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The application of cell-free DNA methylation patterns in critical illnesses protocol

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We use this protocol and it's working

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Abstract

The use of liquid biomarkers in clinical medicine are typically employed to gauge severity of disease, prognosis and to monitor response to treatment. While various biomarkers have been employed in clinical medicine with variable performance characteristics, the use of cell-free DNA (cfDNA) have gained increased traction as a novel biomarker in a wide range of disease states such as cancer and trauma. While the quantification of cfDNA have been correlated with disease severity, the use of methylation patterns of cfDNA can be used to localize the site of injury that may have implications regarding prognosis and therapeutics. We propose a procedure using samples in a swine model of cardiac arrest where carbon monoxide is being used as a therapeutic to demonstrate our method and feasibility to obtain plasma cfDNA methylation patterns to help identify tissue origin with potential application in critical care medicine.

Perioperative procedures and monitoring

- 1 Ventilator settings were as follows: tidal volume 10–11 mL/kg, positive end-expiratory pressure 5 cm H₂O and respiratory rate titrated to achieve end-tidal CO₂ 38–42 mmHg to minimize potential confounding changes in cerebral blood flow and acid–base status. The right femoral artery and vein were cannulated for arterial pressure monitoring and central venous access. Isoflurane was weaned to 0.5–1% to simulate human anesthetic protocols and minimize confounding toxicity and cerebral blood flow changes associated with higher doses of isoflurane. A rectal temperature probe was placed. A 48 cm percutaneous tunneled catheter was placed into the right internal jugular vein to allow sampling of blood and administration of intravenous medication during the survivor period. All data were recorded with PowerLab 16/35 LabChart 8 Pro software from ADInstruments (Sydney, Australia). Sedation through the experiment was maintained with the use of fentanyl (5 µg/kg/h) and dexmedetomidine (2 µg/kg/h) during the prescribed CO gas exposure with minimal use of isoflurane once the exposure was initiated.

Cardiac arrest protocol

- 2 Our cardiac arrest protocol consisted of 8 min of untreated VF followed by standardized Advanced Cardiac Life Support (ACLS) consisting of cardiopulmonary resuscitation (CPR) with first defibrillation taking place 2 min after CPR is initiated (10 min after the start of the VF arrest) every two min until return of spontaneous circulation (ROSC) or until 20 min of ACLS. Animals that achieved ROSC were maintained under general anesthesia to a PaO₂ 60–100 mmHg and a PaCO₂ 35–45 mmHg with predefined hemodynamic targets with IV fluids to achieve adequate intravascular volume status and norepinephrine to achieve target mean arterial pressure (MAP). Normothermia and continuous hemodynamic monitoring was maintained throughout the post resuscitation experimental period. If no ROSC was achieved, resuscitation was continued for an additional 10 min for a total of 20 min of CPR. If ROSC was achieved, CO treatment with 200 ppm was administered for 2 hr for a total of 3 hr post-ROSC based on a previous protocol publication.

Carbon monoxide treatment protocol

- 3 The assigned CO dose was administered with a CO tank (244 cf) at 0–10 L/min using a regulator with flow meter from Airgas (Radnor Township, PA, USA) for 200 ppm. Medical air was administered for controls. The CO concentration entering the endotracheal tube was

monitored using an Inspector CO detector with a 0–2000 ppm range (Sensorcon, New York, USA). Sedation was maintained with the use of fentanyl (5 µg/kg/h) and dexmedetomidine (2 µg/kg/h) during the entire experiment. Our previous prior work has utilized CO doses of 400 ppm and 2000 ppm.

Blood collection, cell-free DNA extraction, and bisulfite conversion

- 4 **To illustrate our protocol leveraging our established swine model of cardiac arrest with the use of CO as a therapy**, blood samples were then collected following a standard protocol using cfDNA/cfRNA Preservative Norgen tubes (Norgen biotek, Canada). Briefly, a total of 20 ml of whole blood placement into the Norgen tubes, each tube was then gently inverted 5 times to allow gentle mixture of the preservative and the blood samples. Samples were then centrifuged at 1,600 x g for 10 min at 4°C to obtain plasma on the upper layer. For cfDNA, the plasma was then centrifuged at 16,000 x g at RT to remove cell debris. The supernatant was then stored at -80°C before cfDNA extraction. cfDNA extraction was then performed with the NextPrep-Mag cfDNA isolation kit (PerkinElmer, MA), following the manufacturer's protocol. CfDNA quantification was performed using Qubit 1x dsDNA HS assay kit with the Qubit 4 fluorometer (Thermo Fisher Scientific). Swine cortex (PG-212) and blood (PG-705) genomic DNA (gDNA) samples were acquired from Zyagen as reference genomes. Samples were bisulfite converted using EZ DNA methylation-lightning kit (Zymo Research) before sequencing and data processing. Libraries for the WGBS were created from the bisulfite-converted samples using the NEBNext Enzymatic Methylation Kit (New England Biolabs) according to the manufacturer's protocol. Libraries were quantified with the Qubit 1x dsDNA high sensitivity assay kit and the Qubit 4 fluorometer. The WGBS was conducted on the NovaSeq 6000 platform (Illumina) producing 2 × 150 paired end reads at 30 x coverage. Sequencing reads were analyzed as follows: quality checked, trimmming, and aligned to the pig reference genome assembly, respectively. Bismark methylation extraction was applied to extract the methylation call for CpGs in each sample.

Differential Methylation Analysis.

- 5 We used the Seqmonk analytic workflow to identify DMRs among tissue types (<https://www.bioinformatics.babraham.ac.uk/projects/seqmonk/>). All DMRs were based on a genomic window size of 25 CpGs. Methylation values were quantitated by the "bisulphite methylation over features" pipeline in Seqmonk. The following criteria were applied for the selection of the following DMRs. Brain-specific DMRs were defined as those regions in which methylation in the cortex differed from methylation in blood by more than 50% (differential (diff) methylation >50%). Positive diff methylation indicates relative hypermethylation of the brain-specific DMR relative to blood, whereas negative diff methylation indicates relative hypomethylation, with an FDR < 0.00005.



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