SUPPLEMENTARY APPENDIX

The effect of iron loading and iron chelation on the innate immune response and subclinical organ injury during human endotoxemia: a randomized trial

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Online supplementary Design and Methods

Experimental human endotoxemia

All subjects gave written informed consent before participation and were thoroughly screened, including a full medical history, physical exam, ECG, and routine laboratory blood tests including markers of kidney and liver function and HIV and Hepatitis-B serology.

Subjects had refrained from the ingestion of alcohol and caffeine for 24 hrs prior to the test, and had fasted since 10 pm the night before. Subjects were placed in a bed in supine position, and received a venous catheter in an antecubital vein, and an arterial catheter in the brachial artery that enabled continuous arterial pressure monitoring, blood sampling, and the infusion of vasoactive medication for the assessment of vascular reactivity by forearm blood flow measurements. Heart rate was continuously monitored throughout the entire experiment using a 5 lead ECG. Subjects were pre-hydrated with 1.5 L NaCl 0.45%/glucose 0.45%.[1] At T=0, 2 ng/kg purified *E.Coli* endotoxin (*Escherichia coli* O:113, Clinical Center Reference Endotoxin, National Institute of Health (NIH), Bethesda, MD, USA) was administered intravenously, followed by 5 mL NaCl 0.9% to ensure complete delivery.

Blood sampling for the measurement of oxidative stress (malondialdehyde, MDA), iron parameters, cytokines and leukocyte count was carried out at baseline (before the start of iron or iron chelation therapy), and serially after the administration of endotoxin. From the morning of the test until 24 hrs after endotoxin administration all urine was collected for the measurement of markers of subclinical renal injury.

At T=8 hrs, after the final blood sampling, arterial and venous canulas were removed and subjects left the hospital. The following morning, subjects returned for final blood sampling by vena puncture and collection of urine samples.

Iron homeostasis analysis

Serum iron, ferritin and transferrin were automatically determined (Immulite 2000, Siemens healthcare Diagnostics, Deerfield, USA) at baseline (T=-2 h) and serially thereafter. Total iron binding capacity (TIBC) was calculated as: TIBC(µmol)=25.0 x transferrin (g/L). Serum concentration of soluble transferrin receptor (sTfR) was measured immunonephelometrically on a BN II System (Dade Behring Marburg GmbH, Marburg, Germany) at -2, 0, 3, 6 and 24 h post LPS.

Serum hepcidin concentrations were measured by a combination of weak cation exchange chromatography and time-of-flight mass spectrometry (WCX-TOF MS) using an internal standard (synthetic hepcidin-24; Peptide International Inc., Louisville, Kentucky USA) for quantification.

Peptide spectra were generated on a Microflex LT matrix-enhanced laser desorption/ionisation TOF MS platform (Bruker Daltonics, Billerica, Massachusettes, USA). Serum hepcidin-25 concentrations were expressed as nmol/L(nM) with a lower limit of detection of 0.5 nM.[2,3]

Growth differentiation factor 15 (GDF-15) concentrations were measured with DuoSet (R&D Systems, Minneapolis, MN, USA) enzyme linked immunosorbent assay (ELISA) for human GDF15 according to the manufacturer's protocol.

Labile plasma iron (LPI) was quantified by measuring the iron-specific redox activity in serum (FeROS^{TM} LPI kit, Aferrix Ltd., Tel-Aviv, Israel) as was essentially described before.[4] Briefly, a reducing agent (ascorbic acid) and an oxidizing agent (atmospheric O2), cause labile iron in the tested sample to oscillate between its oxidized (Fe3+) and reduced (Fe2+) forms, generating Reactive Oxygen Species (ROS) via the Fenton reaction. The ROS are detected by an oxidation-sensitive probe (DHR), which becomes fluorescent when oxidized by ROS.

Oxidative stress

Plasma concentrations of malondialdehyde (MDA), were measured in duplicate as a measure of lipid peroxidation as previously described.[5] Briefly, 50 µL of lithium heparin anti-coagulated plasma, or standard solution was added to 1 mL reagens, consisting of 10 mmol/L 2-thiobarbituric acid in phosphate buffer (0.1 mol/L, pH 3). The solution was vortexed and incubated for 1 hr at 96°C. Thereafter, samples were placed on ice for 5 min, after which 2 mL of butanol was added. The mixture was shaken twice for 1 min to extract the TBA-MDA adduct, and then centrifuged at 1500x g for 5 min at 4°C. For fluometric measurement of the supernatant a Shimadzu RFF-500 spectrofluorometer (Shimadzu Scientific Instruments Inc., Columbia, MD, USA) was used (excitation 515 nm and emission 552 nm). The results were quantified by comparison with the standard curve obtained with 1,1,3,3-tetramethoxy-propane (TMP; Fluka Chemika/Biochemika Buchs , Switzerland) ranging from 0-10 µmol/L. The CV for this assay was <10 % for both intra- and interassay variations.

Cytokines

To determine the concentration of the various cytokines, EDTA anti-coagulated blood was processed immediately by centrifugation at 2000×g at 4°C for 10 min and was stored at -80°C until analysis. Concentrations of the pro-inflammatory cytokines TNF-α and IL-6, the anti-inflammatory cytokines IL-10 and IL-1ra, and markers of endothelial activation Vascular Cell Adhesion Molecule (VCAM) and Intercellular Adhesion Molecule (ICAM) were determined using a simultaneous Luminex assay (Milliplex, Millipore, Billerica, MA, USA).

Subclinical organ injury

Subclinical endothelial injury was measured by the changes in response of the forearm vasculature to the infusion of vasoactive medication into the brachial artery. Changes in forearm blood flow (FBF) were measured by venous occlusion plethysmography according to methods described previously.[6] Briefly, after having received the prehydration fluid, and before iron (chelation) treatment and endotoxemia, the baseline response of the forearm vasculature to acetylcholine, nitroglycerin and norepinephrine was determined. Venous occlusion was achieved by inflating a cuff around the upper arm to 45 mmHg. A strain gauge was placed on the forearm and connected to a plethysmograph (Filtrass Angio; Domed, Munich, Germany) to measure changes in forearm volume in response to inflation of the venous-congesting cuff. From the changes in forearm volume, the blood flow in the forearm was derived using specifically designed software (Filtrass Angio; Domed, Munich, Germany). FBF measurements were carried out before and during the incremental intra-arterial administration of acetylcholine (0.5; 2; 8 $\mu g \cdot mL^{-1} \cdot dL^{-1}$), nitroglycerine (1.25; 2.5; 5 $\mu g \cdot mL^{-1} \cdot dL^{-1}$) and norepinephrine (30; 100; 300 $ng \cdot mL^{-1} \cdot dL^{-1}$) to assess vascular reactivity, as a measure of vascular function. FBFs and drug infusions were normalized to forearm volume as measured with the water displacement method and expressed in mL per min per deciliter forearm volume ($mL \cdot min^{-1} \cdot dL^{-1}$). Each dose-response curve started with a 5-min period of baseline measurements, followed by 2 min intra-arterial infusion, and 4 min of recording FBF. FBF data are the mean of measurements obtained during the last 3 min of each infusion (steady state). Four hrs after LPS administration, the analysis was repeated to determine the LPS-induced effects on vascular reactivity.

For the determination of subclinical renal tubular damage, urinary Glutathione S-Transferase Alpha-1-1 (GSTA1-1) and Glutathione S-Transferase-Pi-1-1 (GSTP1-1) were determined, reflecting proximal and distal tubular damage, respectively.[7,8] Immediately following collection, urine was stored in a 10% (v/v) buffer solution, containing 1M HEPES, pH 7.5, containing 5% (w/v) bovine serum albumin, 1% (w/v) sodium azide, 1% (v/v) Tween-20 and 10% (v/v) glycerol. The urine samples were stored at 4°C after collection. Within 12 hrs urine samples were frozen at -80°C until assayed. Urinary GSTA1-1 and GSTP1-1 levels were measured in duplicate by ELISA, as previously described.[7,8] The CV for these assays was < 10 % for both intra- and interassay variations.

Statistical analysis

Forearm blood flow (FBF) results are expressed as relative changes in FBF in the infusion arm compared with baseline measurement. The FBF-ratios are plotted as dose-response curves for each vasoactive agent. These dose-response curves were repeated 4 hrs after endotoxin administration. The endotoxin-induced differences on vascular reactivity were analyzed by comparing the curve before and after endotoxemia using repeated measures two way ANOVA. Differences between

groups were analyzed by calculating AUCs of the dose response curve before and after endotoxemia for each subject. Relative changes of the AUCs during endotoxemia were expressed as percentage from baseline for each subject. These percentages were compared between the treatment groups to investigate the treatment effects. A p-value<0.05 was considered to indicate significant differences.

We performed a power calculation showing that with a relevant difference of 30% in peak levels of TNF-α, an expected standard deviation of 25% and a power of 80-90%, nine to twelve subjects were needed per group to find a statistically significant difference.

Reference List

1. Dorresteijn MJ, van Eijk LT, Netea MG, Smits P, van der Hoeven JG, Pickkers P. Iso-osmolar prehydration shifts the cytokine response towards a more anti-inflammatory balance in human endotoxemia. J Endotoxin Res. 2005;11(5):287-93.

2. Swinkels DW, Girelli D, Laarakkers C, Kroot J, Campostrini N, Kemna EH, et al. Advances in quantitative hepcidin measurements by time-of-flight mass spectrometry. PLoSONE. 2008;3(7):e2706.

3. Kroot JJ, Laarakkers CM, Geurts-Moespot AJ, Grebenchtchikov N, Pickkers P, van Ede AE, et al. Immunochemical and mass-spectrometry-based serum hepcidin assays for iron metabolism disorders. Clin Chem. 2010;56(10):1570-79.

4. Esposito BP, Breuer W, Sirankapracha P, Pootrakul P, Hershko C, Cabantchik ZI. Labile plasma iron in iron overload: redox activity and susceptibility to chelation. Blood. 2003;102(7):2670-7.

5. Conti M, Morand PC, Levillain P, Lemonnier A. Improved fluorometric determination of malonaldehyde. Clin Chem. 1991;37(7):1273-75.

6. Christ F, Bauer A, Brugger D, Niklas M, Gartside IB, Gamble J. Description and validation of a novel liquid metal-free device for venous congestion plethysmography. J Appl Physiol. 2000;89(4):1577-83.

7. Mulder TP, Peters WH, DA C, Jansen JB. Sandwich ELISA for glutathione S-transferase Alpha 1-1: plasma concentrations in controls and in patients with gastrointestinal disorders. Clin Chem. 1996;42(3):416-19.

8. Mulder TP, Peters WH, Wobbes T, Witteman BJ, Jansen JB. Measurement of glutathione S-transferase P1-1 in plasma: pitfalls and significance of screening and follow-up of patients with gastrointestinal carcinoma. Cancer. 1997;80(5):873-80.