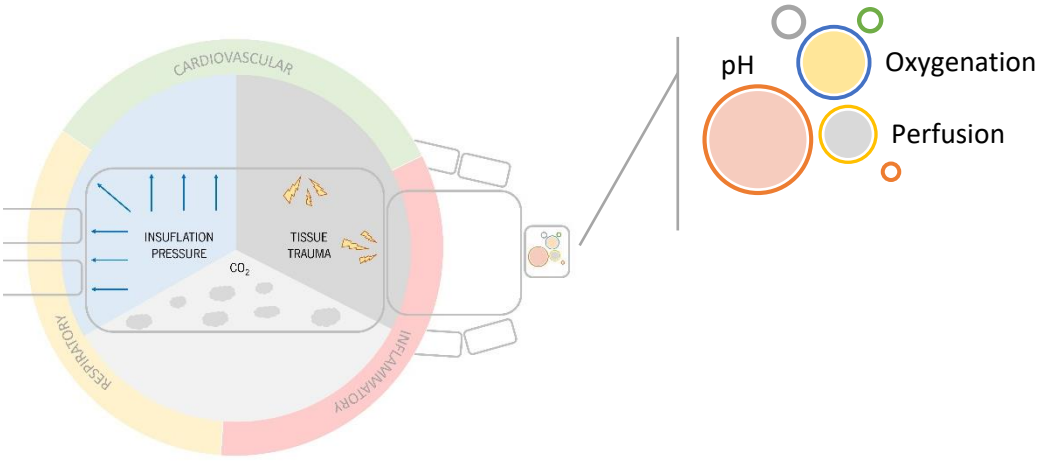


FIGURE 4 BRAIN AFFECTED EVENTS BY THE MECHANICAL AND CHEMICAL IMPACT OF CO_2-INSUFFLATION DURING MIS

CHAPTER 2.
Scope of the thesis

2.1. Motivation and objectives

Surgery is always applied in the perspective of improving patient's condition. Any peri-, intra- or post-operatory factor that adversely affect this primary purpose may potentially negate the advantages of surgery. Some of those factors are the intra-operatory care, anesthesia and analgesia, and the post-operative complications such as inflammation and infection. Therefore, all those factors are being identified and extensively investigated since many years. The surgical associated factors that most significantly concern health professionals are those that affect the brain since their outcomes can adversely affect patient's quality of live. However, the brain is an amazingly adaptive organ and present strategies to buffer insults, such as its neoplastic ability. Nevertheless, the avoidance of stress conditions that affect the metabolism of the brain is therefore beneficial in improving cognitive outcomes and it includes avoidance of intraoperative hyperglycemia, hyperthermia, hypotension and hypoxemia.

Ethics on medical care also focus on the continual search for the least invasive method of administering a treatment to a patient, *"Primun non nocere"*, and MIS has been contributing to this common objective among numerous surgical specialties. Its widespread implementation has contributed to a revolution in surgical practice. Pediatric Surgery was not left behind this quest and laparoscopic and thoracoscopic surgical approaches are becoming a standard surgical approach in neonates and infants. The development of impressively small instruments and the advances in pediatric surgical education, namely MIS training courses with artificial and *ex vivo* and *in vivo* animal models, have contributed to the generalization of this surgical approach in children and neonates, even in the most complicated neonatal procedures.

For many years, studies were focused on demonstrating the feasibility of MIS in children and neonates, however, academic studies are shifting from proving feasibility to a more critical evaluation of the outcomes of this surgical approach. After the enthusiasms for minimally invasive surgical techniques associated with small scars, less postoperative pain and economic advantage of an early return to home, there is now the need for ongoing critical and scientific appraisal of its advantages and disadvantages.

Therefore, the aims of this thesis are:

1. To develop two neonatal animal models of CO_2-pneumothorax and CO_2-pneumoperitoneum, endotracheally intubated, with respiratory support and arterial blood gas monitoring
2. To evaluate the impact of neonatal CO_2-pneumoperitoneum on:
    a. the acute peripheral and central cytokine response
    b. neurodevelopmental milestones acquisition by evaluating the impact of different insufflation pressures and times
    c. long-term adult behavior by evaluating the impact of different insufflation pressures and times
3. To evaluate the impact of neonatal CO_2-pneumothorax on:
    a. the acute peripheral and central cytokine response
    b. immune cells in the periphery
    c. the impact on newborn hippocampal cell survival

2.2. Structure of the thesis

The present thesis is organized in eight chapters distributed in three different parts: introduction, results, and discussion and conclusions. Part I includes chapters 1 and 2 and consist on the state of the art and the scope of the thesis, respectively. Part II gathers the major findings of this thesis, in the format of research papers already accepted for publication/published. These research papers are presented from chapter 3 to 5. Part III

gathers the discussion and the main conclusions of this thesis, corresponding to chapters 6 and 7, respectively.

Chapter 1 presents a brief history of pediatric MIS and resumes the major findings from research studies published over the last 15 years on the effects of CO_2-insufflation during minimally invasive procedures (pneumoperitoneum, pneumothorax) over the neonatal and pediatric organ systems. This chapter intends to enhance the insight with respect to pneumothorax and pneumoperitoneum effects over organ systems of children and neonates and, more specifically, aims to (i) find out the most recent concerns of the pediatric community regarding the effects of CO_2-insufflation over the neonatal organ systems; (ii) highlight the most recent findings and technical advances in perioperative care during MIS; (iii) review the contribution of animal models to the study of metabolic, cardiovascular, respiratory and inflammatory responses to CO_2-insufflation.

The motivation, objectives and structure of the present thesis are addressed in Chapter 2 (current chapter).

Chapter 3 reports a novel technique developed for the intubation of the animal model used in the experimental work held during this thesis. This technique was disseminated through the writing of a research paper entitled "Animal facility videoendoscopic intubation station: tips and tricks from mice to rabbits", published in the peer-reviewed journal *Laboratory Animals*.

Chapter 4 presents the research paper "Neurodevelopment impact of CO_2-pneumoperitoneum in neonates: experimental study in a rat model" published in the *Journal of Surgical Research*.

Chapter 5 displays the results regarding the impact of the thoracic CO_2-insufflation in the format of a research paper entitled "Peripheral and central inflammatory response and long-term behavioral assessment after neonatal CO2-Pneumothorax: study in a rodent model" published in the *Journal of Pediatric Surgery.*

The final part of this thesis comprises the two last chapters. Chapter 6 gives an integrative overview of the results and a general discussion and finally, Chapter 8 displays the main conclusions drawn from this work.

References

Adams S, Jobson M, Sangnawakij P, Heetun A, Thaventhiran A, Johal N, Böhning D, Stanton MP. 2016. Does thoracoscopy have advantages over open surgery for asymptomatic congenital lung malformations? An analysis of 1626 resections. *Journal of Pediatric Surgery* 52: 247–251.

Alain JL, Grousseau D, Terrier G. 1991. Extramucosal pyloromyotomy by laparoscopy. *Surgical Endoscopy* 5: 174–175.

Bannister CF, Brosius KK, Wulkan M. 2003. The effect of insufflation pressure on pulmonary mechanics in infants during laparoscopic surgical procedures. *Pediatric Anesthesia* 13: 785–789.

Baroncini S, Gentili A, Pigna A, Fae M, Tonini C, Tognù A. 2002. Anaesthesia for laparoscopic surgery in paediatrics. *Minerva Anestesiologica* 68: 406–413.

Bax NMA. *Endoscopic Surgery in Infants and Children*. 1st Edition. Springer Berlin Heidelberg 2008.

Bax NM. 2004. Karl Storz Lecture. Ten years of maturation of endoscopic surgery in children. Is the wine good? *Journal of pediatric surgery* 39: 146–151.

Bax NM, Ure BM, van der Zee DC, van Tuijl I. 2001. Laparoscopic duodenoduodenostomy for duodenal atresia. 15: 217.

Van Bel F, Lemmers P, Naulaers G. 2008. Monitoring neonatal regional cerebral oxygen saturation in clinical practice: Value and pitfalls. *Neonatology* 94: 237–244.

Berci G, Brooks PG, Paz-Partlow M. 1986. TV laparoscopy. A new dimension in visualization and documentation of pelvic pathology. *The Journal of reproductive medicine* 31: 585–588.

Bergesio R, Habre W, Lanteri C, Sly P. 1999. Changes in respiratory mechanics during abdominal laparoscopic surgery in children. *Anaesthesia and intensive care* 27(3): 245–

248.

Bernheim BM. 1911. Organoscopy: Cystoscopy of the Abdominal Cavity. *Annals of surgery* 53: 764–767.

Bishay M, Giacomello L, Retrosi G, Thyoka M, Nah SA, McHoney M, De Coppi P, Brierley J, Scuplak S, Kiely EM. 2011. Decreased cerebral oxygen saturation during thoracoscopic repair of congenital diaphragmatic hernia and esophageal atresia in infants. *Journal of pediatric surgery* 46: 47–51.

Bishay M, Mb C, Giacomello L, Retrosi G, Thyoka M, Mb BS, Garriboli M, Brierley J, Harding L, Scuplak S, Cross K, Curry J, Kiely E, De Coppi P, Eaton S. Pierro A. 2013. Hypercapnia and Acidosis During Open and Thoracoscopic Repair of Congenital Diaphragmatic Hernia and Esophageal Atresia: Results of a Pilot Randomized Controlled Trial. *Ann Surg* 258: 895–900.

Blackburn S. *Maternal, fetal and neonatal physiology: A clinical perspective*. 4th Edition. Elsevier Saunders 2012.

Blinman T, Ponsky T. 2012. Pediatric Minimally Invasive Surgery: Laparoscopy and Thoracoscopy in Infants and Children. *Pediatrics* 130: 539–549.

Bush RB, Leonhardt H, Bush IM, Landes RR. 1974. Dr. Bozzini's Lichtleiter. *Urology* 3: 119–123.

Casey LC, Balk RA and Bone RC. 1993. Plasma Cytokine and Endotoxin Levels Correlate with Survival in Patients with the Sepsis Syndrome. *Ann Intern Med* 119: 771–778.

Chang CK, Zdon MJ. 2002. Inhibition of tumor necrosis factor-alpha and inducible nitric oxide synthase correlates with the induction of IL-10 in septic rats undergoing laparotomy and laparoscopy. *Surgical Laparoscopy, Endoscopy and Percutaneous Techniques* 12: 247–251.

Chiarenza SF, Bucci V, Conighi ML, Zolpi E, Costa L, Fasoli L, Bleve C. 2017. Duodenal Atresia: Open versus MIS Repair—Analysis of Our Experience over the Last 12 Years. *BioMed*

Research International 2017: 1–7.

Dallemagne B, Weerts JM, Jehaes C, Markiewicz S, Lombard R. 1991. Laparoscopic Nissen fundoplication: preliminary report. *Surg Laparosc Endosc* 138–143.

Ellis H. 2007. The Hopkins rod-lens system. *Journal of perioperative practice* 17: 272-274.

Fervers C. 1933. Die Laparoskopie mit dem Cystoskop. *Mediz Klinik* 31: 1042–1045.

Fracalanza S, Ficarra V, Cavalleri S, Galfano A, Novara G, Mangano A, Plebani M, Artibani W. 2008. Is robotically assisted laparoscopic radical prostatectomy less invasive than retropubic radical prostatectomy? Results from a prospective, unrandomized, comparative study. *BJU International* 101: 1145–1149.

Fujimoto T, Lane GJ, Segawa O, Esaki S, Miyano T. 1999. Laparoscopic extramucosal pyloromyotomy versus open pyloromyotomy for infantile hypertrophic pyloric stenosis: which is better? *Journal of pediatric surgery* 34: 370–372.

Fujimoto T, Segawa O, Lane GJ, Esaki S, Miyano T. 1999. Laparoscopic surgery in newborn infants. *Surgical endoscopy* 13: 773–777.

Gándara V, Vega DS, Escriú N, Zorrilla IG. 1997. Acid-base balance alterations in laparoscopic cholecystectomy. *Surgical Endoscopy* 11: 707–710.

Gans SL, Berci G. 1973. Peritoneoscopy in infants and children. *Journal of Pediatric Surgery* 8: 399–405.

Georgeson KE, Owings E. 2000. Advances in Minimally Invasive Surgery in Children. 362–364.

Gischler SJ, Mazer P, Duivenvoorden HJ, van Dijk M, Bax NMA, Hazebroek FWJ, Tibboel D. 2009. Interdisciplinary structural follow-up of surgical newborns: a prospective evaluation. *Journal of Pediatric Surgery* 44: 1382–1389.

Gogos CA, Drosou E, Bassaris HP, Skoutelis A. 2000. Pro-versus anti-inflammatory cytokine profile in patients with severe sepsis: a marker for prognosis and future therapeutic options.

Journal of Infectious Diseases 181: 176–180.

Gourlay DM, Cassidy LD, Sato TT, Lal DR, Arca MJ. 2009. Beyond feasibility: a comparison of newborns undergoing thoracoscopic and open repair of congenital diaphragmatic hernias. *Journal of Pediatric Surgery* 44: 1702–1707.

Hagen EW, Sadek-Badawi M, Carlton DP, Palta M. 2008. Permissive Hypercapnia and Risk for Brain Injury and Developmental Impairment. *Pediatrics* 122: e583–e589.

Hanly EJ, Aurora AA, Shih SP, Fuentes JM, Marohn MR, De Maio A, Talamini MA. 2007. Peritoneal acidosis mediates immunoprotection in laparoscopic surgery. *Surgery* 142: 357–364.

Hanly EJ, Bachman SL, Marohn MR, Boden JH, Herring AE, De Maio A, Talamini MA. 2005. Carbon dioxide pneumoperitoneum-mediated attenuation of the inflammatory response is independent of systemic acidosis. *Surgery* 137: 559–566.

Hanly EJ, Fuentes JM, Aurora AR, Bachman SL, De Maio A, Marohn MR, Talamini MA. 2006. Carbon dioxide pneumoperitoneum prevents mortality from sepsis. *Surgical endoscopy* 20: 1482–1487.

Harrell AG, Heniford BT. 2005. Minimally invasive abdominal surgery: Lux et veritas past, present, and future. *American Journal of Surgery* 190: 239–243.

Heel KA, Hall JC. 1996. Peritoneal defences and peritoneum-associated lymphoid tissue. *The British journal of surgery* 83: 1031–1036.

Holcomb Iii GW, Rothenberg SS, Bax KMA, Martinez-Ferro M, Albanese CT, Ostlie DJ, van Der Zee DC, Yeung CK. 2005. Thoracoscopic repair of esophageal atresia and tracheoesophageal fistula: a multi-institutional analysis. *Annals of surgery* 242: 422.

Hopkins HH. 1953. On the Diffraction Theory of Optical Images On the diffraction theory of optlcal images. *Proceedings of the Royal Society of London. Series A, Mathematical and Physical Sciences* 217: 408–432.

Howard BM, Muchamuel T, Andrade S. 1993. Interleukin 10 Protects Mice from Lethal

Endotoxemia. *Journal of Experimental Medicine* 177: 1205–1208.

Jacobeus H. 1911. Uber Die Moglichkeit die Zystoskopie bei Untersuchung seroser Hohlunger anzuwerder. *Munch med Wochenschr* 57: 2090–2092.

Jacobi CA, Wenger F, Opitz I, Müller JM. 2002. Immunologic changes during minimally invasive surgery. *Digestive surgery* 19: 459–63.

Kalfa N, Allal H, Raux O, Lopez M, Forgues D, Guibal M-P, Picaud J-C, Galifer R-B. 2005. Tolerance of laparoscopy and thoracoscopy in neonates. *Pediatrics* 116: e785–e791.

Kardos A, Vereczkey G, Pirot L, Nyirady P, Mekler R. 2001. Use of impedance cardiography to monitor haemodynamic changes during laparoscopy in children. *Pediatric Anesthesia* 11: 175–179.

Kopernik G, Avinoach E, Grossman Y, Levy R, Yulzari R, Rogachev B and Douvdevani A. 1998. The effect of a high partial pressure of carbon dioxide environment on metabolism and immune functions of human peritoneal cells-relevance to carbon dioxide pneumoperitoneum. *American journal of obstetrics and gynecology* 179: 1503–1510.

Lacher M, Kuebler JF, Dingemann J, Ure BM. 2014. Minimal invasive surgery in the newborn: Current status and evidence. *Seminars in Pediatric Surgery* 23: 249–256.

Ladilov Y. 2012. Preconditioning with hypercapnic acidosis: Hope for the ischemic brain. *Neuroscience Letters* 523: 1–2.

Lasersohn L. 2011. Anaesthetic considerations for paediatric laparoscopy. *South African journal of surgery. Suid-Afrikaanse tydskrif vir chirurgie* 49: 22–26.

Lavrijsen SW, Uiterwaal CSPM, Stigter RH, de Vries LS, Visser GHA, Groenendaal F. 2005. Severe umbilical cord acidemia and neurological outcome in preterm and full-term neonates. *Biology of the Neonate* 88: 27–34.

Leighton TA, Liu SY, Bongard FS, Stein M. 1993. Comparative cardiopulmonary effects of carbon dioxide versus helium pneumoperitoneum. *Surgery* 113: 527–31.

Leviton A, Allred E, Kuban KCK, Dammann O, O'Shea TM, Hirtz D, Schreiber MD, Paneth N. 2010. Early Blood Gas Abnormalities and the Preterm Brain. *American Journal of Epidemiology* 172: 907–916.

Li L-W, Zhang W, Ai Y-Q, Li L, Peng Z-Q, Wang H-W. 2013. Influence of laparoscopic carbon dioxide pneumoperitoneum on neonate circulation and respiration. *The Journal of international medical research* 41: 889–894.

Li P, Xu Q, Ji Z, Gao Y, Zhang X, Duan Y, Guo Z, Zheng B, Guo X, Wu X. 2005. Comparison of surgical stress between laparoscopic and open appendectomy in children. *Journal of Pediatric Surgery* 40: 1279–1283.

Litynski GS. 1998. Kurt Semm and an automatic insufflator. *Journal of the Society of Laparoendoscopic Surgeons* 2: 197–200.

Lobe TE, Rothenberg S, Waldschmidt J, Stroedter L. 1999. Thoracoscopic Repair of Esophageal Atresia in an Infant: A Surgical First. *Pediatric Endosurgery & Innovative Techniques* 3: 141–148.

Manner T, Aantaa R and Alanen M. 1998. Lung compliance during laparoscopic surgery in paediatric patients. *Paediatric Anaesthesia* 8: 25–29.

Marinis A, Argyra E, Lykoudis P, Brestas P, Theodoraki K, Polymeneas G, Boviatsis E, Voros D. 2010. Ischemia as a possible effect of increased intra-abdominal pressure on central nervous system cytokines, lactate and perfusion pressures. *Critical Care* 14: R31.

Mathew G, Watson DI, Ellis TS, Jamieson GG, Rofe AM. 1999. The role of peritoneal immunity and the tumour-bearing state on the development of wound and peritoneal metastases after laparoscopy. *The Australian and New Zealand journal of surgery* 69: 14–18.

Mattioli G, Montobbio G, Pini Prato A, Repetto P, Carlini C, Gentilino V, Castagnetti M, Leggio S, Della Rocca M, Kotitsa Z, Jasonni V. 2003. Anesthesiologic aspects of laparoscopic fundoplication for gastroesophageal reflux in children with chronic respiratory and

gastroenterological symptoms. *Surgical Endoscopy And Other Interventional Techniques* 17: 559–566.

McCann ME, Schouten ANJ. 2014. Beyond survival; influences of blood pressure, cerebral perfusion and anesthesia on neurodevelopment. *Pediatric Anesthesia* 24: 68–73.

McHoney M, Corizia L, Eaton S, Kiely EM, Drake DP, Tan HL, Spitz L, Pierro A. 2003. Carbon dioxide elimination during laparoscopy in children is age dependent. in *Journal of Pediatric Surgery* 105–110.

McHoney M, Mackinlay G, Munro F, Capek A, Aldridge L. 2008. Effect of patient weight and anesthetic technique on CO2 excretion during thoracoscopy in children assessed by end-tidal CO2. *Journal of Laparoendoscopic & Advanced Surgical Techniques* 18: 147–151.

McHoney MC, Corizia L, Eaton S, Wade A, Spitz L, Drake DP, Kiely EM, Tan HL, Pierro A. 2006. Laparoscopic surgery in children is associated with an intraoperative hypermetabolic response. *Surgical Endoscopy And Other Interventional Techniques* 20: 452–457.

Miyake H, Kawabata G, Gotoh A, Fujisawa M, Okada H, Arakawa S, Kamidono S, Hara I. 2002. Comparison of surgical stress between laparoscopy and open surgery in the field of urology by measurement of humoral mediators. *International Journal of Urology* 9: 329–333.

Moehrlen U, Ziegler U, Boneberg E, Reichmann E, Gitzelmann CA, Meuli M, Hamacher J. 2006. Impact of carbon dioxide versus air pneumoperitoneum on peritoneal cell migration and cell fate. *Surgical Endoscopy and Other Interventional Techniques* 20: 1607–1613.

Mühe E. 1992. Long-Term Follow-Up after Laparoscopic Cholecystectomy. *Endoscopy* 24: 754–758.

Mukhtar AM, Obayah GM, Elmasry A, Dessouky NM. 2008. The Therapeutic Potential of Intraoperative Hypercapnia During Video-Assisted Thoracoscopy in Pediatric Patients. *Anesthesia & Analgesia* 106: 84–88.

Mutoh M, Takeyama K, Nishiyama N, Kunishima Y, Matsukawa M, Takahashi S, Hotta H,

Itoh N, Tsukamoto T. 2004. Systemic inflammatory response syndrome in open versus laparoscopic adrenalectomy. *Urology* 64: 422–425.

Neuhaus SJ, Watson DI. 2004. Pneumoperitoneum and peritoneal surface changes - A review. *Surgical Endoscopy and Other Interventional Techniques* 18: 1316–1322.

Neuhaus SJ, Watson DI, Ellis T, Rofe AM, Mathew G, Jamieson GG. 2000. Influence of gases on intraperitoneal immunity daring laparoscopy in tumor-bearing rats. *World Journal of Surgery* 24: 1227–1231.

Neunhoeffer F, Warmann SW, Hofbeck M, Müller A, Fideler F, Seitz G, Schuhmann MU, Kirschner HJ, Kumpf M, Fuchs J. 2017. Elevated intrathoracic CO2 pressure during thoracoscopic surgery decreases regional cerebral oxygen saturation in neonates and infants—A pilot study. *Paediatric Anaesthesia* 27: 752–759.

Novitsky YW, Litwin DEM, Callery MP. 2004. The net immunologic advantage of laparoscopic surgery. *Surgical Endoscopy and Other Interventional Techniques* 18: 1411–1419.

Nwokoma NJ, Swindells MG, Pahl K, Mathur AB, Minocha A, Kulkarni M, Tsang T. 2009. Pediatric advanced appendicitis: open versus laparoscopic approach. *Surgical laparoscopy, endoscopy & percutaneous techniques* 19: 110–3.

Pacilli M, Pierro A, Kingsley C, Curry JI, Herod J, Eaton S. 2006. Absorption of carbon dioxide during laparoscopy in children measured using a novel mass spectrometric technique. *British Journal of Anaesthesia* 97: 215–219.

Pelizzo G, Bernardi L, Carlini V, Pasqua N, Mencherini S, Maggio G, De Silvestri A, Bianchi L, Calcaterra V. 2017. Laparoscopy in children and its impact on brain oxygenation during routine inguinal hernia repair. *Journal of Minimal Access Surgery* 13: 51.

Pellicer A, Greisen G, Benders M, Claris O, Dempsey E, Fumagalli M, Gluud C, Hagmann C, Hellström-Westas L, Hyttel-Sorensen S, Lemmers P, Naulaers G, Pichler G, Roll C, Van Bel F, Van Oeveren W, Skoog M, Wolf M, Austin T. 2013. The SafeBoosC phase II randomised clinical trial: A treatment guideline for targeted near-infrared-derived cerebral tissue

oxygenation versus standard treatment in extremely preterm infants. *Neonatology* 104: 171–178.

Peter SDS, Holcomb GW, Calkins CM, Murphy JP, Andrews WS, Sharp RJ, Snyder CL, Ostlie DJ. 2006. Open Versus Laparoscopic Pyloromyotomy for Pyloric Stenosis. 244: 363–370.

Pierro A. 2015. Hypercapnia and acidosis during the thoracoscopic repair of oesophageal atresia and congenital diaphragmatic hernia. *Journal of Pediatric Surgery* 50: 247–249.

Rongione AJ, Kusske AM, Ashley SW, Reber HA, McFadden DW. 1997. Interleukin-10 Prevents Early Cytokine Release in Severe Intraabdominal Infection and Sepsis. *Journal of Surgical Research* 70: 107–112.

Rongione AJ, Kusske AM, Kwan K, Ashley SW, Reber HA, McFadden DW. 2000. Interleukin-10 protects against lethality of intra-abdominal infection and sepsis. *Journal of Gastrointestinal Surgery* 4: 70–76.

Rothenberg S. 2002. Laparoscopic duodenoduodenostomy for duodenal obstruction in infants and children. *Journal of Pediatric Surgery* 37: 1088–1089.

Rothenberg SS. 2002. Thoracoscopic repair of tracheoesophageal fistula in newborns. *Journal of Pediatric Surgery* 37: 869–872.

Rothenberg SS. 2015. Developing neonatal minimally invasive surgery: Innovation, techniques, and helping an industry to change. *Journal of Pediatric Surgery* 50: 232–235.

Rotstein OD. 2001. Peritoneal host defenses: modulation by carbon dioxide insufflation. *Surgical infections* 2: 163-8-70.

Ruzic B, Tomaskovic I, Trnski D, Kraus O, Bekavac-Beslin M, Vrkic N. 2005. Systemic stress responses in patients undergoing surgery for benign prostatic hyperplasia. *BJU International* 95: 77–80.

Sakka SG, Huettemann E, Petrat G, Meier-Hellmann A, Schier F and Reinhart K. 2000. Transoesophageal echocardiographic assessment of haemodynamic changes during laparoscopic herniorrhaphy in small children. *British journal of anaesthesia* 84: 330–334.

Salmaso N, Jablonska B, Scafidi J, Vaccarino FM, Gallo V. 2014. Neurobiology of premature brain injury. *Nature Neuroscience* 17: 341–346.

Schechter NL. 1989. The undertreatment of pain in children: an overview. *Pediatric clinics of North America* 36: 781–794.

Shimotakahara A, Kuebler JF, Vieten G, Metzelder ML, Petersen C, Ure BM. 2007. Pleural macrophages are the dominant cell population in the thoracic cavity with an inflammatory cytokine profile similar to peritoneal macrophages. *Pediatric Surgery International* 23: 447–451.

Shimotakahara A, Metzelder M, Vieten G, Ure B, Kuebler JF. 2010. CO2 Modulates the Inflammatory Cytokine Release of Primary Human Pleural Macrophages. *European Journal of Pediatric Surgery* 20: 111–115.

Steiner O. 1924. Abdominoscopy. Surg Gynecol Obstet. 38: 266–269.

Stengl M, Ledvinova L, Chvojka J, Benes J, Jarkovska D, Holas J, Soukup P, Sviglerová J, Matejovic M. 2013. Effects of clinically relevant acute hypercapnic and metabolic acidosis on the cardiovascular system: an experimental porcine study. *Critical Care* 17: R303.

Stolwijk LJ, Lemmers PM, Harmsen M, Groenendaal F, de Vries LS, van der Zee DC, Benders MJN, van Herwaarden-Lindeboom MYA. 2016. Neurodevelopmental Outcomes After Neonatal Surgery for Major Noncardiac Anomalies. *Pediatrics* 137: e20151728–e20151728.

Stolwijk LJ, van der Zee DC, Tytgat S, van der Werff D, Benders MJNL, van Herwaarden MYA, Lemmers PMA. 2017. Brain Oxygenation During Thoracoscopic Repair of Long Gap Esophageal Atresia. *World Journal of Surgery* 41: 1384–1392.

Sümpelmann R, Schuerholz T, Marx G, Härtel D, Hecker H, Ure BM, Jesch NK. 2006. Haemodynamic, acid-base and blood volume changes during prolonged low pressure pneumoperitoneum in rabbits. *British journal of anaesthesia* 96: 563–568.

Sümpelmann R, Schuerholz T, Marx G, Jesch NK, Osthaus WA, Ure BM. 2006.

Hemodynamic changes during acute elevation of intra-abdominal pressure in rabbits. *Paediatric anaesthesia* 16: 1262–1267.

Taura P, Lopez A, Lacy AM, Anglada T, Beltran J, Fernandez-Cruz L, Targarona E, Garcia-Valdecasas JC, Marin JL. 1998. Prolonged pneumoperitoneum at 15 mmHg causes lactic acidosis. *Surgical Endoscopy* 12: 198–201.

Tobias JD. 2002. Anaesthesia for minimally invasive surgery in children. *Best practice & research. Clinical anaesthesiology* 16: 115–130.

Tregub PP, Malinovskaya NA, Kulikov VP, Salmina AB, Nagibaeva ME, Zabrodina AS, Gertsog GE, Antonova SK. 2016. Inhibition of Apoptosis is a Potential Way to Improving Ischemic Brain Tolerance in Combined Exposure to Hypercapnia and Hypoxia. *Bulletin of Experimental Biology and Medicine* 161: 666–669.

Truchon R. 2004. Anaesthetic considerations for laparoscopic surgery in neonates and infants: A practical review. *Best Practice and Research: Clinical Anaesthesiology* 18: 343–355.

Tsypin LE, Mikhel'son VA, Chusov KP, Kazharskaia EI, Lazarev VV, Prokop'ev GG and Shchukin V V. 2007. Central and cerebral hemodynamics during gynecological laparoscopic interventions in children. *Anesteziologiia i reanimatologiia* 1: 30–32.

Tuna AT, Akkoyun I, Darcin S, Palabiyik O. 2016. Effects of carbon dioxide insufflation on regional cerebral oxygenation during laparoscopic surgery in children: a prospective study. *Revista brasileira de anestesiologia* 66: 249–253.

Tytgat SH, van Herwaarden MYA, Stolwijk LJ, Keunen K, Benders MJNL, de Graaff JC, Milstein DMJ, van der Zee DC, Lemmers PMA. 2016. Neonatal brain oxygenation during thoracoscopic correction of esophageal atresia. *Surgical Endoscopy* 30: 2811–2817.

Tytgat SH, Stolwijk LJ, Keunen K, Milstein DMJ, Lemmers PMA, van der Zee DC. 2015. Brain Oxygenation During Laparoscopic Correction of Hypertrophic Pyloric Stenosis. *Journal of Laparoendoscopic & Advanced Surgical Techniques* 25: 352–357.

Tytgat SH, Van Der Zee DC, Ince C, Milstein DM. 2013. Carbon dioxide gas pneumoperitoneum induces minimal microcirculatory changes in neonates during laparoscopic pyloromyotomy. *Surgical Endoscopy and Other Interventional Techniques* 27: 3465–3473.

Ure BM, Niewold TA, Bax NMA, Ham M, van der Zee DC, Essen GJ. 2002. Peritoneal, systemic, and distant organ inflammatory responses are reduced by a laparoscopic approach and carbon dioxide vs air. *Surgical Endoscopy and Other Interventional Techniques* 16: 836–842.

Ure BM, Suempelmann R, Metzelder MM, Kuebler J. 2007. Physiological responses to endoscopic surgery in children. *Seminars in Pediatric Surgery* 16: 217–223.

Veress. 1938. Neues Instrument zur Ausführung von Brust- oder Bauchpunktionen und Pneumothoraxbehandlung. *Deutsch Med Wochenschr* 40: 1480–1481.

Vijayalakshmi I, Syamasundar RP, Chugh R. *A Comprehensive Approach to Congenital Heart Diseases*. 1st Edition. Jaypee Brothers Medical Publishers 2013.

Volpe JJ. 2009. Brain injury in premature infants: a complex amalgam of destructive and developmental disturbances. *The Lancet Neurology* 8: 110–124.

De Waal EEC, Kalkman CJ. 2003. Haemodynamic changes during low-pressure carbon dioxide pneumoperitoneum in young children. *Pediatric Anesthesia* 13: 18–25.

Walker K, Halliday R, Badawi N, Stewart J, Holland AJA. 2013. Early developmental outcome following surgery for oesophageal atresia. *Journal of Paediatrics and Child Health* 49: 467–470.

Walker K, Holland AJA, Winlaw D, Sherwood M and Badawi N. 2006. Neurodevelopmental outcomes and surgery in neonates. *Journal of Paediatrics and Child Health* 42: 749–751.

Wang L, Qin W, Tian F, Zhang G, Yuan J, Wang H. 2009. Cytokine responses following laparoscopic or open pyeloplasty in children. *Surgical Endoscopy* 23: 544–549.

West MA, Baker J and Bellingham J. 1996. Kinetics of decreased LPS-stimulated cytokine

release by macrophages exposed to CO2. *The Journal of surgical research* 63: 269–74.

West MA, Hackam DJ, Baker J, Rodriguez JL, Bellingham J, Rotstein OD. 1997. Mechanism of decreased in vitro murine macrophage cytokine release after exposure to carbon dioxide: relevance to laparoscopic surgery. *Annals of surgery* 226: 179–90.

Wood JH, Partrick DA, Johnston RB. 2010. The inflammatory response to injury in children. *Current Opinion in Pediatrics* 22: 315–320.

van der Zee DC. 2017. Endoscopic surgery in children – the challenge goes on. *Journal of Pediatric Surgery* 52: 207–210.

Zollikofer R. 1924. Zur Laparoskopie. Schweiz Med Wochenschr. 5: 264–265.

PART II | Results

CHAPTER 3.
Animal model

Animal facility videoendoscopic intubation station: tips and tricks from mice to rabbits

Miranda A, Pêgo JM, Correia-Pinto J

Laboratory Animals 2017; 51:204-207

doi: 10.1177/0023677216652342

Lab Anim OnlineFirst, published on May 26, 2016 as doi:10.1177/0023677216652342

Short Report

Laboratory Animals
0(0) 1–4

DOI: 10.1177/0023677216652342
la.sagepub.com

Animal facility videoendoscopic intubation station: tips and tricks from mice to rabbits

Alice Miranda[1,2], José M Pêgo[1,2,*] and Jorge Correia-Pinto[1,2,3,*]

Abstract

Endotracheal intubation of laboratory animals is a common procedure shared by several research fields for different purposes, such as mechanical ventilation of anaesthetized animals, instillation of cytotoxic nanoparticles, infectious agents or tumour cells for induction of disease models, and even for diagnostic and therapeutic purposes. These different research purposes, achieved in different animal models, require technical expertise and equipment that suits every research need from animal facilities. In this short report we propose a videoendoscopic intubation station that could be shared among the most common laboratory animals, namely the mouse, rat, guinea pig and rabbit, from neonates to adult animals. This report aims to contribute to the reduction of animals excluded from experiments due to false paths during direct and blind intubations and to the refinement of procedures by replacing surgical approaches such as tracheotomy.

Keywords

endotracheal intubation, videoendoscopy, reduction, refinement

Anaesthesia of laboratory animals is a generalized procedure in laboratory animal science; however, endotracheal intubation and mechanical ventilation is often impossible to perform due to a lack of specialized equipment and an absence of trained personnel in laboratory animal intubation.

Appropriate ventilation of anaesthetized laboratory animals may be imperative when fundamental physiological processes are being studied and normal blood pH, oxygen saturation (sO_2) and partial pressure of carbon dioxide ($PaCO_2$) are desirable.[1] Additionally, it allows resuscitation when respiratory arrest occurs, especially when drugs that induce marked respiratory depression are being used.[2]

Endotracheal intubation also allows the delivery of inhalation anaesthesia, a method preferred in large laboratory animal anaesthesia since it combines the advantages of mechanical ventilation with the benefits of inhalation anaesthesia. Studies on endotracheal intubation with inhalation anaesthesia in mice and rats have also been published, suggesting their feasibility in small laboratory animals.[3,4]

The endotracheal route is also used for the induction of pulmonary diseases such as specific occupational diseases contracted by exposure to cytotoxic nanoparticles,[5] and instillation of tumour cells for lung cancer research,[6] and is even used as a route of infection in pulmonary infection models such as tuberculosis.[7] Furthermore, this route has recently been explored for new therapeutic strategies.[8]

Due to the different body sizes of laboratory animals and their specific anatomic features, strategies described for videoendoscopic intubation require specie-specific tips and equipment.[1,3,4,9,10] By combining and optimizing these specie-specific strategies, we present a videoendoscopic intubation station that can be shared among the most commonly used species housed in an animal facility, by applying the same intubation technique.

[1]Life and Health Sciences Research Institute (ICVS), School of Health Sciences, University of Minho, Braga, Portugal
[2]ICVS/3Bs – PT Government Associate Laboratory, Braga/Guimarães, Portugal
[3]Department of Pediatric Surgery, Hospital de Braga, Braga, Portugal
[*]These authors contributed equally to the study.

Corresponding author:
Alice Miranda: Escola de Ciências da Saúde, Universidade do Minho, Campus de Gualtar, 4710-057 Braga, Portugal.
Email: alicemiranda@ecsaude.uminho.pt

Animals used in this work were included in research projects underway in the institution, with each project being approved by the animal ethics committee of the Institution and by Direção Geral de Alimentação e Veterinária (DGAV), the competent national authority for animal protection. All personnel involved in the procedures were approved as competent for animal experimentation by DGAV.

Two animals of each species were used: *Mus musculus*, *Rattus norvegicus*, *Cavia porcellus* and *Oryctolagus cuniculus*. Animals were anaesthetized intraperitoneally with a combination of ketamine and medetomidine.

All species were intubated with the help of a videoendoscopic system with a 1.9 mm diameter, 10 cm length, 30° endoscope (HOPKINS II Telescope; Karl Storz GmbH, Tuttlingen, Germany) and a Telepack X System (Karl Storz GmbH) comprising a light source and an endoscopic camera (Figure 1).

For the right-handed operator, animals were positioned in right lateral recumbency, the endoscope was handled with the left hand and the endotracheal tube with the right hand via the following steps (Figure 2):

1. The endoscope tip was introduced into the mouth above the tongue with the bevel pointed dorsally, allowing visualization of the palate.
2. The endoscope was advanced until visualization of palatine veins was achieved (Figure 2, column 1). By following the palatine veins, the edge of the soft palate was visualized covering the epiglottis and closing the laryngeal opening.
3. Gentle pressure was applied on the soft palate with the tip of the endoscope and the palate was displaced dorsally allowing the epiglottis to fall, thereby allowing visualization of the laryngeal opening (Figure 2, column 2). (If the epiglottis is already ventral to the soft palate, this step is no longer needed.)
4. The endoscope was withdrawn slightly and vocal cords were visualized. An endotracheal tube was inserted into the mouth, parallel to the scope (Figure 2, column 3).
5. The endotracheal tube was then aligned with the laryngeal opening and advanced through the vocal cords during inspiration, taking advantage of the opening of the glottis. Spinning movements of the endotracheal tube helped its progression (Figure 2, column 4).

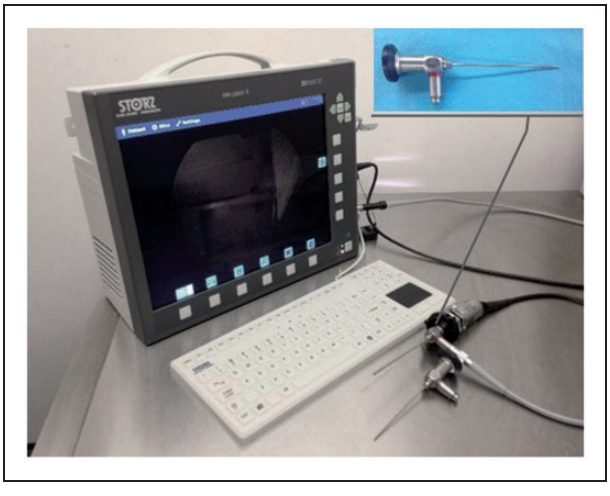


Figure 1. Videoendoscopic intubation station consisting of a 1.9 mm, 10 cm, 30° endoscope and a Telepack X System with a light source and endoscopic camera.

This side-by-side technique uses an endoscope with a very small diameter (1.9 mm) as a laryngoscope to lower the base of the tongue and the epiglottis. The 30° angled tip favours endoscope progression and allows a wide view over the soft palate. The endoscope enters the mouth laterally to the large incisors and the animal's lateral position favours this approach. The camera head can be supported at the operating table while progressing through the mouth, allowing a more stable image. The animal's lateral position has advantages over the dorsal position since the tongue does not fall over the endoscope, nor does it need to be held during endoscope progression and intubation. During intubation, the scope remains in the oral cavity and only the endotracheal tube progresses through the vocal cords. Therefore, this technique can be applied in animals, such as mice and neonatal rats, which require endotracheal tubes with smaller diameters than the scope, as long as their mouth opening allows the progression of the scope (1.9 mm). For these reasons, only one person is required, and no mouth gags are needed for mouth opening or platforms for specific body positions.

Guinea pigs present a unique anatomy with a soft palate continuous with the palatoglossal arches and the base of the tongue.[11] Only a small palatal ostium is visualized (Figure 2, column 3) and the soft palate must be displaced in order to visualize the epiglottis and the small laryngeal opening (Figure 2, row C). The choice of endotracheal tubes should be made according to the species, and adjustments must be made according to the size of the animals within the species:

- *Mus musculus* (26 ± 1 g): 24 G intravenous catheter
- *Rattus norvegicus* (360 ± 15 g): 18 G intravenous catheter
- *Cavia porcellus* (380 ± 12 g): 18 G intravenous catheter
- *Oryctolagus cuniculus* (2300 ± 152 g): 2.5 mm internal diameter

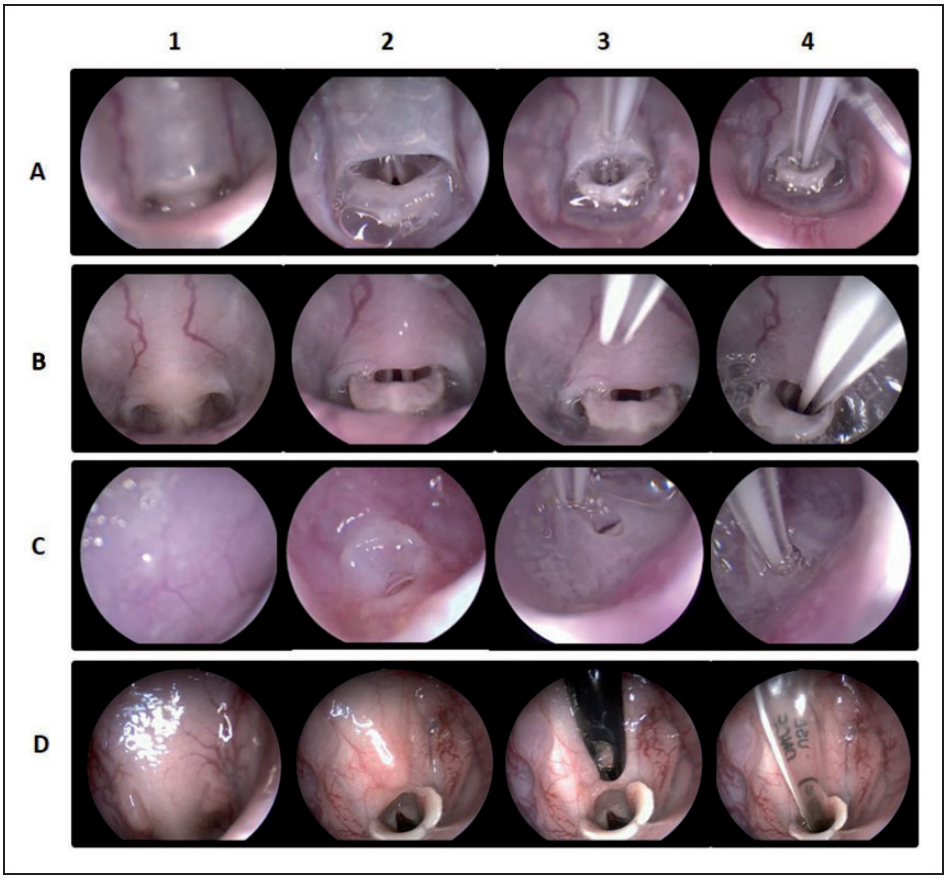


Figure 2. Videoendoscopic intubation of laboratory animals using a 1.9 mm and 30° Hopkins rigid endoscope. View of glottis of the mouse (row A); rat (row B); guinea pig (row C) and rabbit (row D). Visualization of the palatine veins and the soft palate (column 1); visualization of the epiglottis and vocal cords after dorsal displacement of the soft palate (column 2) – except for the guinea pig in which the epiglottis is behind the palatal ostium which makes it difficult to lower and exteriorize; introduction of the endotracheal tube side-by-side with the endoscope (column 3); Introduction of the endotracheal tube throw the vocal cords, inside the trachea. (column 4).

Although the initial investment in a videoendoscopic station by the animal facility may be higher than the cost of the equipment used for direct intubation, this cost is not prohibitive and may be justified by the general usage by researchers in a greater variety of animal models, for the most diverse research fields and for procedures requiring endotracheal access.

Acknowledgment

The authors would like to thank Karl Storz GmbH.

Declaration of conflicting interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Funding

The author(s) disclosed receipt of the following financial support for the research, authorship, and/or publication of this article: This work was granted by Project ON.2 SR&TD Integrated Program (NORTE-07-0124-FEDER-000017), co-funded by the North Portugal Regional Operational Program (ON.2-O Novo Norte), under the National Strategic Reference Framework, through the European Regional Development Fund (ERDF). This work was also supported by FEDER funds through the Operational Programme Competitiveness Factors – COMPETE and National Funds through FCT – Foundation for Science and Technology under the project POCI-01-0145-FEDER-007038. A Miranda was supported by an FCT grant (SFRH/BD/52059/2012).

References

1. Fuentes JM, Hanly EJ, Bachman SL, Aurora AR, Marohn MR and Talamini MA. Videoendoscopic endotracheal intubation in the rat: a comprehensive rodent model of laparoscopic surgery. *J Surg Res* 2004; 122: 240–248.
2. Roelofs S, Manjeri GR, Willems PH, Scheffer GJ, Smeitink JA and Driessen JJ. Isoflurane anesthetic hypersensitivity and progressive respiratory depression in a mouse model with isolated mitochondrial complex I deficiency. *J Anesth* 2014; 28: 807–814.

3. Konno K, Itano N, Ogawa T, Hatakeyama M, Shioya K and Kasai N. New visible endotracheal intubation method using the endoscope system for mice inhalational anesthesia. *J Vet Med Sci* 2014; 76: 863–868.
4. Konno K, Shiotani Y, Itano N, et al. Visible, safe and certain endotracheal intubation using endoscope system and inhalation anesthesia for rats. *J Vet Med Sci* 2014; 76: 1375–1381.
5. Jacobsen NR, Stoeger T, van den Brule S, et al. Acute and subacute pulmonary toxicity and mortality in mice after intratracheal instillation of ZnO nanoparticles in three laboratories. *Food Chem Toxicol* 2015; 85: 84–95.
6. Buckle T and van Leeuwen FWB. Validation of intratracheal instillation of lung tumour cells in mice using single photon emission computed tomography/computed tomography imaging. *Lab Anim* 2010; 44: 40–45.
7. de Steenwinkel JEM, ten Kate MT, de Knegt GJ, et al. Course of murine tuberculosis and response to first-line therapy depends on route of infection and inoculum size. *Int J Tuberc Lung Dis* 2011; 15: 1478–1484.
8. Walter K, Steinwede K, Aly S, et al. Cathepsin G in experimental tuberculosis: relevance for antibacterial protection and potential for immunotherapy. *J Immunol* 2015; 195: 3325–3333.
9. Vergari A, Polito A, Musumeci M, Palazzesi S and Marano G. Video-assisted orotracheal intubation in mice. *Lab Anim* 2003; 37: 204–206.
10. Clary EM, O'Halloran EK, de la Fuente SG and Eubanks S. Videoendoscopic endotracheal intubation of the rat. *Lab Anim* 2004; 38: 158–161.
11. Johnson DH. Endoscopic intubation of exotic companion mammals. *Vet Clin North Am Exot Anim Pract* 2010; 13: 273–289.

CHAPTER 4.
CO_2-pneumoperitoneum

Neurodevelopment impact of CO2-pneumoperitoneum in neonates: experimental study in a rat model

Miranda A, Roque S, Pêgo JM, Correia-Pinto J

J Surg Res 2017: [Epub ahead of print]

https://doi.org/10.1016/j.jss.2017.09.014

Available online at www.sciencedirect.com

ScienceDirect

journal homepage: www.JournalofSurgicalResearch.com

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

Alice Miranda, DVM,[a,b,*] *Susana Roque, PhD,*[a,b] *José M. Pêgo, MD, PhD,*[a,b] *and Jorge Correia-Pinto, MD, PhD*[a,b,c]

[a] *Life and Health Sciences Research Institute (ICVS), School of Medicine, University of Minho, Braga, Portugal*
[b] *ICVS/3B's - PT Government Associate Laboratory, Braga, Guimarães, Portugal*
[c] *Department of Pediatric Surgery, Hospital de Braga, Braga, Portugal*

ARTICLE INFO

Article history:
Received 2 April 2017
Received in revised form
10 July 2017
Accepted 12 September 2017
Available online xxx

Keywords:
Laparoscopy
Carbon-dioxide pneumoperitoneum
Neurodevelopment
Cytokine
Interleukin

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]

* *Corresponding author*. Life and Health Sciences Research Institute (ICVS), School of Medicine, University of Minho, Campus de Gualtar, 4710-057, Braga, Portugal. Tel./fax: +351 253 604 834.
E-mail address: alicemiranda@med.uminho.pt (A. Miranda).
0022-4804/$ – see front matter
https://doi.org/10.1016/j.jss.2017.09.014

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)

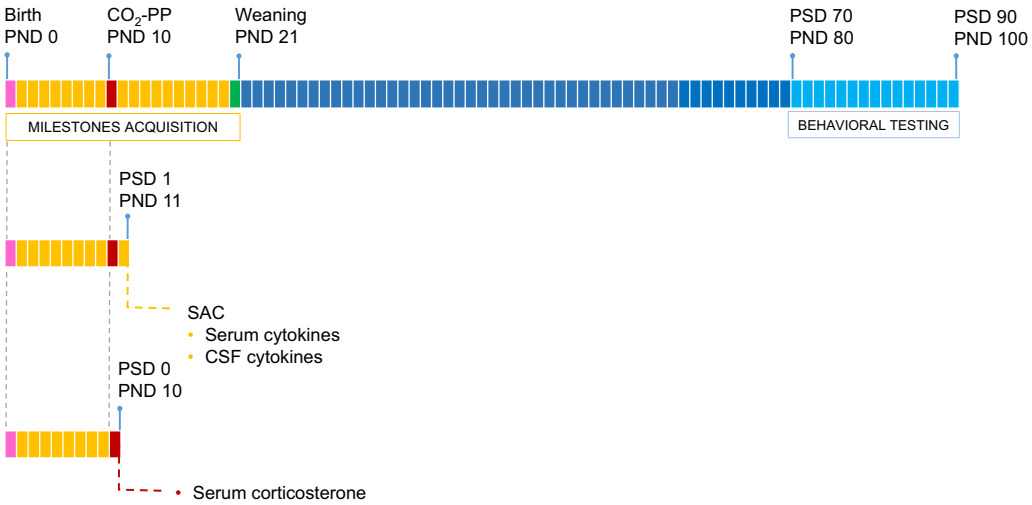


Fig. 1 – Experimental timeline: birth (pink); insufflation day (red); weaning (green). PSD = postsurgical day; PND = postnatal day. (Color version of figure is available online.)

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

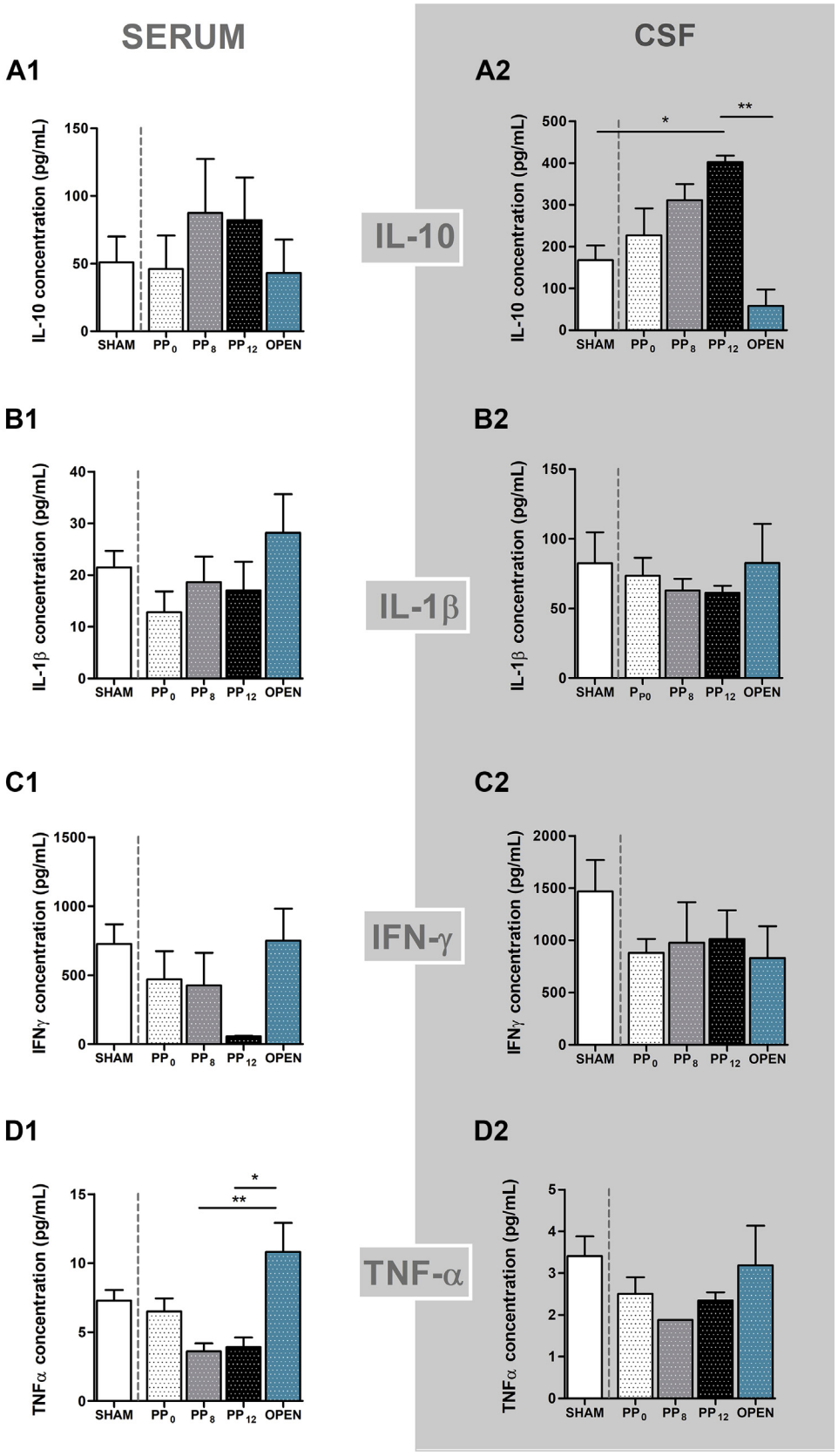


Fig. 2 – IL-10 (A), IL-1β (B), IFN-γ (C), and TNF-α (D) concentrations 24h after CO_2-insufflation (P30). Serum concentrations (A1, B1, C1, D1) in SHAM (white bars; $n = 17$), PP_0 (light gray bars; $n = 8$), PP_8 (dark gray bars; $n = 5$), PP_{12} (black bars; $n = 5$), and OPEN (blue bars $n = 6$) 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.)

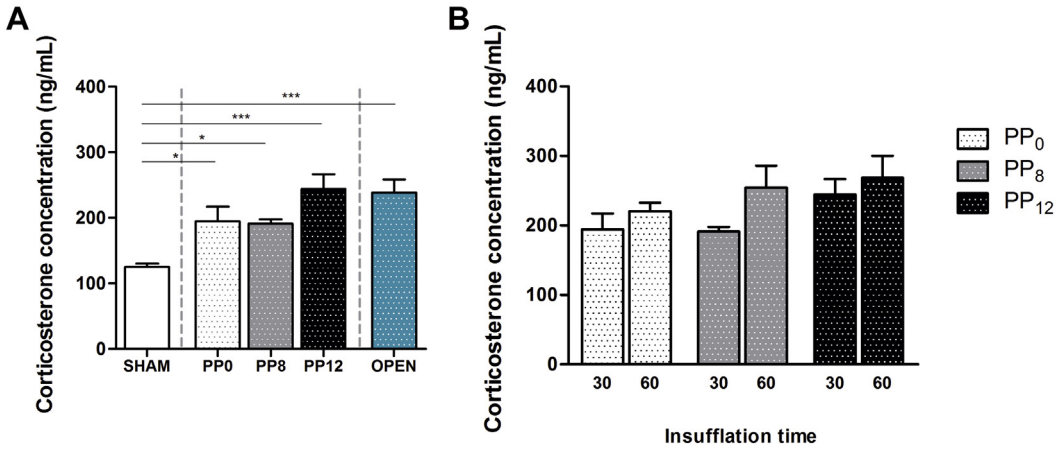


Fig. 3 – (A) Serum corticosterone levels after 30-minute CO_2-insufflation (P30) in SHAM (white bars; $n = 8$), PP_0 (light gray bars; $n = 6$), PP_8 (dark gray bars; $n = 7$), PP_{12} (black bars; $n = 6$), and OPEN (blue bars; $n = 6$) groups. Group means were compared by one-way ANOVA followed by 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

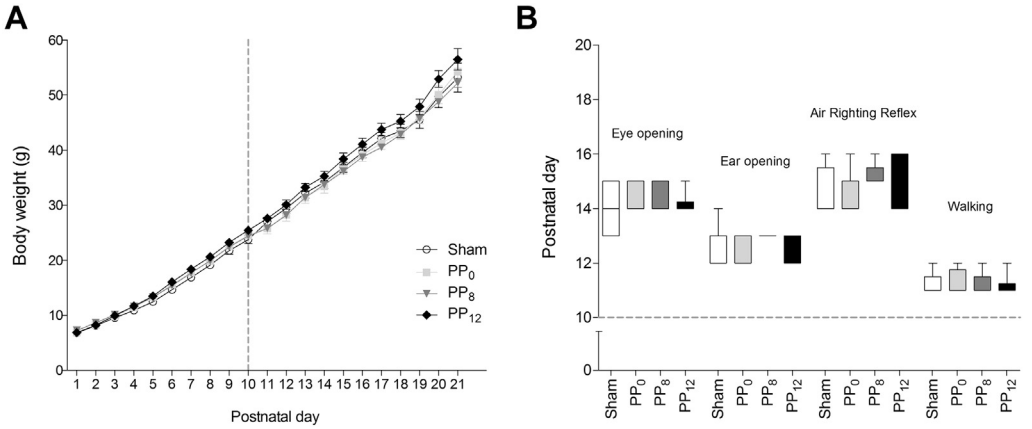


Fig. 4 – Developmental milestones after 30-minute CO_2-insufflation (P30) in SHAM (white symbols); PP_0 (light gray symbols); PP_8 (dark gray symbols); and PP_{12} (black symbols). Dashed line corresponds to insufflation day. (A) Body weight gain from birth until weaning. (B) Somatic development and 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.

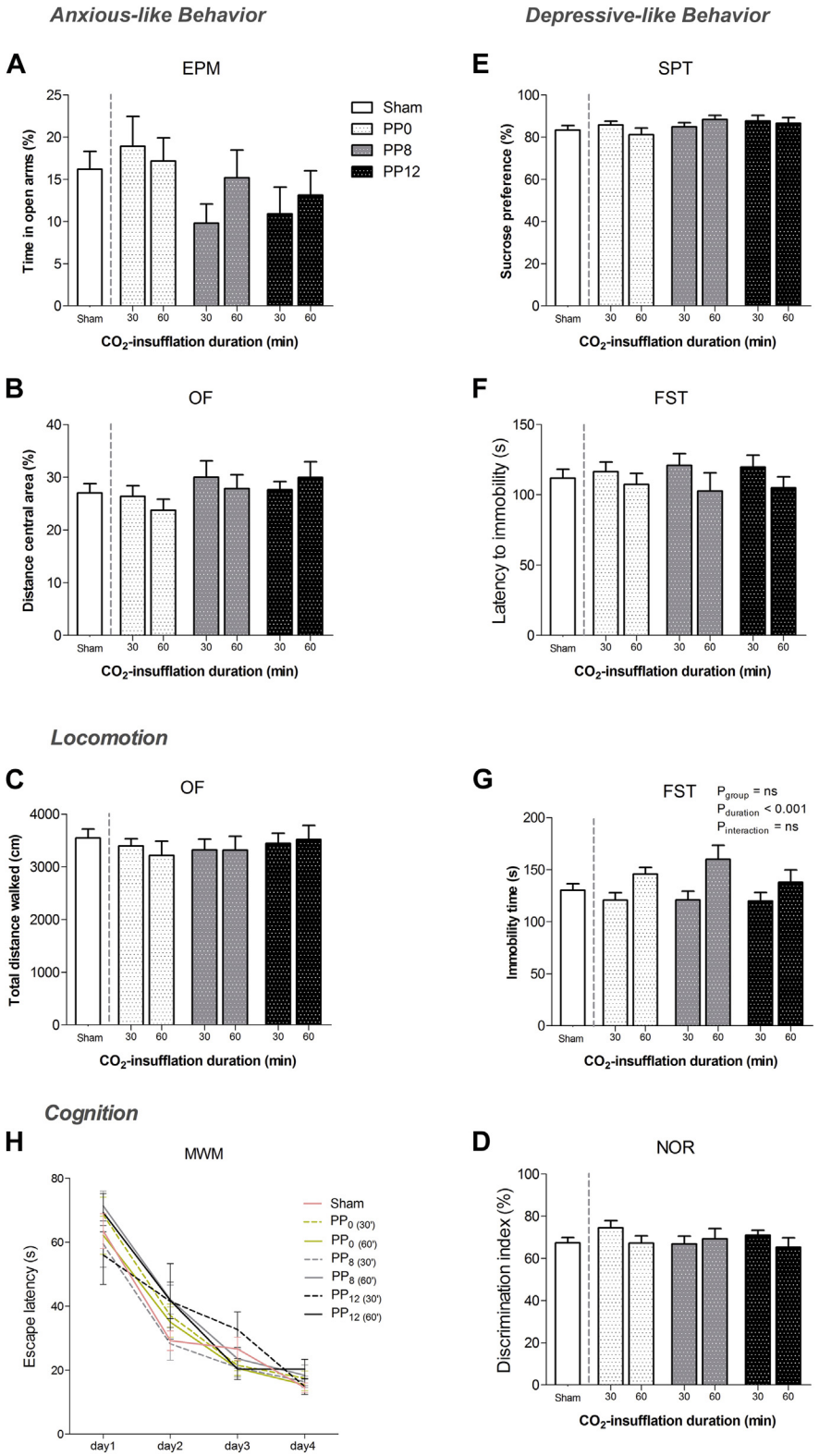


Fig. 5 – Behavioral tests performed in adult animals, 3 months after CO_2-insufflation: SHAM animals (white bars; $n = 43$;); PP_0 (light gray bars; $n_{30min} = 23$; $n_{60min} = 13$); PP_8 (dark gray bar; $n_{30min} = 16$; $n_{60min} = 16$); and PP_{12} (black bars; $n_{30min} = 19$; $n_{60min} = 15$). (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 non-neurological 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.

Acknowledgment

Funding: This work has been funded by FEDER, through the Competitiveness Factors Operational Programme (COMPETE), and by National funds, through the Foundation for Science and Technology (FCT), under the project POCI-01-0145-FEDER-007038; and by the project NORTE-01-0145-FEDER-000013, supported by the Northern Portugal Regional Operational Programme (NORTE 2020), under the Portugal 2020 Partnership Agreement, through the European Regional Development Fund (FEDER). A Miranda and S. Roque were supported by FCT grants (SFRH/BD/52059/2012) and (SFRH/BPD/72710/2010), respectively. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript. The authors would like to thank Karl Storz GmbH for equipment and technical support.

Authors' contribution: A.M. conducted animal experiments, data analysis and interpretation, and manuscript writing. J.C.P. and J.M.P. designed experiments and contributed to the writing of the manuscript.

Disclosure

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

REFERENCES

1. Abbas TO, Hayati A, Ali M. Role of laparoscopy in non-trauma emergency pediatric surgery: a 5-year, single center experience a retrospective descriptive study with literature review. *BMC Res Notes*. 2012;5:550.
2. Blinman T, Ponsky T. Pediatric minimally invasive surgery: laparoscopy and Thoracoscopy in infants and children. *Pediatrics*. 2012;130:539–549.
3. Castillo R, Buckel E, León F, et al. Effectiveness of learning Advanced laparoscopic Skills in a Brief Intensive laparoscopy training Program. *J Surg Educ*. 2015;72:648–653.
4. Gourlay DM, Cassidy LD, Sato TT, Lal DR, Arca MJ. Beyond feasibility: a comparison of newborns undergoing thoracoscopic and open repair of congenital diaphragmatic hernias. *J Pediatr Surg*. 2009;44:1702–1707.
5. Fujimoto T, Segawa O, Lane GJ, Esaki S, Miyano T. Laparoscopic surgery in newborn infants. *Surg Endosc*. 1999;13:773–777.
6. Rappaport BA, Suresh S, Hertz S, Evers AS, Orser BA. Anesthetic neurotoxicity - clinical Implications of animal models. *N Engl J Med*. 2015;372:796–797.
7. Andropoulos DB, Greene MF. Anesthesia and developing brains — Implications of the FDA Warning. *N Engl J Med*. 2017;376:905–907.
8. Sanders RD, Hassell J, Davidson AJ, Robertson NJ, Ma D. Impact of anaesthetics and surgery on neurodevelopment: an update. *Br J Anaesth*. 2013;110:i53–i72.
9. Tytgat SHAJ, Stolwijk LJ, Keunen K, Milstein DMJ, Lemmers PMA, van der Zee DC. Brain oxygenation during laparoscopic Correction of Hypertrophic Pyloric Stenosis. *J Laparoendosc Adv Surg Tech*. 2015;25:352–357.
10. Pelizzo G, Bernardi L, Carlini V, et al. Laparoscopy in children and its impact on brain oxygenation during routine inguinal hernia repair. *J Minim Access Surg*. 2017;13:51–56.
11. Tuna AT, Akkoyun I, Darcin S, Palabiyik O. Effects of carbon dioxide insufflation on regional cerebral oxygenation during laparoscopic surgery in children: a prospective study. *Rev Bras Anestesiol*. 2016;66:249–253.
12. Maesani M, Pares F, Michelet D, et al. Haemodynamic and cerebral oxygenation during paediatric laparoscopy in fluid optimized patients. *Br J Anaesth*. 2016;116:564–566.
13. Bromander S, Anckarsäter R, Kristiansson M, et al. Changes in serum and cerebrospinal fluid cytokines in response to non-neurological surgery: an observational study. *J Neuroinflammation*. 2012;9:242.
14. Hovens IB, van Leeuwen BL, Mariani MA, Kraneveld AD, Schoemaker RG. Postoperative cognitive dysfunction and neuroinflammation; Cardiac surgery and abdominal surgery are not the same. *Brain Behav Immun*. 2016;54:178–193.
15. Hovens IB, Schoemaker RG, van der Zee EA, Absalom AR, Heineman E, van Leeuwen BL. Postoperative cognitive dysfunction: Involvement of neuroinflammation and neuronal functioning. *Brain Behav Immun*. 2014;38:202–210.
16. Scott DA, Evered LA, Silbert BS. Cardiac surgery, the brain, and inflammation. *J Extra Corpor Technol*. 2014;46:15–22.
17. Wan Y, Xu J, Ma D, Zeng Y, Cibelli M, Maze M. Postoperative impairment of cognitive function in rats: a possible role for cytokine-mediated inflammation in the hippocampus. *Anesthesiology*. 2007;106:436–443.
18. Kálmán J, Juhász A, Bogáts G, et al. Elevated levels of inflammatory biomarkers in the cerebrospinal fluid after coronary artery bypass surgery are predictors of cognitive decline. *Neurochem Int*. 2006;48:177–180.
19. Harrison NA, Brydon L, Walker C, Gray MA, Steptoe A, Critchley HD. Inflammation causes mood changes through alterations in subgenual cingulate activity and mesolimbic connectivity. *Biol Psychiatry*. 2009;66:407–414.
20. Martin N, Bossenmeyer-Pourié C, Koziel V, et al. Non-injurious neonatal hypoxia confers resistance to brain senescence in aged male rats. *PLoS One*. 2012;7:e48828.
21. Miranda A, Pêgo JM, Correia-Pinto J. Animal facility videoendoscopic intubation station: tips and tricks from mice to rabbits. *Lab Anim*. 2016;51:204–207.
22. Spear LP. Neurobehavioral assessment during the early postnatal period. *Neurotoxicol Teratol*. 1990;12:489–495.
23. Pêgo JM, Morgado P, Pinto LG, Cerqueira JJ, Almeida OFX, Sousa N. Dissociation of the morphological correlates of stress-induced anxiety and fear. *Eur J Neurosci*. 2008;27:1503–1516.
24. Porsolt RD, Le Pichon M, Jalfre M. Depression: a new animal model sensitive to antidepressant treatments. *Nature*. 1977;266:730–732.
25. Cohen J. *Statistical power analysis for the behavioral Sciences*. NewYork: NY: RoutledgeAcademic; 1988.
26. Cibelli M, Fidalgo AR, Terrando N, et al. Role of interleukin-1β in postoperative cognitive dysfunction. *Ann Neurol*. 2010;68:360–368.
27. Aurora AR, Fuentes JM, Hanly EJ, De Maio A, Talamini MA. CO2 pneumoperitoneum attenuates IL-6 production in peritoneal macrophages. *J Surg Res*. 2004;121:336.
28. West MA, Baker J, Bellingham J. Kinetics of decreased LPS-stimulated cytokine release by macrophages exposed to CO2. *J Surg Res*. 1996;63:269–274.
29. Fuentes JM, Hanly EJ, Aurora AR, et al. CO2 abdominal insufflation pretreatment increases survival after a lipopolysaccharide-contaminated laparotomy. *J Gastrointest Surg*. 2006;10:32–38.
30. Machado MCC, Coelho AMM, Martins JO, et al. CO2 abdominal insufflation decreases local and systemic inflammatory response in experimental acute pancreatitis. *Pancreas*. 2010;39:175–181.
31. Hanly EJ, Fuentes JM, Aurora AR, et al. Carbon dioxide pneumoperitoneum prevents mortality from sepsis. *Surg Endosc*. 2006;20:1482–1487.
32. Hanly EJ, Aurora AA, Shih SP, et al. Peritoneal acidosis mediates immunoprotection in laparoscopic surgery. *Surgery*. 2007;142:357–364.
33. Kastin AJ, Akerstrom V, Pan W. Interleukin-10 as a CNS therapeutic: the obstacle of the blood-brain/blood-spinal cord barrier. *Mol Brain Res*. 2003;114:168–171.
34. Raison CL, Capuron L, Miller AH. Cytokines sing the blues: inflammation and the pathogenesis of depression. *Trends Immunol*. 2006;27:24–31.
35. Goehler LE, Gaykema RPA, Hansen MK, Anderson K, Maier SF, Watkins LR. Vagal immune-to-brain communication: a visceral chemosensory pathway. *Auton Neurosci Basic Clin*. 2000;85:49–59.
36. Quan N, Banks WA. Brain-immune communication pathways. *Brain Behav Immun*. 2007;21:727–735.
37. Maier SF, Goehler LE, Fleshner M, Watkins LR. The role of the vagus nerve in cytokine-to-brain communication. Intergovernmental Panel on Climate Change, editor. *Ann N Y Acad Sci*. 1998;840:289–300.
38. Gidron Y, Kupper N, Kwaijtaal M, Winter J, Denollet J. Vagus-brain communication in atherosclerosis-related

inflammation: a neuroimmunomodulation perspective of CAD. *Atherosclerosis*. 2007;195:e1–e9.
39. Hirsch J, Vacas S, Terrando N, et al. Perioperative cerebrospinal fluid and plasma inflammatory markers after orthopedic surgery. *J Neuroinflammation*. 2016;13:211.
40. Tsamis D, Theodoropoulos G, Stamopoulos P, et al. Systemic inflammatory response after laparoscopic and conventional colectomy for cancer: a matched case-control study. *Surg Endosc Other Interv Tech*. 2012;26:1436–1443.
41. Kvarnström A, Swartling T, Kurlberg G, Bengtson J-P, Bengtsson A. Pro-inflammatory cytokine release in Rectal surgery: comparison between laparoscopic and open surgical Techniques. *Arch Immunol Ther Exp (Warsz)*. 2013;61:407–411.
42. Roque S, Mesquita AR, Palha JA, Sousa N, Correia-Neves M. The behavioral and Immunological impact of Maternal Separation: a Matter of timing. *Front Behav Neurosci*. 2014;8:192.
43. Mesquita AR, Pêgo JM, Summavielle T, Maciel P, Almeida OFX, Sousa N. Neurodevelopment milestone abnormalities in rats exposed to stress in early life. *Neuroscience*. 2007;147:1022–1033.
44. Lim SH, Park E, You B, et al. Neuronal synapse formation induced by microglia and interleukin 10. *PLoS One*. 2013;8:1.
45. Wang J, Xie L, Yang C, et al. Activated regulatory T cell regulates neural stem cell proliferation in the subventricular zone of normal and ischemic mouse brain through interleukin 10. *Front Cell Neurosci*. 2015;9:361.
46. Hovens IB, Schoemaker RG, van der Zee EA, Heineman E, Izaks GJ, van Leeuwen BL. Thinking through postoperative cognitive dysfunction: How to bridge the gap between clinical and pre-clinical perspectives. *Brain Behav Immun*. 2012;26:1169–1179.
47. Van Harten AE, Scheeren TWL, Absalom AR. A review of postoperative cognitive dysfunction and neuroinflammation associated with cardiac surgery and anaesthesia. *Anaesthesia*. 2012;67:280–293.
48. Dilger RN, Johnson RW. Aging, microglial cell priming, and the discordant central inflammatory response to signals from the peripheral immune system. *J Leukoc Biol*. 2008;84:932–939.
49. Wynne AM, Henry CJ, Godbout JP. Immune and behavioral consequences of microglial reactivity in the aged brain. *Integr Comp Biol*. 2009;49:254–266.
50. Terrando N, Monaco C, Ma D, Foxwell BMJ, Feldmann M, Maze M. Tumor necrosis factor triggers a cytokine cascade yielding postoperative cognitive decline. *Proc Natl Acad Sci*. 2010;107:20518–20522.
51. Jiang P, Ling Q, Liu H, Tu W. Intracisternal administration of an interleukin-6 receptor antagonist attenuates surgery-induced cognitive impairment by inhibition of neuroinflammatory responses in aged rats. *Exp Ther Med*. 2015;9:982–986.
52. Barrientos RM, Hein AM, Frank MG, Watkins LR, Maier SF. Intracisternal Interleukin-1 receptor antagonist prevents postoperative cognitive decline and neuroinflammatory response in aged rats. *J Neurosci*. 2012;32:14641–14648.

CHAPTER 5.
CO_2-pneumothorax

Peripheral and central inflammatory response and long-term behavioral assessment after neonatal CO2-Pneumothorax: study in a rodent model

Miranda A, Roque S, Serre-Miranda C, Pêgo JM, Correia-Pinto J

J Pediatr Surg 2017: [Epub ahead of print]

doi: 10.1016/j.jpedsurg.2017.08.016

Journal of Pediatric Surgery xxx (2017) xxx–xxx

Contents lists available at ScienceDirect

Journal of Pediatric Surgery

journal homepage: www.elsevier.com/locate/jpedsurg

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

Alice Miranda [a,b,*], Susana Roque [a,b], Cláudia Serre-Miranda [a,b], José Miguel Pêgo [a,b], Jorge Correia-Pinto [a,b,c]

[a] Life and Health Sciences Research Institute (ICVS), School of Medicine, University of Minho, Braga, Portugal
[b] ICVS/3B's - PT Government Associate Laboratory, Braga/Guimarães, Portugal
[c] Department of Pediatric Surgery, Hospital de Braga, Braga, Portugal

ARTICLE INFO

Article history:
Received 9 April 2017
Received in revised form 12 August 2017
Accepted 14 August 2017
Available online xxxx

Key words:
Neonate
Minimally invasive surgery
Pneumothorax
CO_2-insufflation
Inflammation

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.

* Corresponding author at: Life and Health Sciences Research Institute (ICVS), School of Medicine, *Campus* de Gualtar, University of Minho, 4710-057 Braga, Portugal. Tel.: +351 253 604 834.
E-mail address: alicemiranda@med.uminho.pt (A. Miranda).

http://dx.doi.org/10.1016/j.jpedsurg.2017.08.016

Please cite this article as: Miranda A, et al, Inflammatory response and long-term behavioral assessment after neonatal CO_2-pneumothorax: study in a rodent model..., J Pediatr Surg (2017), http://dx.doi.org/10.1016/j.jpedsurg.2017.08.016

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

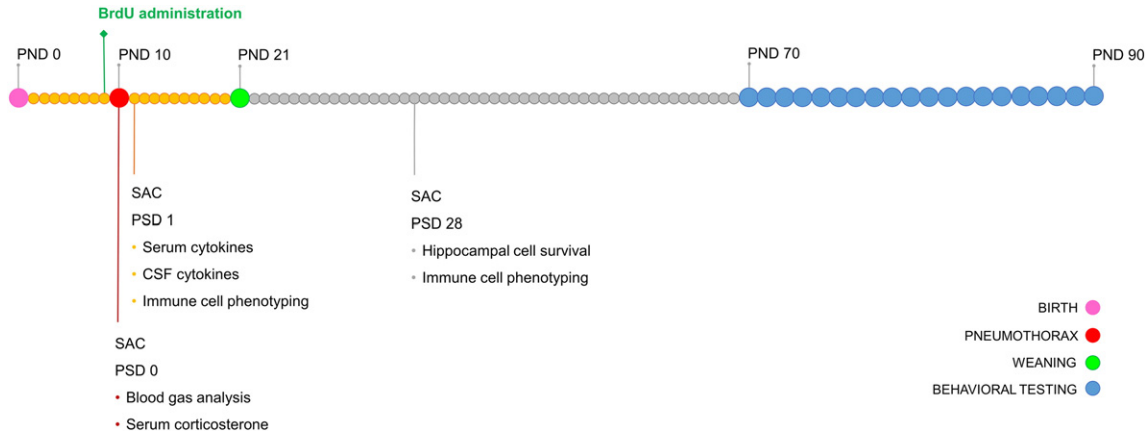


Fig. 1. Experimental timeline: Postnatal day (PND); post-surgical day (PSD); 5-bromo-2′-deoxyuridine (BrdU).

Please cite this article as: Miranda A, et al, Inflammatory response and long-term behavioral assessment after neonatal CO_2-pneumothorax: study in a rodent model..., J Pediatr Surg (2017), http://dx.doi.org/10.1016/j.jpedsurg.2017.08.016

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.

Please cite this article as: Miranda A, et al, Inflammatory response and long-term behavioral assessment after neonatal CO_2-pneumothorax: study in a rodent model..., J Pediatr Surg (2017), http://dx.doi.org/10.1016/j.jpedsurg.2017.08.016

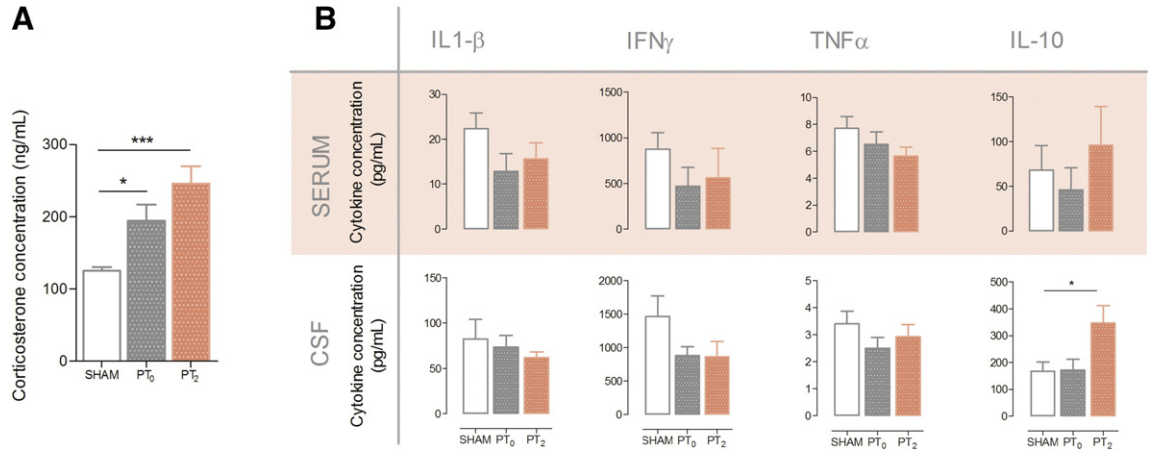


Fig. 2. Serum corticosterone **(A)** immediately after thoracic CO_2-insufflation in SHAM (white bars; n = 8), PT_0 (gray bars; n = 6) and PT_2 (pink bars; n = 6) groups. Peripheral and central IL-1β, IFN-γ, TNF-α and IL-10 **(B)** concentrations after thoracic CO_2-insufflation in SHAM (n = 11), PT_0 (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

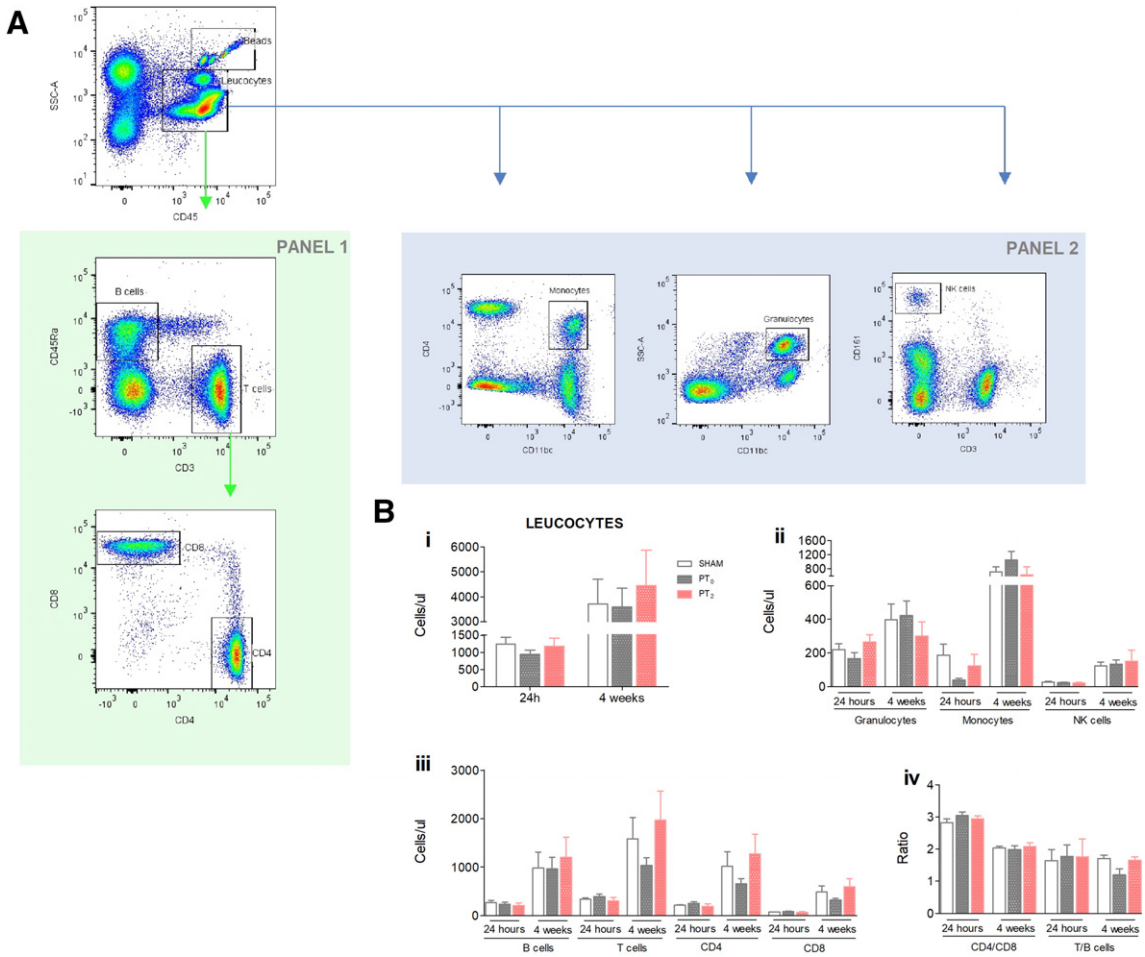


Fig. 3. Blood was processed for flow cytometry and labeled with specific antibodies for general leukocyte identification. Singlets were selected by plotting FCS-H to FSC-A, and leucocyte subset discrimination was obtained by applying the gating strategy represented in (**A**). Groups were compared by 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.

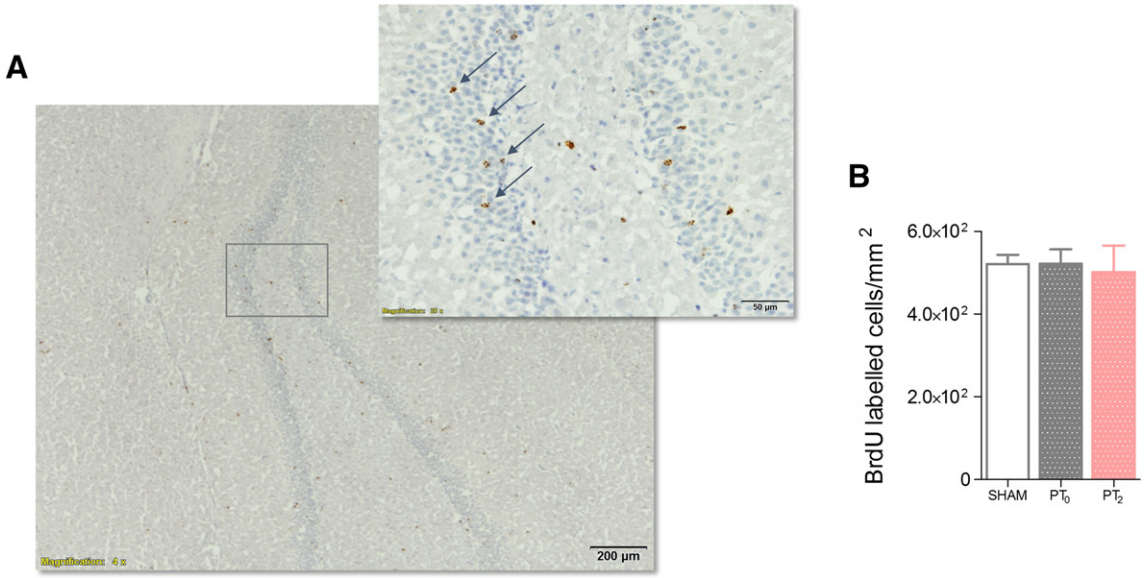


Fig. 4. Coronal sections were taken at the level of the dorsal hippocampus, and BrdU-labeled cells (gray arrows as some examples) were identified in the subgranular zone (SGZ) and granular cell layer (GCL) of the dentate gyrus with optical microscopy (**A**). The total number of BrdU + cells per mm^2 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.

Please cite this article as: Miranda A, et al, Inflammatory response and long-term behavioral assessment after neonatal CO_2-pneumothorax: study in a rodent model..., J Pediatr Surg (2017), http://dx.doi.org/10.1016/j.jpedsurg.2017.08.016

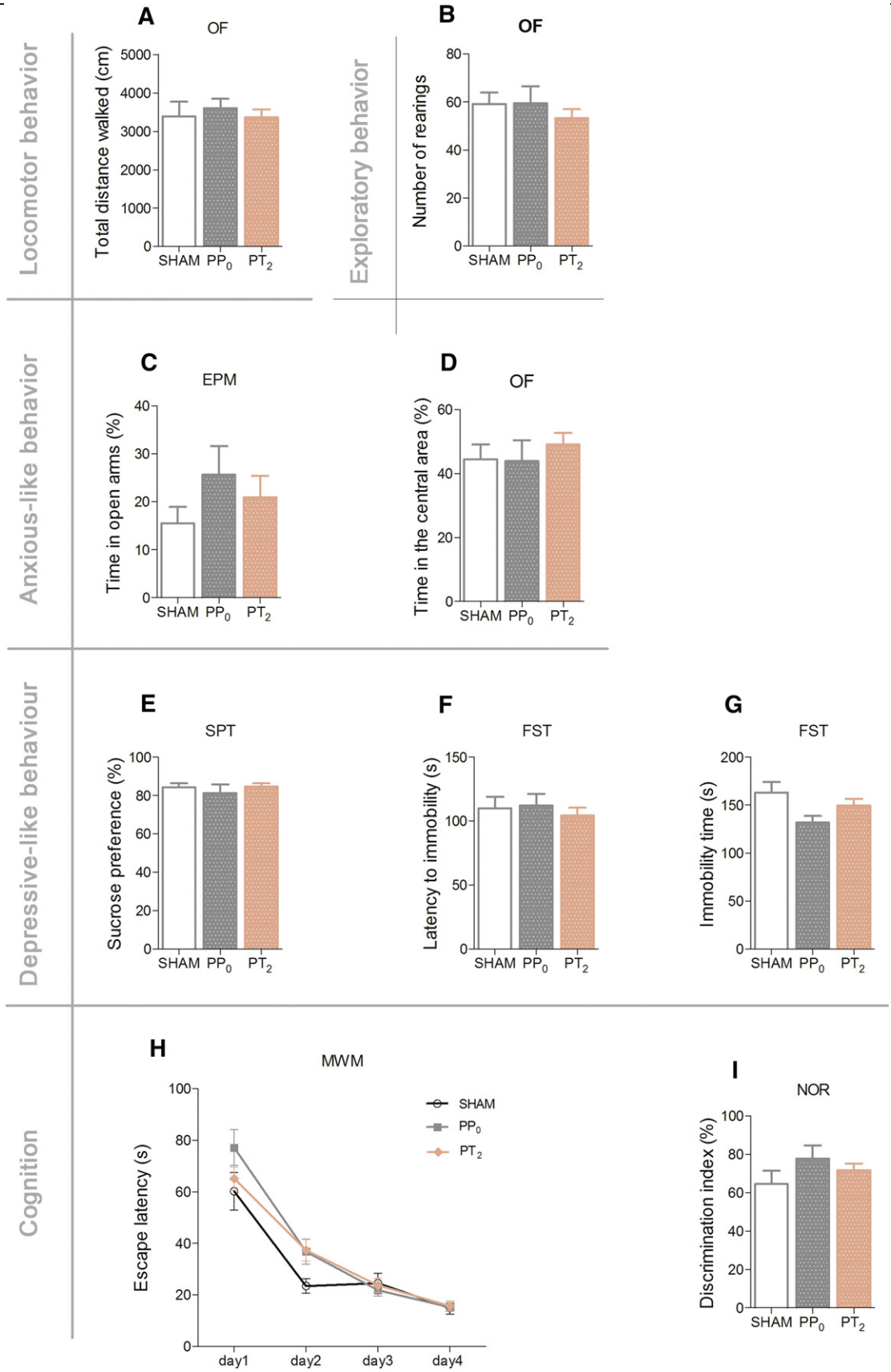


Fig. 5. Adult behavior of rats submitted to CO_2 pneumothorax in neonatal period of life: SHAM animals (white bars); PT_0 (gray bars); PT_2 (pink bars). **Locomotor activity**: (A) Total distance traveled in the open field (OF) arena; **Exploratory behavior**: (B) Number of rearings in the OF arena; **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.

Please cite this article as: Miranda A, et al, Inflammatory response and long-term behavioral assessment after neonatal CO_2-pneumothorax: study in a rodent model..., J Pediatr Surg (2017), http://dx.doi.org/10.1016/j.jpedsurg.2017.08.016

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

Please cite this article as: Miranda A, et al, Inflammatory response and long-term behavioral assessment after neonatal CO_2-pneumothorax: study in a rodent model..., J Pediatr Surg (2017), http://dx.doi.org/10.1016/j.jpedsurg.2017.08.016

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.

Funding

This work was granted by FEDER through the Operational Programme Competitiveness Factors - COMPETE and National Funds through the Foundation for Science and Technology -FCT under the project POCI-01-0145-FEDER-007038; and by the project NORTE-01-0145-FEDER-000013, supported by Norte Portugal Regional Operational Programme (NORTE 2020), under the PORTUGAL 2020 Partnership Agreement, through the European Regional Development Fund (ERDF). We also acknowledge the FCT for providing a doctoral fellowship to *A. Miranda* (SFRH/BD/52059/2012) and to C. Serre-Miranda (SFRH/BD/112494/2015) and a post-doctoral fellowship to S. Roque (SFRH/BPD/72710/2010). The funders have not been involved in manuscript writing or the decision to submit the article for publication.

Acknowledgments

The authors would like to thank Karl Storz GmbH for technical support and equipment.

The authors would like to acknowledge Doctor Mónica Morais for technical support in behavioral testing and immunohistochemical techniques.

References

[1] Holcomb GW. Thoracoscopic surgery for esophageal atresia. Pediatr Surg Int 2017;33:475–81.
[2] Holcomb Iii GW, Rothenberg SS, Bax KMA, et al. Thoracoscopic repair of esophageal atresia and tracheoesophageal fistula: a multi-institutional analysis. Ann Surg 2005;242:422.
[3] Tytgat SHaJ, van Herwaarden MYa, Stolwijk LJ, et al. Neonatal brain oxygenation during thoracoscopic correction of esophageal atresia. Surg Endosc 2016;30:2811–7.
[4] Bishay M, Giacomello L, Retrosi G, et al. Decreased cerebral oxygen saturation during thoracoscopic repair of congenital diaphragmatic hernia and esophageal atresia in infants. J Pediatr Surg 2011;46:47–51.
[5] Stolwijk LJ, van der Zee DC, Tytgat S, et al. Brain oxygenation during Thoracoscopic repair of long gap esophageal atresia. World J Surg 2017;41:1384–92.
[6] Stolwijk LJ, Tytgat SHaJ, Keunen K, et al. The effects of CO2-insufflation with 5 and 10 mmHg during thoracoscopy on cerebral oxygenation and hemodynamics in piglets: an animal experimental study. Surg Endosc 2015;29:2781–8.
[7] Pierro A. Hypercapnia and acidosis during the thoracoscopic repair of oesophageal atresia and congenital diaphragmatic hernia. J Pediatr Surg 2015;50:247–9.
[8] Talmor M, Hydo L, Barie PS. Relationship of systemic inflammatory response syndrome to organ dysfunction, length of stay, and mortality in critical surgical illness: effect of intensive care unit resuscitation. Arch Surg 1999;134:81–7.
[9] McAdams RM, Juul SE. The role of cytokines and inflammatory cells in perinatal brain injury. Neurol Res Int 2012;2012:1–15.
[10] Miranda A, Pêgo JM, Correia-Pinto J. Animal facility videoendoscopic intubation station: tips and tricks from mice to rabbits. Lab Anim 2017;51:204–7.
[11] Bessa JM, Ferreira D, Melo I, et al. The mood-improving actions of antidepressants do not depend on neurogenesis but are associated with neuronal remodeling. Mol Psychiatry 2009;14:764–73 [739].
[12] Bekris S, Antoniou K, Daskas S, et al. Behavioural and neurochemical effects induced by chronic mild stress applied to two different rat strains. Behav Brain Res 2005;161:45–59.
[13] Walf AA, Frye CA. The use of the elevated plus maze as an assay of anxiety-related behavior in rodents. Nat Protoc 2007;2:322–8.
[14] Gould TD, Dao DT, Kovacsics CE. Mood and anxiety related phenotypes in mice. Neuromethods 2009;42:1–20.
[15] Prut L, Belzung C. The open field as a paradigm to measure the effects of drugs on anxiety-like behaviors: a review. Eur J Pharmacol 2003;463:3–33.
[16] Bevins R a, Besheer J. Object recognition in rats and mice: a one-trial non-matching-to-sample learning task to study "recognition memory". Nat Protoc 2006;1:1306–11.
[17] Castagné V, Moser P, Roux S, et al. Rodent models of depression: Forced swim and tail suspension behavioral despair tests in rats and mice. Curr. Protoc. Pharmacol. Hoboken, NJ, USA: John Wiley & Sons, Inc.; 2010 1–14.
[18] Morris R. Developments of a water-maze procedure for studying spatial learning in the rat. J Neurosci Methods 1984;11:47–60.
[19] Vorhees CV, Williams MT. Forms of learning and memory. Nat Protoc 2006;1:848–58.
[20] Cohen J. Statistical power analysis for the behavioral sciences. 2nd ed. Hillsdale: NJ: Lawrence Earlbaum Associates; 1988.
[21] Dobbing J, Sands J. Comparative aspects of the brain growth spurt. Early Hum Dev 1979;3:79–83.
[22] Semple BD, Blomgren K, Gimlin K, et al. Brain development in rodents and humans: identifying benchmarks of maturation and vulnerability to injury across species. Prog Neurobiol 2013;106–107:1–16.
[23] Rice D, Barone S. Critical periods of vulnerability for the developing nervous system: evidence from humans and animal models. Environ Health Perspect 2000;108:511–33.
[24] Avital S, Itah R, Szomstein S, et al. Correlation of CO2 pneumoperitoneal pressures between rodents and humans. Surg Endosc 2009;23:50–4.
[25] Leister I, Schüler P, Vollmar B, et al. Microcirculation and excretory function of the liver under conditions of carbon dioxide pneumoperitoneum. Surg Endosc 2004;18:1358–63.
[26] Walker K, Halliday R, Badawi N, et al. Early developmental outcome following surgery for oesophageal atresia. J Paediatr Child Health 2013;49:467–70.
[27] Aite L, Bevilacqua F, Zaccara A, et al. Short-term neurodevelopmental outcome of babies operated on for low-risk esophageal atresia: a pilot study. Dis Esophagus 2014;27:330–4.
[28] Gischler SJ, Mazer P, Duivenvoorden HJ, et al. Interdisciplinary structural follow-up of surgical newborns: a prospective evaluation. J Pediatr Surg 2009;44:1382–9.
[29] Yuki K, Matsunami E, Tazawa K, et al. Pediatric perioperative stress responses and anesthesia. Transl Perioper Pain Med 2017;2(1):1–12.
[30] Desborough JP. The stress response to trauma and surgery. Br J Anaesth 2000;85:109–17.
[31] Asteriou C, Lazopoulos A, Rallis T, et al. Video-assisted thoracic surgery reduces early postoperative stress. A single-institutional prospective randomized study. Ther Clin Risk Manag 2016;12:59–65.
[32] Liu H-F, Gao L, Liu T, et al. Comparison of acute phase reaction and postoperative stress in pigs undergoing video-assisted thoracoscopic versus thoracotomy pneumonectomy. Acta Vet Scand 2016;58:75.
[33] Liu X, Wu Y, Zhu J, et al. Totally thoracoscopic repair of atrial septal defect reduces systemic inflammatory reaction and myocardial damage in initial patients. Eur J Med Res 2014;19:13.
[34] Jones RO, Anderson NH, Murchison JT, et al. Innate immune responses after resection for lung cancer via video-assisted thoracoscopic surgery and thoracotomy. Innovations (Phila) 2014;9:93–103.
[35] Ng CS, Wan IY, Yim AP. Impact of video-assisted Thoracoscopic major lung resection on immune function. Asian Cardiovasc Thorac Ann 2009;17:426–32.
[36] Bromander S, Anckarsäter R, Kristiansson M, et al. Changes in serum and cerebrospinal fluid cytokines in response to non-neurological surgery: an observational study. J Neuroinflammation 2012;9:242.
[37] Hirsch J, Vacas S, Terrando N, et al. Perioperative cerebrospinal fluid and plasma inflammatory markers after orthopedic surgery. J Neuroinflammation 2016;13:211.
[38] Shiozaki T, Hayakata T, Tasaki O, et al. Cerebrospinal fluid concentrations of anti-inflammatory mediators in early-phase severe traumatic brain injury. Shock 2005;23:406–10.
[39] Kirchhoff C, Buhmann S, Bogner V, et al. Cerebrospinal IL-10 concentration is elevated in non-survivors as compared to survivors after severe traumatic brain injury. Eur J Med Res 2008;13:464–8.
[40] Woiciechowsky C, Volk HD. Increased intracranial pressure induces a rapid systemic interleukin-10 release through activation of the sympathetic nervous system. Acta Neurochir Suppl 2005:373–6.
[41] Hayakata T, Shiozaki T, Tasaki O, et al. Changes in Csf S100b and cytokine concentrations in early-phase severe traumatic brain injury. Shock 2004;22:102–7.
[42] Perez-Barcena J, Ibáñez J, Brell M, et al. Lack of correlation among intracerebral cytokines, intracranial pressure, and brain tissue oxygenation in patients with traumatic brain injury and diffuse lesions. Crit Care Med 2011;39:533–40.

Please cite this article as: Miranda A, et al, Inflammatory response and long-term behavioral assessment after neonatal CO_2-pneumothorax: study in a rodent model..., J Pediatr Surg (2017), http://dx.doi.org/10.1016/j.jpedsurg.2017.08.016

PART III | Discussion and Conclusions

CHAPTER 6.
General discussion

With this thesis we aimed to contribute to unravel the impact of CO_2-insufflation over the central nervous system of neonates by using a neonatal rodent model. As a first step, we performed a systematic bibliographic review on the respiratory, cardiovascular and inflammatory response during the CO_2-insufflation and in what way this response may affect the brain related events (Chapter 1). From the abovementioned review it is evident that minimally invasive surgical procedures in neonates and small infants are technically feasible and an increasing number of conditions can benefit from this surgical approach (Lacher *et al.*, 2014). However, recent reports have raised questions about the safety of this surgical approach, especially on the impact of CO_2-insufflation on immature organs and organ systems (Bishay *et al.*, 2011; Tytgat *et al.*, 2015, 2016; Stolwijk *et al.*, 2017; Zani *et al.*, 2017) with no studies evaluating the long-term consequences of this specific early-life event (Bishay *et al.*, 2013; Tytgat *et al.*, 2013). We first describe the development of an endotracheal intubation technique that can be applied in neonatal rats in a safe and reliable way (Chapter 3). The development of this technique allowed the development of an adequate neonatal *in vivo* model to study how CO_2-insufflation influence immunological and neurodevelopmental parameters, which are addressed in Chapter 4 and 5. These topics address the influence of different insufflation-pressures and insufflation-times, as well as different body cavities, thoracic and peritoneal.

6.1. Neonatal rodent endotracheal intubation

Human studies on the impact of CO_2-pneumoperitoneum and -pneumothorax are frequently performed under diverse confounding factors like the anaesthetic protocol, ventilatory management, cavity accessed, insufflation pressure, duration of surgery, age of patients, intravascular volume status, degree of Trendelenburg position, and even the influence of

pathological conditions or anatomical defects being corrected (Veekash *et al.*, 2010). All these factors can lead to misleading conclusions and for that reason, studies on animal models represent a great advantage for the evaluation of the acute and long-term impact of CO_2-insufflation, with pig, rabbit, guinea-pig, rat and mouse being the most used animal models (Hazebroek *et al.*, 2002; Molinas *et al.*, 2004; Fuh *et al.*, 2005; Sümpelmann *et al.*, 2006; Bourdel *et al.*, 2007; Stolwijk *et al.*, 2015; Liu *et al.*, 2016). However, most studies on the CO_2-insufflation effects are performed in adult animals, with only a few using neonatal models (Fuh *et al.*, 2005; Stolwijk *et al.*, 2015). Neonates have specific anatomical and physiological characteristics such an increased peritoneal surface area to mass ratio, low peritoneal and pleural thickness and narrow distance between vessels and serous surface (Kalfa *et al.*, 2005; Lasersohn, 2011). All these factors contribute to higher CO_2-absorption in neonatal patients, which is less obtained when using adult animal models. Another important factor is the ventilatory support in anesthetized animals, which was shown to be essential for the maintenance of physiological blood gas partial pressures and pH, being even more important during CO_2-insufflation, as mechanical ventilation is a key player in the elimination of the absorbed CO_2 and in the maintenance of physiological acid-base status. In anesthetized, spontaneously breathing animals, acidosis, hypercapnia and mild hypoxia inevitably install, and the induction of CO_2-pneumoperitoneum or -pneumothorax in non-ventilated animals exacerbates these events, resulting in severe hypercapnia, acidosis, hypoxia and even death (Hazebroek *et al.*, 2002; Heijnen *et al.*, 2002; Mynbaev *et al.*, 2002; Molinas *et al.*, 2004). The purpose of experimental studies is to extrapolate experimental findings to daily clinical practice and studies may be negatively biased due to adverse cardiorespiratory consequences of CO_2-insufflation in spontaneously breathing animals. In anesthetized laboratory animals, the mechanical ventilation is many times neglected due to technical difficulties associated with false path during bling intubations or with the difficult direct intubations due to the small animal size and limited mouth opening.

In this work, there was the need to endotracheal intubate neonatal rats, so they could be properly mechanical ventilated and therefore result in adequate animal models for further

evaluation of the impact of CO_2-insufflation. Only videoendoscopic systems would allow the safe and reliable intubation of neonatal animals with such a narrow mouth opening. The first videoendoscopic intubation technique was described by Worthley *et al.* (Worthley *et al.*, 2000) which used a 10 mm fibre-optic, 0° scope to intubate rabbits. Vergary *et al.* (Vergari *et al.*, 2003) used a 1.7 mm, 0° arthroscope to intubate mice, by applying a guide-wire in the trachea before inserting the endotracheal tube (cannula). To intubate rats, Fuentes *et al.* (Fuentes *et al.*, 2004) used a 3 mm, 30° endoscope, while Clary *et al.* (Clary *et al.*, 2004) chose a 2 mm, 0° endoscope. These two rodent studies used a technique in which the endotracheal tube progresses side-by-side with the endoscope. The supine positioned animals presented their head and neck extended, and a stylet was used to direct the cannula. The described techniques present some difficulties since many tasks must be simultaneously accomplished and are hardly obtained by a single operator: the stable position of the animal, simultaneous camera holding and non-traumatic tongue retraction, and coordinated movements of cannula progression and stylet retraction. All these procedures seem complex to perform in such a limited space as the oral cavity of the neonatal rat. More recently, studies have described over-the-endoscope techniques, which consists in covering the endoscope with the cannula, which progresses once the endoscope is aligned with the laryngeal opening (Konno *et al.*, 2014a; Konno *et al.*, 2014b). However, this technique can only be applied to animals requiring endotracheal tubes with internal diameters greater than the outer diameter of the scopes. In our study, the intubation of PND 10 rats required the optimization of the videoendoscopic intubation techniques already described in the literature, in order to limit the accessory instruments and reduce the number of persons required to successfully perform the technique. Due to the very small internal diameter of the cannula (0,6 mm), over-the-endoscope techniques could not be applied and only the side-by-side techniques could be adopted.

In chapter 3 of this thesis we describe a new videoendoscopic intubation method that can be used to endotracheal intubate neonatal rats. While optimizing our technique, three characteristics were considered as advantageous for the choice of the endoscope: (i) the

smallest outer diameter available, so it can easily progress above the tongue without excessive mouth opening nor oral cavity injury; (ii) 30° endoscope, allowing a wide view of the oral cavity while progressing; and (iii) shorter length possible, so the operator could coordinate camera movements without resulting in huge image changes. The endoscope that joined these three characteristics was a 1.9 mm, 10 cm length, 30° endoscope. Being a 30° endoscope can gently progress above the tongue similarly to a laryngoscope, and its tip can lower the epiglottis, which was hardly obtained with a 0° endoscope. This movement will make the epiglottis to fall and allow the visualization of the laryngeal opening. Regarding the animal position during intubation, our technique allowed the achievement of the following aims: (i) non-traumatic tongue retraction or no retraction at all; (ii) no need for camera head holding and (iii) stable image during cannula progression. The position that combined all these aims was the right lateral recumbency (for the right-handed operator) as the tongue falls by itself to the right side of the animal and no tongue retraction is needed, and the camera head can be hold in the table allowing a stable image. With this technique we could successfully endotracheal intubate PND10 rats, which were further mechanically ventilated. Since this technique was reliable for the intubation of such a small animal model, we hypothesized that other laboratory animal species could benefit from this endotracheal intubation technique for the most varied research purposes (anesthesia delivery, induction of toxic or infectious pulmonary diseases, instillation of tumour cells and even for therapeutic strategies). This technique was then successfully applied to mice, adult rats, guinea pigs and rabbits. We believe that the described technique could be of great value for the refinement of procedures during laboratory animal anesthesia and contribute to the development of better animal models.

6.2. CO_2-pneumoperitoneum and -pneumothorax

The endotracheal intubation of PND 10 rats allowed the proper mechanical ventilation of our neonatal animal model. Arterial blood gas concentrations, pH and oxygen saturation was evaluated and adjusted to physiological ranges by applying respiratory frequency and tidal

volume adjustments in all CO_2-pneumoperitoneum conditions under study, which included different insufflation-pressures and insufflation-times. The insufflation pressures applied in our work were based on rodent and human CO_2-pneumoperitoneum correlation studies highlighting that the application of human standard working pressures in the rat model will correspond to abnormally high insufflation pressures in the rat. Avital *et al.* observed that insufflation pressures > 8 mmHg result in declined CO_2 diffusion through the rat peritoneum due to pressure occlusion of peritoneal capillaries, not simulating routine working pressures employed in humans (Avital *et al.*, 2009). Blobner *et al.* have also demonstrated in the porcine model that peritoneal CO_2-absorption rate is directly correlated with the intrabdominal pressure, but only up to a maximum insufflation pressure of 16 mmHg. The author found that intrabdominal pressures above 16 mmHg in the porcine model caused a reduction of CO_2-absorption rate due to pressure occlusion of peritoneal capillaries (Blobner *et al.*, 1999) and similar findings were obtained in human patients submitted to CO_2-insufflation pressures between 14 to 20 mmHg (Blobner *et al.*, 1993). The insufflation pressures chosen in our study were therefore lower than the standard human insufflation pressures. In our CO_2-pneumoperitoneum groups, the applied insufflation pressures of 8 mmHg and 12 mmHg were chosen as equivalent to high and very high-insufflation pressures in humans, respectively.

During surgery, the body's mounts a reaction to achieve a new state of homeostasis which is reflected by neuroendocrine, immunologic and metabolic changes. One of the manifestations of the surgical stress response is an increased corticosterone production, and the interplay between corticosterone levels and several parameters of the immune system during the first days of life has been shown to impact adult behavior (Roque *et al.*, 2014). 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 (Mesquita *et al.*, 2007). In chapter 4 of this thesis, the acute impact of CO_2-pneumoperitoneum on corticosterone production and on the peripheral and central cytokine response was evaluated. We observed that although

corticosterone levels were significantly increased in all groups when compared to SHAM animals, this was secondary to an acute and single stressful event in neonatal life, which seemed to induce no changes in neurodevelopmental milestones acquisition. Moreover, in CO_2-pneumoperitoneum groups, the surgical stress does not seem to be aggravated by duplicating the insufflation time from 30 minutes to 60 minutes.

Regarding the peripheral inflammatory changes, open surgery led to significant disturbances in serum TNFα levels when compared to CO_2-pneumoperitoneum groups. Since no differences were observed between those experimental groups and the controls, this finding may result from the combination of increased TNFα levels in the open surgery group and a possible reduction in TNFα levels in animals submitted to CO_2-pneumoperitoneum. *In vitro* studies have supported our findings, as LPS-stimulated macrophages derived from rats exposed to CO_2-pneumoperitomeum presented a reduced macrophage TNFα production when compared to macrophages derived from animals submitted to gasless or helium laparoscopy and laparotomy (Mathew *et al.*, 1999; Neuhaus *et al.*, 2000). Additionally, *in vivo* studies have reported that a CO_2-insufflation pre-treatment significantly reduces serum TNFα levels in acute pancreatitis model (Machado *et al.*, 2010) and Jacobi *et al.* have demonstrated decreased TNFα plasma levels in rats undergoing laparoscopy with CO_2-insufflation, as compared to controls or helium laparoscopy (Jacobi *et al.*, 1998). Although most studies were performed with sepsis models, leading to an exacerbation of the inflammatory modifying effects of CO_2-insufflation, similar findings were obtained in our work performed in aseptic conditions. Regarding the serum levels of the anti-inflammatory cytokine IL-10, although the differences were not statistically significant between groups, the CO_2-pneumoperitoneum groups presented a tendency in increased concentrations of this anti-inflammatory cytokine. This tendency is in good accordance with Hanly *et al.* and Jacobi *et al.* works that have shown increased serum IL-10 levels in LPS-sepsis and non-sepsis animal models submitted to CO_2-pneumoperitoneum when compared to helium or anesthesia control groups (Jacobi *et al.*, 1998; Hanly *et al.*, 2006). Interestingly, this anti-inflammatory profile was reflected in the CSF of animals submitted to CO_2-

pneumoperitoneum, with significantly increased levels in the group exposed to higher insufflation pressures.

No experimental or clinical studies have evaluated the central inflammatory response after MIS, and the upstream mechanisms of the central cytokine profile found in our study need further investigation to clarify whether this finding is a consequence specific of the gas or if it results from the mechanical effects of the increased intracavity pressure on brain homeostasis, regardless the type of gas used. Based on current literature, there is no clear evidence whether a relation between CSF IL-10 levels and variations in ICP or brain oxygenation/perfusion exists. As most studies correlating central cytokine response and intracranial pressure are performed in traumatic brain injured (TBI) patients (Hayakata *et al.*, 2004; Shiozaki *et al.*, 2005; Kirchhoff *et al.*, 2008; Perez-Barcena *et al.*, 2011), the extrapolation to the context of MIS may not be ideal since TBI patients usually have severe intracranial damages, many times associated with additional extracranial injuries. Additionally, the time of hospital admission and CFS collection after the TBI may result in contradictory findings. For those reasons, further studies specifically addressing CSF cytokine concentrations after MIS must be correlated with intraoperative parameters, as brain oxygenation, perfusion and intracranial pressure. Unlike other cytokines (Pan *et al.*, 1999), IL-10 does not seem to cross the intact blood brain barrier (BBB) (Kastin *et al.*, 2003). The observed increase in CSF IL-10 levels in animals exposed to CO_2-insufflation can result from (i) peripheral circulating IL-10 acting directly in the brain through regions devoided of the blood brain barrier, such as the choroid plexus and the circumventricular organs and (Raison *et al.*, 2006); (ii) peripherally produced cytokines that 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 (Maier *et al.*, 1998; Goehler *et al.*, 2000; Gidron *et al.*, 2007; Quan *et al.*, 2007); (iii) activation of microglia by a given external stimuli, which result in the production of several pro-inflammatory mediators, favouring the BBB permeabilization and, ultimately, driving to the infiltration of peripheral cells into the CNS (including macrophages and T cells). IL-10,

similarly to other immune mediators present within the CNS in the context of disease, can be produced by brain-infiltrating immune cells. Macrophages arrival to the CNS will contribute, together with microglia, to the cytokine production or indirectly through the modulation of the tissue microenvironment. Similarly, infiltrating T cells may alter the cytokine *milieu* and thus reprogram microglia responses (González *et al.*, 2014). Although this BBB permeabilization phenomenon is described as occurring in the context of disease, little is known about the possible disruption of BBB during MIS, and weather the mechanical and chemical effects of CO_2-insufflation contributes to changes in BBB integrity and therefore explain the macromolecular circulation of cytokines and cells from those two compartments. Studies on the CNS inflammatory reactions elicited by peripheral surgery have been published, and CSF cytokine levels have been evaluated in patients submitted to peripheral surgery (not involving the CNS or areas of the body innervated by the *vagus* nerve or other afferent cranial nerves) as in knee arthroplasty and hip surgery. Results indicate a lack of correlation between peripheral and central cytokine levels, suggesting that they are separately regulated in response to surgical trauma. These findings support another involved mechanism than the increased BBB permeability and the migration of phagocytic cells into the CNS. However, a subgroup of patients presented increased baseline CSF/serum albumin ratios preoperatively, suggesting that some individuals can present different BBB permeability and be particularly prone to exaggerated inflammatory responses following peripheral surgery (Bromander *et al.*, 2012). In our work, no investigation on BBB permeability changes during CO_2-insufflation was done but further evaluation of BBB integrity should be performed and correlated with IL-10 CSF and serum levels. We observed a much greater increase in CSF IL-10 levels than in serum, and similar phenomena with other cytokines was observed in human patients submitted to non-neurological surgery, suggesting that an inflammatory reaction in CNS could be elicited independently of the systemic one (Bromander *et al.*, 2012; Hirsch *et al.*, 2016). Furthermore, it cannot be excluded that the cytokine turnover in the CNS is slower than in the periphery, which might explain the

differences in cytokine levels between those two compartments 24 h after the CO_2-insufflation.

The finding from this thesis suggest that a prolonged central anti-inflammatory environment seems to remain in individuals submitted to MIS. This finding may have implications in other well studied phenomena as the postoperative cognitive decline (POCD). Cognitive impairment, including the conditions of postoperative delirium and POCD, is frequently observed after major surgery (Deiner *et al.*, 2009) and, although risk factors such as advanced age and neurodegenerative processes have been identified, its pathophysiology is still unclear (Moller *et al.*, 1998). It has been demonstrated that surgery causes a persistent and possibly irreversible decrement in memory and learning in a murine model of Alzheimer disease, primarily through a transient activation of neuroinflammation (Tang *et al.*, 2013). Increased expression of pro-inflammatory cytokines in a rodent's hippocampus following surgery was associated with cognitive decline (Rosczyk *et al.*, 2008; Cibelli *et al.*, 2010) and preclinical studies suggest that inflammation is a possible pathogenic mechanism for POCD (Cibelli *et al.*, 2010; Terrando *et al.*, 2010, 2011). The central anti-inflammatory profile observed in our experimental groups submitted to CO_2-insufflation may be a forerunner for the study of the incidence of POCD after MIS *versus* open surgery.

An additional assessment of the functional consequences on adult behavior was performed in our rat model submitted in the neonatal period to different CO_2 insufflation-pressures and insufflation-times. No differences were observed between groups regarding neurodevelopmental milestones acquisition nor in body weight. No insufflation-pressure, insufflation-time or interaction effect was detected in the long-term behavioral evaluation in any of the behavioral domains analysed. Only one study have evaluated the long-term behavioral impact of neonatal CO_2-insufflation in the guinea-pig model and no behavioral deficits were detected in adulthood (Fuh *et al.*, 2005). However, being a precocial animal model (born with a high degree of neuromuscular coordination and neurobehavioral competence, being highly mobile, capable of thermoregulation and independent feeding at birth) the guinea pig is much less likely to be affected by neonatal brain insults compared

with the less mature neonatal rat model. When brain vulnerability is being under study, the different timing of the brain growth spurt of different species must be taken into consideration. The timing of brain development is different in relation to birth in different species and, being the guinea-pigs prenatal brain developers, this factor must be taken in to account when extrapolating results from one species to another (Dobbing *et al.*, 1979). Interestingly, a study performed in pregnant guinea-pigs exposed to CO_2-pneumoperitoneum have shown post-natal hyperactivity in the offspring, traduced in increased locomotor activity when compared to controls (De La Fuente *et al.*, 2003). Our animal model choice was based on the above mentioned factors, as rodents at postnatal day 7–10 are considered equivalent to a term human infant (Dobbing *et al.*, 1979) and 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 (Semple *et al.*, 2013) being considered a good *in vivo* model for developmental brain injury studies.

Studies in children have shown that, despite the lower insufflation pressures usually applied in thoracoscopic approaches when comparing to laparoscopic ones, the derangement in respiratory function is more pronounced during thoracoscopy (Pacilli *et al.*, 2006; McHoney *et al.*, 2008). Additionally, studies suggest that brain oxygenation might be affected by the hemodynamic disturbances induced by CO_2-insufflation, and authors associated those periods of transient decrease in $rScO_2$ as being significantly more frequent during thoracoscopy than in abdominal open surgery (Neunhoeffer *et al.*, 2017), suggesting potentially deleterious impact of these events over the developing brain (Bishay *et al.*, 2011; Stolwijk *et al.*, 2015). Therefore, and after exploring the influence of the insufflation-pressure and insufflation-time during CO_2-pneumoperitoneum, we decided to evaluate if the same observations were shared between thoracic and peritoneal CO_2-insufflation regarding the possible functional consequences on adult behavior. Additionally, the peripheral and central cytokine response that accompanied the corticosterone production was analysed in order to

understand if similar phenomena of a central anti-inflammatory profile occurs regardless the cavity being insufflated.

In our work, and similarly with the findings obtained in the pneumoperitoneum study, no functional alterations were detected in any of the behavioral domains analysed, and no impact on the survival of hippocampal newborn cells was observed. Corticosterone levels were increased in anesthetized and mechanically ventilated animals, suggesting that these intraoperative factors contribute to some degree of surgical stress. Additionally, the CO_2-pneumothorax did not result in significant disturbances in the peripheral pro- and anti-inflammatory cytokines evaluated in this work but interestingly, the increased CSF concentration of the anti-inflammatory cytokine IL-10 was observed, showing that the same phenomena is occurring during CO_2-pneumoperitoneum and -pneumothorax.

Studies on the impact of MIS are mainly focused in highlighting the benefits of MIS over traditional open surgical approaches with respect to postoperative morbidity. However no studies evaluated the immune system of a given patient before and after CO_2-insufflation in MIS, or evaluated how the innate and adaptive immune system is modulated by the CO_2-insufflation. Studies are mainly focused in comparing thoracotomy with thoracoscopic approaches, most of them concluding that MIS results in better preservation of postoperative immune function (Ng *et al.*, 2009; Jones *et al.*, 2014; Liu *et al.*, 2014). Therefore, the immune system was evaluated in our pneumothorax model and we observed that CO_2-insufflation has no impact on cellular immune system balance 24h and 4 weeks after the insufflation, as no differences in the main leucocyte population was detected in the blood. This finding suggests a balance between pro- and anti-inflammatory response during CO_2-insufflation and a well preserved immune function, equivalent to non insufflated animals.

The potential lasting neurotoxic effects of agents used to induce general anesthesia and sedation could not be left behind in neonatal surgical studies. Anaesthetic agents administered to young children have been focus of concern for many years (Andropoulos *et al.*, 2017) and the Food and Drug Administration and the American Academy of Paediatrics' recently stated that surgical procedures performed under anesthesia should be avoided in

children younger than 3 years old (Rappaport *et al.*, 2015). Since laparoscopic and thoracoscopic procedures in neonates are considered life-saving surgeries, it is assumed that the risk-benefit is highly analysed, and the cumulative effect of anaesthesia is unavoidable in these young patients. *In vitro* and *In vivo* studies have demonstrated that both N-methyl-D-aspartate (NMDA) antagonists and gamma-aminobutyric acid (GABA) agonists induce functional effects over brain development (Sanders *et al.*, 2013). However, human studies have found that a single exposure to general anesthesia was not associated with poorer neurodevelopmental outcomes (Davidson *et al.*, 2016; Sun *et al.*, 2016) and that the extent of injury induced by anaesthetic agents is related to the pediatric patient's age and with the cumulative anaesthetic dose (Jevtovic-Todorovic *et al.*, 2013). In our neurodevelopmental study, the choice of anaesthetic agent was based on current knowledge on neurotoxic effects but more importantly, regardless the anaesthetic agent chosen, the need to differentiate between the adverse effects of CO_2-insufflation and those associated with the use of anaesthetic agents demanded the inclusion of a SHAM and an anesthetized control group. In this way, we believe that our study was able to evaluate the isolated effects of CO_2-insufflation, rather than evaluating the cumulative effects of anesthesia and CO_2-insufflation.

While no enlightening human studies on the neurodevelopmental impact of MIS are published, our findings give evidence that the neonatal exposure to CO_2-insufflation, either peritoneal or thoracic, do not result in functional long-term deficits. Further studies exploring the functional consequences of the central anti-inflammatory environment developed after the CO_2-exposure will help to understand if the well-known benefits of MIS over traditional open surgical approaches also result from this central anti-inflammatory response.

CHAPTER 7.
Main conclusions

In this thesis we demonstrated a new safe and reliable endotracheal intubation method that can be used from neonatal rats to rabbit models, allowing the adequate mechanical ventilation of many animal surgical models or for the adequate tracheal access for substance delivery.

This is the first study demonstrating a pronounced anti-inflammatory reaction centrally (as reflected in CSF) after MIS. The traditional paradigm of an immunologically privileged CNS is being overturned as experimental and clinical studies have been showing accumulating evidence that the CNS isolation from inflammatory reactions (immune privilege) may not be sustained perioperatively. The functional consequences of our findings in neonatal and pediatric patients submitted to MIS need further preclinical and clinical studies, and the implications of these findings in other surgical associated conditions must be explored. This work demonstrates that CO_2-insufflation does not disturb the peripheral inflammatory cytokine response nor modulates the immune cell function, and that derangements in the immune function after MIS may result from intraoperative factors other than the CO_2-insufflation.

Minimally invasive surgical procedures in neonates and infants are usually limited to those preserving life of children with congenital anomalies. However, survival alone is no longer sufficient for the successful surgical treatment of congenital abnormalities. Morbidity, risk of impaired growth, and developmental delays are factors under scrupulous monitoring by multidisciplinary follow-up teams, but human longitudinal studies are still lacking. In this thesis we demonstrate the absence of functional consequences in adult behavior after the neonatal exposure to CO_2-insufflation, which make us believe that it is safe to continue performing laparoscopic and thoracoscopic surgery in neonatal and pediatric patients. This thesis contributes to the transverse understanding of the impact of MIS in neonates and small infants.

References

Andropoulos DB, Greene MF. 2017. Anesthesia and Developing Brains — Implications of the FDA Warning. *New England Journal of Medicine* 376: 905–907.

Avital S, Itah R, Szomstein S, Rosenthal R, Inbar R, Sckornik Y, Weinbroum A. 2009. Correlation of CO2 pneumoperitoneal pressures between rodents and humans. *Surgical Endoscopy and Other Interventional Techniques* 23: 50–54.

Bishay M, Giacomello L, Retrosi G, Thyoka M, Nah SA, McHoney M, De Coppi P, Brierley J, Scuplak S, Kiely EM. 2011. Decreased cerebral oxygen saturation during thoracoscopic repair of congenital diaphragmatic hernia and esophageal atresia in infants. *Journal of pediatric surgery* 46: 47–51.

Bishay M, Giacomello L, Retrosi G, Thyoka M, Garriboli M, Brierley J, Harding L, Scuplak S, Cross K, Curry J, Kiely E, De Coppi P, Eaton S, Pierro A 2013. Hypercapnia and Acidosis During Open and Thoracoscopic Repair of Congenital Diaphragmatic Hernia and Esophageal Atresia: Results of a Pilot Randomized Controlled Trial. *Ann Surg* 258: 895–900.

Blobner M, Bogdanski R, Jelen-Esselborn S, Henke J, Erhard W, Kochs E. 1999. Viszerale Resorption von intra-abdominell insuffliertem Kohlen-dioxid beim Schwein. *ains · Anästhesiologie · Intensivmedizin · Notfallmedizin · Schmerztherapie* 34: 94–99.

Blobner M, Felber AR, Gögler S, Feussner H, Weigl EM, Jelen G, Jelen-Esselborn S. 1993. [The resorption of carbon dioxide from the pneumoperitoneum in laparoscopic cholecystectomy]. *Der Anaesthesist* 42: 288–294.

Bourdel N, Matsuzaki S, Bazin J-E, Pouly J-L, Mage G, Canis M. 2007. Peritoneal tissue-oxygen tension during a carbon dioxide pneumoperitoneum in a mouse laparoscopic model with controlled respiratory support. *Human Reproduction* 22: 1149–1155.

Bromander S, Anckarsäter R, Kristiansson M, Blennow K, Zetterberg H, Anckarsäter H, Wass CE. 2012. Changes in serum and cerebrospinal fluid cytokines in response to non-

neurological surgery: an observational study. *Journal of neuroinflammation* 9: 242.

Cibelli M, Fidalgo AR, Terrando N, Ma D, Monaco C, Feldmann M, Takata M, Lever IJ, Nanchahal J, Fanselow MS, Maze M. 2010. Role of interleukin-1β in postoperative cognitive dysfunction. *Annals of Neurology* 68: 360–368.

Clary EM, O'Halloran EK, de la Fuente SG, Eubanks S. 2004. Videoendoscopic endotracheal intubation of the rat. *Laboratory animals* 38: 158–61.

Davidson AJ, Disma N, de Graaff JC, Withington DE, Dorris L, Bell G, Stargatt R, Bellinger DC, Schuster T, Arnup SJ, Hardy P, Hunt RW, Takagi MJ, Giribaldi G, Hartmann PL, Salvo I, Morton NS, von Ungern Sternberg BS, Locatelli BG, Wilton N, Lynn A, Thomas JJ, Polaner D, Bagshaw O, Szmuk P, Absalom AR, Frawley G, Berde C, Ormond GD, Marmor J, McCann ME. 2016. Neurodevelopmental outcome at 2 years of age after general anaesthesia and awake-regional anaesthesia in infancy (GAS): an international multicentre, randomised controlled trial. *The Lancet* 387: 239–250.

Deiner S, Silverstein JH. 2009. Postoperative delirium and cognitive dysfunction. *British Journal of Anaesthesia* 103: 41–46.

Dobbing J, Sands J. 1979. Comparative aspects of the brain growth spurt. *Early human development* 3: 79–83.

Fuentes JM, Hanly EJ, Bachman SL, Aurora AR, Marohn MR, Talamini MA. 2004. Videoendoscopic endotracheal intubation in the rat: A comprehensive rodent model of laparoscopic surgery. *Journal of Surgical Research* 122: 240–248.

Fuh E, De La Fuente S, Shah MK, Okodiko DK, Cummings TJ, Eubanks WS, Reynolds JD. 2005. Long-term behavioral assessment of guinea pigs following neonatal pneumoperitoneum. *Surgical Endoscopy And Other Interventional Techniques* 19: 715–719.

Gidron Y, Kupper N, Kwaijtaal M, Winter J, Denollet J. 2007. Vagus-brain communication in atherosclerosis-related inflammation: A neuroimmunomodulation perspective of CAD.

Atherosclerosis. 195: 1–9.

Goehler LE, Gaykema RPA, Hansen MK, Anderson K, Maier SF, Watkins LR. 2000. Vagal immune-to-brain communication: A visceral chemosensory pathway. *Autonomic Neuroscience: Basic and Clinical*. 85: 49–59.

González H, Elgueta D, Montoya A, Pacheco R. 2014. Neuroimmune regulation of microglial activity involved in neuroinflammation and neurodegenerative diseases. *Journal of Neuroimmunology* 274: 1–13.

Hanly EJ, Fuentes JM, Aurora AR, Bachman SL, De Maio A, Marohn MR, Talamini MA. 2006. Carbon dioxide pneumoperitoneum prevents mortality from sepsis. *Surgical endoscopy* 20: 1482–1487.

Hayakata T, Shiozaki T, Tasaki O, Ikegawa H, Inoue Y, Toshiyuki F, Hosotubo H, Kieko F, Yamashita T, Tanaka H, Shimazu T, Sugimoto H. 2004. Changes In Csf S100b And Cytokine Concentrations In Early-Phase Severe Traumatic Brain Injury. *Shock* 22: 102–107.

Hazebroek EJ, Haitsma JJ, Lachmann B, Steyerberg EW, Bruin RWF, Bouvy ND, Bonjer HJ. 2002. Impact of carbon dioxide and helium insufflation on cardiorespiratory function during prolonged pneumoperitoneum in an experimental rat model. *Surgical Endoscopy and Other Interventional Techniques* 16: 1073–1078.

Heijnen BHM, Elkhaloufi Y, Straatsburg IH, Van Gulik TM. 2002. Influence of acidosis and hypoxia on liver ischemia and reperfusion injury in an in vivo rat model. *Journal Of Applied Physiology* 93: 319–323.

Hirsch J, Vacas S, Terrando N, Yuan M, Sands LP, Kramer J, Bozic K, Maze MM, Leung JM. 2016. Perioperative cerebrospinal fluid and plasma inflammatory markers after orthopedic surgery. *Journal of Neuroinflammation* 13: 211.

Jacobi CA, Wenger F, Sabat R, Volk T, Ordemann J, Müller JM. 1998. The impact of laparoscopy with carbon dioxide versus helium on immunologic function and tumor growth in a rat model. *Digestive surgery* 15: 110–116.

Jevtovic-Todorovic V, Absalom AR, Blomgren K, Brambrink A, Crosby G, Culley DJ, Fiskum G, Giffard RG, Herold KF, Loepke AW, Ma D, Orser BA, Planel E, Slikker W, Soriano SG, Stratmann G, Vutskits L, Xie Z, Hemmings HC. 2013. Anaesthetic neurotoxicity and neuroplasticity: an expert group report and statement based on the BJA Salzburg Seminar. *British Journal of Anaesthesia* 111: 143–151.

Jones RO, Anderson NH, Murchison JT, Brittan M, Simon EJ, Casali G, Simpson AJ, Walker WS. 2014. Innate immune responses after resection for lung cancer via video-assisted thoracoscopic surgery and thoracotomy. *Innovations: Technology and Techniques in Cardiothoracic and Vascular Surgery* 9: 93–103.

Kalfa N, Allal H, Raux O, Lopez M, Forgues D, Guibal M-P, Picaud J-C, Galifer R-B. 2005. Tolerance of laparoscopy and thoracoscopy in neonates. *Pediatrics* 116: e785–e791.

Kastin AJ, Akerstrom V, Pan W. 2003. Interleukin-10 as a CNS therapeutic: The obstacle of the blood-brain/blood-spinal cord barrier. *Molecular Brain Research* 114: 168–171.

Kirchhoff C, Buhmann S, Bogner V, Stegmaier J, Leidel BA, Braunstein V, Mutschler W, Biberthaler P. 2008. Cerebrospinal IL-10 concentration is elevated in non-survivors as compared to survivors after severe traumatic brain injury. *European journal of medical research* 13: 464–468.

Konno K, Itano N, Ogawa T, Hatakeyama M, Shioya K, Kasai N. 2014a. New Visible Endotracheal Intubation Method Using the Endoscope System for Mice Inhalational Anesthesia. *Journal of Veterinary Medical Science* 76: 863–868.

Konno K, Shiotani Y, Itano N, Ogawa T, HAtakeyama M, Shioya K, Kasai N. 2014b. Visible, Safe and Certain Endotracheal Intubation Using Endoscope System and Inhalation Anesthesia for Rats. *Journal of Veterinary Medical Science* 76: 1375–1381.

De La Fuente S, Pinheiro J, Gupta M, Eubanks WS, Reynolds JD. 2003. Early postnatal behavior deficits after maternal carbon dioxide pneumoperitoneum during pregnancy: A study in a guinea pig model. *Surgical Endoscopy and Other Interventional Techniques* 17:

1823–1825.

Lacher M, Kuebler JF, Dingemann J, Ure BM. 2014. Minimal invasive surgery in the newborn: Current status and evidence. *Seminars in Pediatric Surgery* 23: 249–256.

Lasersohn L. 2011 Anaesthetic considerations for paediatric laparoscopy. *South African Journal of Surgery* 49: 22–26.

Liu H-F, Gao L, Liu T, Yan-Jiang, Wang H-B. 2016. Comparison of acute phase reaction and postoperative stress in pigs undergoing video-assisted thoracoscopic versus thoracotomy pneumonectomy. *Acta Veterinaria Scandinavica* 58: 75.

Liu X, Wu Y, Zhu J, Lv X, Tang Y, Sun J, Zhang S. 2014. Totally thoracoscopic repair of atrial septal defect reduces systemic inflammatory reaction and myocardial damage in initial patients. *European journal of medical research* 19: 13

Machado MCC, Coelho AMM, Martins JO, Sampietre SN, Molan NAT, Patzina RA, Machado MAC, Jancar S. 2010. CO2 Abdominal Insufflation Decreases Local and Systemic Inflammatory Response in Experimental Acute Pancreatitis. *Pancreas* 39: 175–181.

Maier SF, Goehler LE, Fleshner M, Watkins LR. The role of the vagus nerve in cytokine-to-brain communication. *Annals of the New York Academy of Sciences* 1998; Edited by Intergovernmental Panel on Climate Change. Cambridge: Cambridge University Press. 840: 289–300.

Mathew G, Watson DI, Ellis TS, Jamieson GG, Rofe AM. 1999. The role of peritoneal immunity and the tumour-bearing state on the development of wound and peritoneal metastases after laparoscopy. *The Australian and New Zealand journal of surgery* 69: 14–18.

Mesquita AR, Pêgo JM, Summavielle T, Maciel P, Almeida OFX, Sousa N. 2007. Neurodevelopment milestone abnormalities in rats exposed to stress in early life. *Neuroscience* 147: 1022–1033.

McHoney M, Mackinlay G, Munro F, Capek A, Aldridge L. 2008. Effect of patient weight and

anesthetic technique on CO2 excretion during thoracoscopy in children assessed by end-tidal CO2. *Journal of Laparoendoscopic & Advanced Surgical Techniques* 18: 147–151.

Molinas CR, Tjwa M, Vanacker B, Binda MM, Elkelani O, Koninckx PR. 2004. Role of CO2 pneumoperitoneum-induced acidosis in CO2 pneumoperitoneum-enhanced adhesion formation in mice. *Fertility and Sterility* 81: 708–711.

Moller JT, Cluitmans P, Rasmussen LS, Houx P, Rasmussen H, Canet J, Rabbitt P, Jolles J, Larsen K, Hanning CD, Langeron O, Johnson T. 1998. Long-term postoperative cognitive dysfunction in the elderly: ISPOCD1 study. *Lancet* 351: 857–861.

Mynbaev OA, Molinas CR, Adamyan L V., Vanacker B, Koninckx PR. 2002. Pathogenesis of CO2 pneumoperitoneum-induced metabolic hypoxemia in a rabbit model. *Journal of the American Association of Gynecologic Laparoscopists* 9: 306–314.

Neuhaus SJ, Watson DI, Ellis T, Rofe AM, Mathew G, Jamieson GG. 2000. Influence of gases on intraperitoneal immunity daring laparoscopy in tumor-bearing rats. *World Journal of Surgery* 24: 1227–1231.

Neunhoeffer F, Warmann SW, Hofbeck M, Müller A, Fideler F, Seitz G, Schuhmann MU, Kirschner HJ, Kumpf M, Fuchs J. 2017. Elevated intrathoracic CO2 pressure during thoracoscopic surgery decreases regional cerebral oxygen saturation in neonates and infants—A pilot study. *Paediatric Anaesthesia* 27: 752–759.

Ng CS, Wan IY, Yim AP. 2009. Impact of Video-Assisted Thoracoscopic Major Lung Resection on Immune Function. *Asian Cardiovascular and Thoracic Annals* 17: 426–432.

Pacilli M, Pierro A, Kingsley C, Curry JI, Herod J, Eaton S. 2006. Absorption of carbon dioxide during laparoscopy in children measured using a novel mass spectrometric technique. *British Journal of Anaesthesia* 97: 215–219.

Pan W, Kastin AJ. 1999. Penetration of neurotrophins and cytokines across the blood-brain/blood-spinal cord barrier. *Advanced Drug Delivery Reviews* 36: 291–298.

Perez-Barcena J, Ibáñez J, Brell M, Crespí C, Frontera G, Llompart-Pou JA, Homar J, Abadal

JM. 2011. Lack of correlation among intracerebral cytokines, intracranial pressure, and brain tissue oxygenation in patients with traumatic brain injury and diffuse lesions. *Critical care medicine* 39: 533–540.

Quan N, Banks WA. 2007. Brain-immune communication pathways. *Brain, Behavior, and Immunity* 21: 727–735.

Raison CL, Capuron L, Miller AH. 2006. Cytokines sing the blues: inflammation and the pathogenesis of depression. *Trends in Immunology* 27: 24–31.

Rappaport B a, Suresh S, Hertz S, Evers AS, Orser B. 2015. Anesthetic Neurotoxicity - Clinical Implications of Animal Models. *The New England journal of medicine* 372: 796–797.

Roque S, Mesquita AR, Palha JA, Sousa N, Correia-Neves M. 2014. The Behavioral and Immunological Impact of Maternal Separation: A Matter of Timing. *Frontiers in Behavioral Neuroscience* 8: 192.

Rosczyk HA, Sparkman NL, Johnson RW. 2008. Neuroinflammation and cognitive function in aged mice following minor surgery. *Experimental Gerontology* 43: 840–846.

Sanders RD, Hassell J, Davidson AJ, Robertson NJ, Ma D. 2013. Impact of anaesthetics and surgery on neurodevelopment: an update. *British Journal of Anaesthesia* 110: i53–i72.

Semple BD, Blomgren K, Gimlin K, Ferriero DM, Noble-Haeusslein LJ. 2013. Brain development in rodents and humans: Identifying benchmarks of maturation and vulnerability to injury across species. *Progress in Neurobiology* 106–107: 1–16.

Shiozaki T, Hayakata T, Tasaki O, Hosotubo H, Fuijita K, Mouri T, Tajima G, Kajino K, Nakae H, Tanaka H, Shimazu T, Sugimoto, H. 2005. Cerebrospinal Fluid Concentrations of Anti-Inflammatory Mediators in Early-Phase Severe Traumatic Brain Injury. *Shock* 23: 406–410.

Stolwijk LJ, Tytgat SHAJ, Keunen K, Suksamanapan N, van Herwaarden MYA, Groenendaal F, Lemmers PMA, van der Zee DC. 2015. The effects of CO2-insufflation with 5 and 10 mmHg during thoracoscopy on cerebral oxygenation and hemodynamics in piglets: an

animal experimental study. *Surgical Endoscopy* 29: 2781–2788.

Stolwijk LJ, van der Zee DC, Tytgat S, van der Werff D, Benders MJNL, van Herwaarden MYA, Lemmers PMA. 2017. Brain Oxygenation During Thoracoscopic Repair of Long Gap Esophageal Atresia. *World Journal of Surgery* 41: 1384–1392.

Sümpelmann R, Schuerholz T, Marx G, Härtel D, Hecker H, Ure BM, Jesch NK. 2006. Haemodynamic, acid-base and blood volume changes during prolonged low pressure pneumoperitoneum in rabbits. *British journal of anaesthesia* 96: 563–568.

Sun LS, Li G, Miller TLK, Salorio C, Byrne MW, Bellinger DC, Ing C, Park R, Radcliffe J, Hays SR, DiMaggi CJ, Cooper TJ, Rauh V, Maxwell LG, Youn A, McGowan FX. 2016. Association Between a Single General Anesthesia Exposure Before Age 36 Months and Neurocognitive Outcomes in Later Childhood *JAMA* 315: 2312–2320.

Tang JX, Mardini F, Janik LS, Garrity ST, Li RQ, Bachlani G, Eckenhoff RG, Eckenhoff MF. 2013. Modulation of Murine Alzheimer Pathogenesis and Behavior by Surgery. *Annals of Surgery* 257: 439–448.

Terrando N, Eriksson LI, Kyu Ryu J, Yang T, Monaco C, Feldmann M, Jonsson Fagerlund M, Charo IF, Akassoglou K, Maze M. 2011. Resolving postoperative neuroinflammation and cognitive decline. *Annals of Neurology* 70: 986–995.

Terrando N, Monaco C, Ma D, Foxwell BMJ, Feldmann M, Maze M. 2010. Tumor necrosis factor triggers a cytokine cascade yielding postoperative cognitive decline. *Proceedings of the National Academy of Sciences* 107: 20518–20522.

Tytgat SH, van Herwaarden MYA, Stolwijk LJ, Keunen K, Benders MJNL, de Graaff JC, Milstein DMJ, van der Zee DC, Lemmers PMA. 2016. Neonatal brain oxygenation during thoracoscopic correction of esophageal atresia. *Surgical Endoscopy* Springer US. 30: 2811–2817.

Tytgat SH, Stolwijk LJ, Keunen K, Milstein DMJ, Lemmers PMA, van der Zee DC. 2015. Brain Oxygenation During Laparoscopic Correction of Hypertrophic Pyloric Stenosis. *Journal*

of Laparoendoscopic & Advanced Surgical Techniques 25: 352–357.

Tytgat SH, Van Der Zee DC, Ince C, Milstein DM. 2013. Carbon dioxide gas pneumoperitoneum induces minimal microcirculatory changes in neonates during laparoscopic pyloromyotomy. *Surgical Endoscopy and Other Interventional Techniques* 27: 3465–3473.

Veekash G, Wei LX, Su M. 2010. Carbon dioxide pneumoperitoneum, physiologic changes and anesthetic concerns. *Ambulatory Surgery* 16: 41–46.

Vergari A, Polito A, Musumeci M, Palazzesi S, Marano G. 2003. Video-assisted orotracheal intubation in mice. *Laboratory Animals* 37: 204–206.

Worthley SG, Roque M, Helft G, Soundararajan K, Siddiqui M, Reis ED. 2000. Rapid oral endotracheal intubation with a fibre-optic scope in rabbits: a simple and reliable technique. *Laboratory animals* 34: 199–201.

Zani A, Lamas-Pinheiro R, Paraboschi I, King SK, Wolinska J, Zani-Ruttenstock E, Eaton S, Pierro A. 2017. Intraoperative acidosis and hypercapnia during thoracoscopic repair of congenital diaphragmatic hernia and esophageal atresia/tracheoesophageal fistula. *Paediatric Anaesthesia* 27: 841–848.