ORIGINAL PAPERS – Supplemental Data

Furosemide in the treatment of phosgene induced acute lung injury

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SUPPLEMENTAL DATA

RNA extraction and RT-qPCR

Total RNA was extracted from bronchial biopsy and alveolar specimens using the Trizol reagent kit (Invitrogen, Paisley, United Kingdom) and samples treated with RNase-free DNase (Ambion, Huntingdon, United Kingdom) to eliminate contaminating genomic DNA. Total RNA (0.2µg), random hexamers (100ng), and M-MLV reverse transcriptase (120U; Promega, Southampton, United Kingdom) were used for cDNA production.

For quantitative analysis of genes of interest; glutathione reductase (GSR), glutathione peroxidase 2 (GPX2), glutathione s-transferase a2 (GSTA2), superoxide dismutase (extracellular) (SOD) (LOC780439), Interleukin 8 (IL-8), keratinocyte growth factor (KGF), V-akt murine thymoma viral oncogene homolog 1 (AKT1), Toll-like receptor 3 (TLR3) and Caspase-8 (CASP8) real-time PCR was performed using gene-specific primers and a gene-specific fluorogenic probe with an iCycler (BioRad, Hercules, Calif), with glucose phosphate isomerase (GPI) and pepdidyproplyl isomerase A (PPIA) as normalising genes (PrimerDesign, Southampton, United Kingdom). Selection of normalising genes was performed following analysis of 11 potential candidates using a geNorm housekeeping selection kit (PrimerDesign, Southampton, United Kingdom) and software. [32,33]. Analysis of RT-qPCR data was performed by using the ΔΔCt method. The primer sequences were as follows:

GSTA2 – forward CAAGTACAACCTCTACGGGAAG, reverse GGCAACAGCAAGATCATTTCAC;

GSR – forward CAATTGGCGGGTTATAAAGGAAA, reverse CTATGTGGGACTTGGTCAGATT;

SOD – forward CCGAGCCCAACAGCACCAG, reverse ACCTCCAGCGGGTTGTAGTG;

IL-8 – forward GCA ACAACAACAGCAGTAACAA, reverse CAGCACAGGAATGAGGCATAG;

KGF – forward GAGGGGATATAAGAGTGAGAAGAC, reverse TGATTTCCATGATGTTGTAATTGTTC;

AKT1 – forward TCCGAGAAGAACGTGGTGTAT, reverse AGAGCCCGAAGTCAGTGATC;

TLR3 – forward GAGAATCTATCCCTGAGCAACAT, reverse AGGAATCATTACCAATCACACTTAAG;

Angiopoeitin-like 2 (ANGPTL2) – forward GCTCCGTCAACTTCTTTAGGAA, reverse TCGTCAGCCAGTAAATGTTCTC;

CASP8 – forward AGACAGACTCAGAACAGACAGA, reverse GCTTCGGTAAGAAACACAGTTG;

GPX2 – forward CATCAACATCGAGCCTGACAT, reverse CACTGAATGCTAAGGATGGATACT.

Antioxidant capacity assays

The antioxidant capacity of furosemide was measured by assays comparing two different known antioxidant mechanisms, namely hydrogen atom transfer (HAT) and electron transfer (ET). The Oxygen Radical Absorbance assay (ORAC) measures HAT activity and furosemide was compared to the ability of Vitamin C (Vit C) and N-acetyl cysteine (NAC) in equimolar amounts to prevent the quenching of fluorescein fluorescence by the stable radical 2,2'-azinobis-3-ethylbenzothiazoline-6-sulfonic acid using a fluorimeter over time [34]. ET reactions were measured via the ability of antioxidants to cause the reduction of copper II to copper I in the presence of the copper I chelator neocuprione (CUPRAC assay). Chelation of copper I leads to an increase in absorbance at 450nm which was measured in a spectrophotometer and compared to equimolar amounts of Vit C and NAC [35]. Each assay was repeated 3 times.

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