Reduction of perioperative inflammatory reaction with exogenous methane

Ph.D. Thesis

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FULL PAPERS RELATED TO THE SUBJECT OF THE THESIS

1. Bari G, Érces D, Varga G, Szűcs S, Varga Z, Bogáts G, Boros M. Methane inhalation reduces the systemic inflammatory response in a large animal model of extracorporeal circulation. Eur J Cardiothoracic Surgery 2019. doi:10.1093/ejcts/ezy453. **IF: 3.5**

2. Bari G, Érces D, Varga G, Szűcs S, Bogáts G. A pericardialis tamponád kórélettana, klinikuma és állatkísérletes vizsgálati lehetőségei.
[Pathophysiology, clinical and experimental possibilities of pericardial tamponade]. Orv Hetil. 2018. 159:163–167 **IF: 0.3**

3. Bari G, Szűcs S, Érces D, Ugocsai M, Bozsó N, Balog D, Boros M, Varga G. A cardiogen sokk modellezése pericardialis tamponáddal [Experimental model for cardiogenic shock with pericardial tamponade]. Magy Seb. 2017. 70:297–302. **IF: 0**

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5. Bari G, Szűcs S, Érces D, Boros M, Varga G. Experimental pericardial tamponade - translation of a clinical problem to its large animal model. Turk J Surg. 2018. 34(3):205-211. **IF: 0.1**

ABSTRACTS RELATED TO THE SUBJECT OF THE THESIS

I. **Bari G**, Varga G, Szűcs S, Gules M, Kaszaki J, Boros M, Érces D. Methane inhalation decreases mucosal mast cell degranulation in a large animal model of cardiogenic shock. Abstract Book, 17th Congress of the European Shock Society, Paris, 13–15 September 2017.

II. **Bari G**, Érces D, Szűcs S, Gules M, Gyaraki P, Szilágyi ÁL, Hartmann P, Boros M, Varga G. Improved platelet function after methane inhalation in a large animal model of obstructive circulatory shock. Abstract Book, 53rd ESSR Congress, Madrid, 2018, p. 87 (https://www.essr2018.com/abstracts)

1. INTRODUCTION

1.1. Gasotransmitters and methane

Many gases in the Earth's atmosphere, such as oxygen (O_2) and carbon dioxide (CO_2), are physiologically important or biologically irreplaceable. A number of other gases that occur naturally, such as nitric oxide (NO), carbon monoxide (CO) and hydrogen sulphide (H_2S), are considered "gasotransmitters", with diverse effects on the basal functions of aerobic cells. According to current views, a member of this category of gas must adhere to four characteristics (simplicity, availability, volatility and effectiveness) and meet six criteria: (a) small molecules, (b) freely permeable to membranes, (c) endogenously generated in mammalian cells with specific substrates and enzymes, (d) serve well-defined specific functions at physiologically relevant concentrations, (e) functions can be mimicked by their exogenously applied counterparts, and (f) have specific cellular and molecular targets (R. Wang et al. 2014). In general, gasotransmitters play vital regulatory roles in maintaining homeostasis in the human body through distinct biochemical pathways. Biotic CH_4 formation takes place through anaerobic fermentation by a unique class of prokaryotes (archaea), and the catalyzing enzyme of this pathway is methyl co-enzyme M reductase, with the use of CO_2 and H_2 as main substrates (Eckburg et al. 2005). An anti-inflammatory potential for CH_4 was first reported in a canine model of intestinal ischaemia-reperfusion (I-R) (Boros et al. 2012), and thereafter other reports have confirmed the antioxidant and anti-apoptotic effects of exogenous CH_4 administration in various *in vitro* and *in vivo* settings (Ye et al. 2015; Liu et al. 2016; Chen et al. 2016; Li et al., 2019). The exact mechanism of action is not fully clarified, but it has been shown that the anti-inflammatory action is mediated through the inhibition of polymorphonuclear (PMN) leukocyte activation and reduced reactive oxygen species (ROS) production (Boros et al. 2012). Other experimental data have also shown that the biological effects are related to the inhibition of xanthine oxidoreductase (XOR) activity as well, thus lowering the amount of superoxide radical formation (Poles et al. 2018), The available data on the possible pathways of CH_4 effects (i.e. anti-inflammatory, antioxidant and anti-apoptotic) in various *in vitro* and *in vivo* experimental models were recently reviewed (Mészáros, 2017).

1.2. The inflammatory consequences of cardiac surgery

Low cardiac output (CO) states, such as pericardial tamponade (PT), post-cardiotomy cardiac stunning and perioperative myocardial infarction, often lead to cardiogenic shock, with a high risk of morbidity and mortality. Nevertheless, permanently low CO, even without definite signs of cardiogenic shock, can induce microcirculatory impairment in peripheral tissues, triggering local and later systemic inflammatory responses. Low CO states like PT have a strong impact on mesenteric circulation by

triggering local splanchnic hypoxia due to endogenous vasoconstriction (Kaszaki et al. 1989). A diverse system of cascades, including pro-inflammatory cytokines and complement factors, enhances mucosal leukocyte recruitment and amplifies the local inflammatory response.

Despite the safety of modern CPB circuits, extracorporeal circulation (ECC) is still associated with some degree of post-operative inflammatory activation, similar to the septic systemic inflammatory response (SIRS) (Day et al. 2005; Millar et al. 2016). It has been shown that blood contact with the foreign surface of the CPB circuit activates humoral and cellular factors (Millar et al. 2016), leading to the intrinsic activation of the coagulation cascade. Due to the routine use of heparin before initiation of ECC, thrombin and fibrin complexes are not formed, but the first steps of the coagulation cascade are activated along with other pro-inflammatory cascades, such as the kinin–kallikrein system or the complement system, triggering a wide variety of cellular systems, such as endothelial cells, leukocytes, thrombocytes and mast cells (Day et al. 2005). If these cascade activations are dysregulated due to prolonged CPB time or metabolic changes, significant tissue and organ damage can occur in sensitive organs such as the kidneys and intestines. Therefore, a search for novel therapeutic strategies to reduce post-CPB inflammatory damage is an important clinical and scientific research goal.

1.3. In vivo models of PT- and CBP-induced SIRS

In a seminal paper, Lluch et al. described a canine model of cardiogenic shock, where the left ventricular function was reduced by coronary embolization (Lluch et al. 1969). Since then, numerous refinements have been made to study the immediate and late macro- and microhaemodynamic consequences, but a reproducible, well-controlled and stable experimental setting is still needed. The *in vivo* reduction of myocardial function can be carried out relatively easily by selective coronary ligation or embolization, but the technique is accompanied by high mortality due to malignant arrhythmias. On the other hand, it is possible to reduce cardiac output by obstructing ventricular filling and lowering the left ventricular stroke volume, a condition that can be achieved by inducing PT. Our research group has previously demonstrated that PT is indeed an appropriate model to study the pathomechanism of cardiogenic shock (Kaszaki et al. 1989). These experiments were carried out on anaesthetized, mechanically ventilated dogs, and it has been shown that peripheral haemodynamics and vasoactive humoral responses could be monitored in sufficient detail (Kaszaki et al. 1989). In this setup, PT is induced by infusion of saline through a cannula placed in the pericardium via thoracotomy, and the progression could be immediately terminated by fluid extraction from the pericardium. This has thus become a standard model for studying the consequences of reversible cardiogenic shock (Starr et al.

1982). The inherent disadvantage of the model is thoracotomy itself and the possibility of impaired lung function.

The technological development of human ECC devices capable of providing sufficient perfusion was based on a variety of *in vivo* experiments and animal trials. Since then, many refinements have been made to perfect the CBP technique for clinical use; however, basic problems, such as inflammatory reactions and cellular damage to circulating blood, have yet to be solved. Besides the technological needs of ECC circuit development, the basic pathophysiological problems require the use of well-established, stable animal models. Due to the high costs of CBP components and supplemental expenses, *in vivo* ECC studies are performed in only few research institutions. Swine, dogs, cows and sheep are usually employed as models (Jungwirth et al. 2010), but the general disadvantage of heavy equipment use and the requirement of a full-scale operative environment remain. It would be possible to perform small animal experiments, using rodents (Fujii et al. 2015), to reduce costs, time and the need for manpower, but there are many other disadvantages, including species differences and the operative challenges in such small scales. In this sense, the porcine model of CPB is still considered the best platform for the translational development of novel organ protection strategies.

2. MAIN GOALS

Cardiac surgery-related perioperative inflammatory activation is a significant clinical problem, and there is a clear need for efficient adjuvant therapies to influence the outcome of such events. Our first aim was to develop and design suitable *in vivo* models, where the characteristics of the postoperative clinical syndrome can be examined under standardized conditions.

1. A main disadvantage of the currently used large animal PT models is thoracotomy itself with impaired lung function and extended wound surface with a high risk of bleeding complication, and lung and tissue injury. Our aim was to design an experimental procedure to model iatrogenic PT, where thoracotomy can be avoided, but the situation is realistic, similar to the clinical appearance.
2. Secondly, we aimed to characterize the ECC-induced haemodynamic and microcirculatory consequences in a clinically relevant larger animal model with anatomical and immunological similarities to humans.
3. Previous evidence demonstrated the anti-inflammatory capacity of CH_4 with the possibility of modulating the pathways leading to oxidative stress. Therefore, our next aim was to outline the intestinal effects of CH_4 inhalation in a standardized large animal model of experimental PT. For this purpose, normoxic CH_4 was administered through

the ventilator of anaesthetized minipigs during PT-induced non-occlusive splanchnic I-R.

4. We also aimed to investigate whether the systemic inflammatory response caused by ECC could be modified with exogenous CH_4. The additional aim of this study was to determine whether CH_4 administration would influence the development of post-CPB kidney dysfunction. We investigated this hypothesis in anaesthetized minipigs with centrally cannulated ECC with normoxic CH_4 added to the oxygenator gas sweep.

3. MATERIALS AND METHODS

3.1. Experimental animals

28 male outbred Vietnamese minipigs (weighing 45±8 kg) were used (n=18 in *Study I*, n=10 in *Study II*). The experiments were performed in accordance with National Institutes of Health guidelines on the handling and care of experimental animals and EU Directive 2010/63 on the protection of animals used for scientific purposes. The studies were approved by the Animal Welfare Committee of the University of Szeged (approval number: V/148/2013).

3.2. Anaesthesia and haemodynamic monitoring

A mixture of ketamine (20 mg kg^{-1}) and azaperone (2 mg kg^{-1}) was administered intramuscularly. Anaesthesia was maintained with a continuous infusion of propofol (50 µl $min^{-1}kg^{-1}$ *iv*; 6 mg $kg^{-1}h^{-1}$; Propofol), midazolam (1.2 mg $kg^{-1}h^{-1}$) and fentanyl (0.02 mg $kg^{-1}h^{-1}$), while muscle relaxation was maintained with pipecuronium (single administration, 4 mg dose). After endotracheal intubation, mechanical ventilation was started with a tidal volume of 10 ml kg^{-1}, and the respiratory rate was adjusted to maintain the end-tidal pressure of CO_2 and partial pressure of CO_2 in the 35–45 mmHg ranges. The left jugular vein was cannulated for fluid and drug administration. The left femoral artery was cannulated for the measurement of mean arterial pressure (MAP), heart rate (HR) and CO by transpulmonary thermodilution (PiCCO Catheters; PULSION Medical Systems, Feldkirchen, Germany). MAP, HR and CO data were recorded, while pressure signals (BPR-02 transducer; Experimetria Ltd, Budapest, Hungary) and superior mesenteric artery (SMA) flow signals (T206 Animal Research Flowmeter; Transonic Systems Inc., Ithaca, NY, USA) were measured continuously and registered with a computerized data-acquisition system. Urine output was assessed by surgically placing a urinary catheter in the bladder via the femoral incision. The urine was collected and measured at the end of the observation period to calculate average hourly diuresis. Ringer's lactate was given at a rate of 10 $mlkg^{-1}h^{-1}$. After a median laparotomy, a flow probe was placed around the SMA

(Transonic Systems Inc., Ithaca, NY, USA). The wound in the abdominal wall was temporarily closed thereafter with clips.

3.3. Surgical interventions and experimental protocol in Study I

The animals were randomly allocated into three experimental groups. The diaphragm was accessed through a median laparotomy. A 3-cm incision was made at the sternal part, avoiding the muscular region of the diaphragm. The pericardium was opened, and a cannula was fixed into the pericardial cavity with a pledgeted purse-string suture. The tamponade was induced and maintained for 60 min by intrapericardial administration of heparinized own blood (100±50 ml), and MAP was maintained at 40–45 mmHg. The abdominal wall was closed with surgical clips during the observation period and between microcirculatory image recordings. Group 1 (n=6) served as a sham-operated control, with the same surgical interventions, time frame and sampling as in Group 2 (n=6) but without the induction of PT. Group 3 received inhaled 2.5% v/v normoxic CH_4 (Linde Gas, Hungary) in the last 5 minutes of PT and for an additional 10 min after PT. In Groups 2 and 3, after the end of 60-min PT, the blood was released from the pericardial sac and the animals were monitored for 180 min. Blood gases and haemodynamic parameters were measured every 30 min. An *in vivo* histological examination on the ileal mucosa and detection of PMN leukocytes were performed at baseline, 30 min after the relief of PT (90 min) and at the end of the experiments (240 min). Tissue biopsies were harvested at the baseline, 30 min after the relief of PT (90 min) and at the end of the experiments (240 min). Blood samples were taken at the baseline 30 min after the relief of PT (90 min) and at the end of the experiments (240 min).

3.4. Surgical interventions and experimental protocol in Study II

The animals (n=10) were randomly allocated into two experimental groups (n=5, each) (Figure 5). A median sternotomy was performed and the pericardium dissected. The aorta was dissected free and circled with tape. After the administration of heparin (2000 IU iv) and activated clotting time (ACT) control (ACT > 500 sec), purse-string sutures were placed in the ascending aorta and the right atrial appendage, and standard central CPB cannulation was performed. Cardiotomy suction was not employed because the aorta or the cardiac chambers were not open. After the baseline haemodynamic measurements, CPB was initiated. In addition, the cessation of the respiration achieved maximal peak blood and cellular CH_4 concentration in the treated animals. In both groups, CPB was maintained for 120 min and adjusted throughout according to the CO calculated for the animals. The non-treated CPB group (n=5) received a standard air–oxygen gas mixture with 0.6 FiO_2 at a flow rate of 2 l min^{-1} to the oxygenator. In the CH_4-treated (CBP+Met) group, 2.5% v/v normoxic CH_4 (Linde Gas, Hungary) was added to the oxygenator gas sweep at a rate of 1

l min^{-1}. FiO_2 values were adjusted to the post-oxygenator PaO_2 values. The CH_4 content of the blood was monitored by near-infrared laser technique-based photoacoustic spectroscopy after 60 min of CH_4 treatment. The duration of CPB and the post-CPB times did not differ between the groups. The post-CPB haemodynamic stability (MAP > 60 mmHg) of the animal was maintained by continuous *iv* volume replacement (Ringer's lactate solution) and by maintaining the systemic vascular resistance with perfusion of norepinephrine if necessary (0.05–0.35 μg kg^{-1} h^{-1}).

3.5. Microcirculatory measurements

An intravital orthogonal polarization spectral imaging technique (Cytoscan A/R; Cytometrics, Philadelphia, PA) was used for non-invasive visualization of the mucosal microcirculation of ileum. Red blood cell velocity (RBCV, μm/s) changes in the capillary were determined in three separate fields by means of a computer-assisted image analysis system (IVM Pictron, Budapest, Hungary).

3.6. In vivo detection of mucosal damage

The extent of damage to the ileal mucosa was evaluated by means of fluorescence confocal laser scanning endomicroscopy (CLSEM) developed for *in vivo* histology. The ileal mucosal structure was recorded after the topical application of the fluorescent dye acriflavine (Sigma-Aldrich Inc., St. Louis, MO, USA). The changes in the mucosal architecture were examined with a semi-quantitative scoring system as described previously (Kovács et al. 2012).

3.7. Whole blood superoxide production and tissue leukocyte accumulation measurement

The chemiluminometric method developed by Zimmermann et al. (Zimmermann et al. 1991) was used for the whole blood superoxide production measurements. Being a marker of tissue leukocyte infiltration, MPO activity was measured on the pellet of the homogenate by Kuebler's method (Kuebler et al.1996).

3.8. XOR activity

XOR activity was determined in the ultrafiltered, concentrated supernatant with a fluorometric kinetic assay based on the conversion of pterine to isoxanthopterine in the presence (total XOR) or absence (XO activity) of the electron acceptor methylene blue (Beckman et al. 1989).

3.9. Histology for light microscopy of leukocytes and mast cells

Full-thickness ileal biopsies taken at the end of the experiments were analysed in each group. The tissue was fixed in 6% buffered formalin, embedded in paraffin, cut into 4-μm-thick sections and stained with haematoxylin and eosin. The infiltration of leukocytes was detected and the number of leukocytes counted in at least 20 fields of view at an original

magnification of 400x. Intestinal biopsy samples for light microscopy were rapidly placed into ice-cold Carnoy's fixative and trimmed along the longitudinal axis. The fixed tissue was attached to a hard cardboard backing to ensure the optimal longitudinal direction of the section. The samples were embedded in paraffin, sectioned and stained with haematoxylin-eosin, acidic toluidine blue (pH 0.5) or alcian blue-safranin O (pH 0.4). MCs stained positively were quantitated in the villi of an average of 20 villus-crypt units. The counting was performed in coded sections at 400 optical magnifications by one investigator.

3.10. Measurement of blood CH_4 content

A near-infrared laser technique-based photoacoustic spectroscopy apparatus (Tuboly et al. 2013) was employed to confirm the presence of exogenous CH_4 in the blood. The CH_4 values were corrected for background levels and expressed in ppm.

3.11. Statistical analysis

Data analysis was performed with a statistical software package (SigmaStat for Windows, Jandel Scientific, Erkrath, Germany). Friedman repeated measures analysis of variance on ranks was applied within groups. Time-dependent differences from the baseline for each group were assessed by Dunn's method. The differences between groups were analysed with the Mann–Whitney test. In the figures, median values and 75^{th} and 25^{th} percentiles are given; p values < 0.05 were considered significant.

4. RESULTS

4.1. Results of Study I

4.1.1. Changes in haemodynamic parameters

MAP remained at 40–45 mmHg as previously planned throughout the 60 min of PT, accompanied by a concomitantly decreased CO and elevated HR (sham: M=2.4; p25=2.3; p75=2.6 L/min/m^2; PT: M=1.2; p25=1.2; p75=1.4 L/min/m^2; PT+CH_4: M=1.1; p25=1.1; p75=1.2 L/min/m^2). After the release of PT, MAP started to increase. However, by the end of the observation period, it was significantly lower (M=73; p25=70; p75=80 Hgmm) compared to the baseline values and to the MAP for the sham-operated group. No significant difference was observed between the PT and PT + CH_4-treated groups. During the post-tamponade phase, the CO returned to the baseline values and there was no significant difference between the Sham (M=2.4; p25=2.1; p75=2.7 L/min/m^2), PT (M=2.1; p25=2; p75=2.3 L/min/m^2) and PT + CH_4-treated groups (M=2.1; p25=1.9; p75=2.2 L/min/m^2). The same changes could be observed in the HR values in the Sham (M=75.8; p25=66.3; p75=85 1/min), PT (M=118.8; p25=108.5; p75=144.1 1/min) and PT + CH_4-treated groups (M=114; p25=108; p75=140.5 1/min). The decreased venous return was evidenced by a significant elevation of CVP throughout the PT phase (*data not shown*). The

SMA flow showed a significant drop in the PT phase as expected in non-treated (M=192.2; p25=168.9; p75=245.9 ml/min) and PT+CH_4-treated groups (M=175.2; p25=138.5; p75=263.5 ml/min) compare to control values and sham group (M= 439.2; p25=400; p75=460.8 ml/min). After the release of PT, the SMA flow dramatically increased but did not reach the baseline values. Differences in SMA flow between CH_4-treated and non-treated groups were not observed.

4.1.2. Changes in microcirculation

The RBCV did not change in the sham group (M=471; p25=411; p75=543 μm/sec), while a significant decrease from the baseline was detected in the PT (M=379; p25=353; p75=488 μm/sec) and PT + CH_4-treated groups (M=400; p25=328; p75=440 μm/sec). At the end of the observation period, the CH_4-treated group showed a significant increase (M=522; p25=463; p75=557 μm/sec) in RBCV compared to the non-treated PT group (M=369; p25=318; p75=402 μm/sec).

4.1.3. Intestinal leukocyte accumulation

The leukocyte accumulation in the ileal tissue samples increased in the PT group (M=55; p25=42; p75=65 leukocyte/viewfield) compared to the sham group (M=30; p25=24; p75=35 leukocyte/viewfield). The CH_4-treated group showed a significant decrease (M=34; p25=27; p75=38 leukocyte/viewfield) in the leukocyte accumulation compared to the non-treated PT group.

4.1.4. Mucosal mast cell degranulation

The mast cell degranulation of the ileal tissue samples significantly increased after 90 minutes in the PT group (M=19.4; p25=17.4; p75=21.3%) compared to sham group (M=10.7; p25=9.7; p75=11.7%) and remained significantly high at the end of the observation period. This increase of degranulation was not observed in the CH_4-treated PT group at 90 minutes (M=10.9; p25=10; p75=13%) or at 240 minutes of the protocol.

4.1.5. In vivo histology

The mucosal morphology was examined with the intravital CLSEM technique in real time. The epithelial morphology of the small intestinal mucosa presented normal morphological patterns in the sham animals and in the baseline samples of the PT group and CH_4-treated group, while 30 min after PT induction longitudinal fissures appeared and partial epithelium defects were detected in the non-treated PT group. The lack of epithelium was extended in the end of observation. This extent of damage was not observed in the CH_4-treated PT group at 90 minutes or at 240 minutes. The scores of the mucosal damage were significantly higher in both non-treated and CH_4-treated groups at 90 min and 240 min of the experiment. However, CH_4 treatment significantly decreased the score of mucosal damage compared to the non-treated PT group.

4.1.6. Whole blood superoxide content

The superoxide content of the blood showed no difference in the baseline values for the three groups. After 30 min of the release of PT, the amount of superoxide significantly increased in the non-treated group (M=28366; p25=16558; p75=42792 RLU) compared to the sham group (M=6523; p25=2546; p75=14432 RLU). In the CH_4-treated group (M=2946; p25=2313; p75=10473 RLU), this increase was not observed. At 240 min significant differences in superoxide content could not be shown between the groups.

4.2. Results of Study II

4.2.1. Changes in renal arterial blood flow and hourly diuresis

A significantly decreased RA flow was detected during the CPB and post-CPB periods. CH_4 addition through the oxygenator resulted in significantly higher renal blood flow (M=77; p25=66; p75=79 ml/min) during the CPB period in contrast to the non-treated group (M=39; p25=36; p75=44 ml/min). The hourly diuresis was significantly higher in the CH_4-treated group (M=1.1; p25=0.7; p75=1.4 ml/kg/h) than in the animals without treatment (M=0.4; p25=0.3; p75=0.6 ml/kg/h).

4.2.2. Changes in blood CH_4 level

The photoacoustic spectroscopy data showed a 1.4 ppm increase in CH_4 concentration in the aortic blood sample above the background CH_4 level. The CH_4 concentration, measured from the samples from the jugular vein, was significantly lower compared to the CH_4 levels in the aortic samples.

4.2.3. Changes in MPO activity

The rate of neutrophil accumulation in the intestinal, cardiac and renal tissue was determined by measurement of MPO activity. Intestinal MPO activity was significantly lower in the CPB–CH_4 group (M=2.2; p25=2.1; p75=2.5 mU/(mg protein)) in contrast to the non-treated group (M=3.3; p25=2.6; p75=3.5 mU/(mg protein)). The MPO level in the cardiac and renal tissue showed no differences between the two groups.

4.2.4. Changes in XOR activity

In the non-treated group, the XOR activity in all examined tissues was significantly higher 2 h after the CPB period (ileal: M=5.4; p25=4.3; p75=10.4 pmol/min/mg; cardiac: M=4.0; p25=2.9; p75=6.0 pmol/min/mg; renal: M=9.7; p25=8.4; p75=21.6 pmol/min/mg) compared to the values for the group with exogenous CH_4 administration (ileal: M=2.6; p25=1.5; p75=2.7 pmol/min/mg; cardiac: M=1.7; p25=1.1; p75=1.9 pmol/min/mg; renal: M=1.1; p25=0.45; p75=1.9 pmol/min/mg).

4.2.5. Changes in blood superoxide production

Whole blood superoxide production was reduced by CH_4 administration at the end of the experiment (M=543; p25=388; p75=728 RLU) in contrast with the control CPB

group (M=1301; p25=1089; p75=1458 RLU).

4.2.6. Norepinephrine demand

During the post-CPB period, norepinephrine was administered to maintain a minimum of 60 mmHg MAP. We found significant differences between the inotropic demands of the 2 experimental groups. The CH_4-treated group required significantly less norepinephrine support (M=0.08; p25=0.06; p75=0.14 µg/kg/min) compared to the non-treated group (M=0.25; p25=0.23; p75=0.33 µg/kg/min).

5. DISCUSSION

5.1. Study I

In the first protocol we investigated the overall characteristics and the mesenteric consequences of temporary mechanical compression of the heart leading to microcirculatory I-R of the splanchnic system. A next, major forward step was the elimination of thoracotomy and the exclusive use of laparotomy for intestinal monitoring and pericardial cannula insertion (Bari et al. 2017). In our new model, pledgets are placed in the pericardial purse-string suture line to seal the possible leaking points. This technique minimizes surgical trauma and may contribute to the stability of the experimental setting. Furthermore, we defined the importance of choosing the proper type of fluid to fill the pericardial sac. In the case of saline, the risk of fluid leakage and thus experimental instability is high; the technique was thus changed to the most relevant colloid, heparinized own blood drawn from the central venous line. The model is stable and repeatable, and these qualities may make it a good tool in the cardiovascular research arsenal. The induction of PT significantly influenced the macrohaemodynamics. MAP and CO decreased, while CVP and HR rose. As a characteristic consequence of low CO, the mesenteric circulation deteriorated and the dramatic decrease of the SMA flow indicated the non-homogenous pattern of circulatory redistribution. The parallel *in viv*o detection of the microcirculation provided evidence for the decreased intestinal intramural perfusion. As expected, the haemodynamic disturbances were improved after the relief of the PT, but MAP and CO did not return to the normal baseline level. This circulatory state can be considered transitory, possibly returning to pre-PT values after longer observation times. We detected no differences after CH_4 treatment in the macrohaemodynamics as compared to the non-treated control group. However, the RBCV in the mucosa significantly improved in the CH_4-treated group 180 min after the release of PT. These changes might be a consequence of the reduced pro-inflammatory activation, including the degranulation of MMCs, which might have an influence on peripheral vascular resistance. Indeed, a higher proportion of activated mast cells were observed in the non-treated PT group 30 min after the relief of PT, and the same

histological picture was seen after 240 min. In parallel, the accumulation of PMN leukocytes was also significant as a direct consequence of I-R. In line with the microcirculatory changes at 30 min after the release of PT, intravital CLSEM data also showed the first signs of structural damage of the mucosa. In line with the reduction of the presence and activation of pro-inflammatory cells, the extent of the structural damage to the mucosa was much lower and the content of superoxide in the blood was also reduced by CH_4 administration. The available data on possible pathways of CH_4 effects (i.e. anti-inflammatory, antioxidant and anti-apoptotic) in various *in vitro* and *in vivo* experimental models were recently reviewed (Mészáros et al. 2017). It has been suggested that many effects can be explained with the physico-chemical properties of the non-polar gas. Until now no specific receptors or enzymes have been found mediating the CH_4 effects. However, there is evidence that CH_4 attenuates ROS production through the activation of antioxidant enzymes via the Nrf2-ARE pathway (L. Wang et al. 2017). As a result of Nrf2-ARE, the activation of downstream enzymes (i.e. haem oxygenase-1, catalase and superoxide dismutase) and lower production of ROS is expected, leading to anti-inflammatory effects. In addition to these intracellular signalling pathways, the mitochondrial electron transport system is believed to be a potential target of CH_4. Strifler et al. reported that leak mitochondrial respiration improved in a medium containing 2.2% CH_4–air mixture (Strifler et al. 2016). These potential effects of CH_4 can be explained if CH_4 dissolves in mitochondrial membranes. It may thus influence membrane rigidity or change the conformity of trans-membrane proteins in the respiratory chain, leading to alterations in ROS production. Therefore, the reduction of the source of ROS, including a decreased level of leucocyte and mast cell activation, may be associated with improved microcirculation and the overall better structural status of the mucosal surface.

5.2. Study II

In our model, we present a novel method of delivering an effective anti-inflammatory gas to improve the consequences of SIRS after CBP. The aim was to simulate a centrally-cannulated extracorporeal bypass circuit for a relatively short period of time. The cannulation of CPB and that of central VA-ECMO are in this case nearly identical, but the air–blood interface in the venous reservoir, the use of an occlusive roller pump, the duration of the extracorporeal circulation, the degree of haemodilution and the lack of heparin-coated circuits rather resemble CPB. However, based on the practical experience that can be derived from the model, we can say that the induction of the inflammatory reaction due to extracorporeal circulation takes less time and the reaction is more pronounced as compared to the use of a less damaging ECMO circuit. We have shown that this technique is stable and repeatable and provides a clinically relevant animal model to

study the consequences of CPB-induced inflammatory reactions. Renal function is frequently affected by CPB and there is therefore a constant search for renal protection methods. In the present study, we observed a significant decrease in renal arterial flow during and after CPB, and, despite a decrease, the flow was significantly higher in the CH_4-treated group than without the addition of CH_4. The increase also had a functional effect, as the hourly diuresis remained in the low normal range in the CH_4-treated group as compared to the oliguria in the animals which had not received CH_4 treatment. According to findings reported by Vogel et al. (Vogel et al. 2015), superoxide is capable of directly increasing Ca^{2+} influx in the smooth muscle cells of renal afferent arterioles, resulting in vasoconstriction, which may cause decreased renal blood flow. The establishment of CPB inevitably leads to a certain degree of hypoperfusion and activation of superoxide-producing enzymes such as NADPH-oxidase and XOR (McDonald et al. 2014). The significance of the effect of CH_4 on oxidative stress enzymes is emphasized by the lower superoxide level in the blood samples in the CH_4-treated group. Another important finding of our study is the decreased XOR activity in the cardiac tissue after the addition of CH_4. During tissue ischaemia and reperfusion, XOR is a significant source of superoxide and a known contributor to oxidative stress. In the present study, CH_4 inhalation significantly decreased renal XOR activity, thus possibly exerting an effect on renal function similar to that of allopurinol. According to arterial and venous data, approximately 2/3 of the administered exogenous CH_4 was distributed across the body. Since decreased XOR activity was detected in the cardiac and small intestinal samples as well, this points to a systemic, non-organ-specific effect of CH_4. We suggest that the tissue-specific differences are due to the barrier nature and more pronounced immune function of the intestinal mucosa. Here, the baseline MPO values are approx. 2 magnitudes higher as compared to those of other tissues. On the other hand, as a shock organ, the GI tract is more exposed to the harmful effects of low flow and hypoxia caused by redistributed circulation during and after CPB; therefore, inflammatory activation may also be present on a much larger scale and in an earlier time frame. It should be noted that the inotropic demand was also significantly lower in CH_4-treated animals. Norepinephrine can influence renal perfusion; however, the doses that were administered in this study should not cause impairment of the renal circulation (Bellomo et al. 2001). Moreover, Schaer et al. (Schaer et al. 1985) reported that norepinephrine treatment increased renal blood flow in dogs until the peak dose of 1.6 mg/kg/min, while the largest dose used in the present study was 0.35 mg/kg/min. Further, the effect of CH_4 treatment on renal flow was already observed 60 min after the start of CPB, thus ruling out the possible effect of inotropic treatment on this parameter.

6. SUMMARY OF NEW FINDINGS

1. We significantly refined a previous experimental technique and designed a new animal model, which resembles iatrogenic PT. For this purpose, the pericardium was accessed through the diaphragm via median laparotomy, and a cannula was fixed into the pericardial cavity with a pledgeted purse-string suture. Tamponade was induced and maintained by intrapericardial administration of heparinized own blood.
2. We designed a simplified, but clinically relevant large animal model of CPB-induced SIRS, where the haemodynamic and microcirculatory consequences of ECC can be studied in sufficient detail.
3. We confirmed the bioactivity of CH_4 in a large animal model of PT. The decreased ileal leukocyte infiltration and mast cell degranulation with the parallel reduction of blood superoxide concentrations demonstrate that exogenous CH_4 efficiently modulates the pathways of oxidative stress leading to reduced structural damage of the mucosa.
4. We demonstrated for the first time that CH_4 treatment is capable of increasing renal blood flow and hourly diuresis in the post-CBP period. We also provided evidence that the transmembrane diffusion of CH_4 using a commercially available hollow fibre membrane oxygenator is able to reduce the inflammatory activation (neutrophil accumulation and XOR activity) in peripheral tissues (i.e. heart, kidney and ileum) and the circulation as well (as evidenced by reduced blood superoxide content), which indicates that systemic CH_4 administration may be a feasible way to modulate ECC-induced ROS production.

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