

Flow cytometry analysis validation

Prior to the above analysis, a series of experiments were conducted to validate the mitochondrial superoxide assay using MitoSOX via flow cytometry. These experiments were performed on cells obtained from kidneys of 6 untreated male Sprague-Dawley rats using the method described above. Flow cytometric acquisition and analysis were performed using the method described previously.

To evaluate the concentration of MitoSOX which will result in optimal signal, kidney cells were stained with MitoSOX at concentrations 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 μM using the method described above. Concentrations up to 3 μM resulted in a steady increase in MitoSOX geometric MFI. This signal plateaued at concentrations between 3-6 μM and declined at concentrations $> 6 \mu\text{M}$ (Figure 5). To minimise risk of cytotoxicity initiated by MitoSOX, which might in turn result in cell and mitochondrial damage, a concentration 1 μM below the concentration that produced the maximal geometric MFI (ie 2 μM) was chosen.

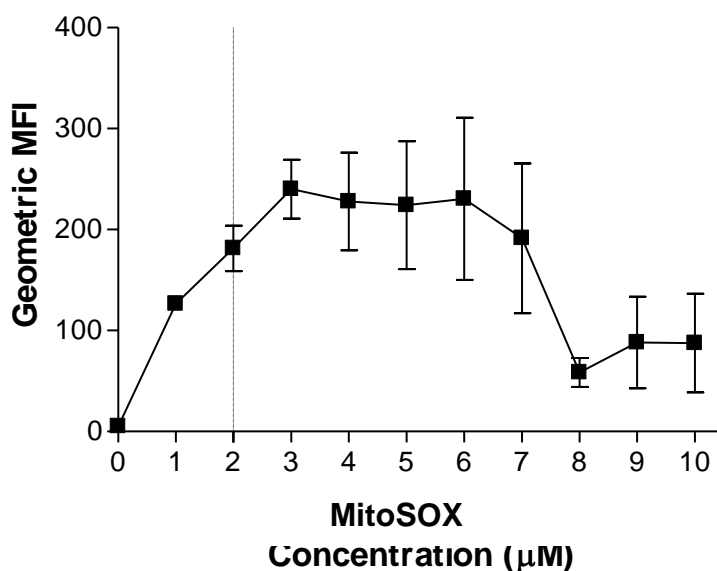


Figure 5: MitoSOX titration curve, $n = 4$.

Antimycin A (Sigma-Aldrich, St Louis, USA), a mitochondrial complex IV inhibitor known to induced mitochondrial superoxide production, was used in establishing a positive control. The kidney cells were treated with Antimycin A (5 μM) prior to staining with MitoSOX and DiIC₁(5). The geometric MFI for both dyes were evaluated using the flow cytometric analysis method described above. Cells pre-treated with 5 μM Antimycin A had significantly higher MitoSOX fluorescence (694 ± 105 , $n = 6$) compared with untreated cells (control, 213 ± 27 , $n = 6$, $P < 0.005$) (Figure 6). Antimycin A at 5 μM did not significantly reduce mitochondrial membrane potential as indicated by DiIC₁(5) geometric MFI (control: 136 ± 49 and Antimycin A: 72 ± 12 , $n = 6$ each, *ns*) (Figure 7).