

## EFFECT OF OXIDATIVE STRESS ON UROTHELIAL-AFFERENT SIGNALLING IN THE MOUSE: POSSIBLE ROLE IN AGED BLADDER DYSFUNCTION

### Hypothesis / aims of study

The incidence of bladder diseases, such as overactive bladder (OAB) syndrome and urgency incontinence (UI) is increased with age. Why bladder function is particularly susceptible to aging is still unclear and the aetiology of OAB and UI are not fully understood. A number of studies suggest that the epithelial layer of the bladder, known as urothelium, together with the sub urothelium and the sensory innervation play a key role in normal bladder function. Therefore, an alteration in this sensory signalling could be a contributing factor to low urinary tract disorders such as OAB and UI. The role of oxidative stress in ageing has been extensively investigated <sup>(1)</sup>. This condition is caused by an imbalance between the productions of pro oxidants (reactive oxygen species, ROS) and antioxidants (catalase CAT, superoxide dismutase SODs, glutathione etc...). There are a number of studies showing that the oxidative stress may play an important role in the development of bladder dysfunction by increasing detrusor muscle contractility and stimulating bladder afferent fibres <sup>(2)</sup>.

The aim of this study was to investigate the effect of oxidative stress on the urothelium and sensory nerve function from the bladder and determine if there is an increase in oxidative stress in the aged urothelium which may drive a cascade of events leading ultimately to bladder dysfunction in ageing.

### Study design, materials and methods

C57/B6 control mice (aged 5 months) and aged mice (24 months) were used in this study. Mice were sacrificed humanely in accordance with UK legislation.

**Intracellular ROS level detection:** urothelial cells from control (N=10) and aged (N=6) mice were cultured as previously described.  $3 \times 10^4$  cells were plated on collagen (96-well) and 40  $\mu$ M dichlorofluorescein (DCF) probe was added. ROS concentration was measured by fluorescence.

**Oxidative DNA damage marker 8-hydroxy-deoxyguanosine (8-OHdG) detection:** DNA was isolated from urothelial/suburothelial tissues harvested from control (N=6) and aged (N=4) mice. EpiQuik 8-OHdG DNA damage quantification direct kit (Epigentek) was used. 8-OHdG was detected fluorimetrically and expressed as percentage relative to a positive control.

**qRT-PCR:** anti-oxidant enzymes catalase (CAT) and superoxide dismutase 2 (SOD2) mRNA expression level were examined to compare levels in control (N= 5) and in aged (N=5) urothelium/sub urothelium. qRT-PCR with TaqMan chemistry was performed. Results are expressed as relative expression after normalization with the housekeeping gene GAPDH (1/ $\Delta$ Ct) and as fold change, using the formula  $2^{-\Delta\Delta Ct}$ . Fold change  $\geq \pm 2$  was considered significant.

**Calcium imaging:** urothelial cells from control mice (N=6) were loaded with the calcium sensitive dye Fura 2-AM and oxidative stress was induced treating the cells with 0.003% hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) for 10 minutes, with or without 1 hour pre-incubation with 10mM anti-oxidant N-acetyl cysteine (NAC). The possible oxidative-stress mediated pathway was investigated by pre-incubation with 1 $\mu$ M TRP channel antagonist Ruthenium Red for 15 minutes before H<sub>2</sub>O<sub>2</sub> exposure.

**Extracellular bladder afferent nerve recording:** the effect of oxidative stress on bladder sensory function was investigated in control mice (N=3). Simultaneous recordings of afferent nerve firing (pelvic and hypogastric nerves) and intravesical pressure during bladder distension, were performed. The response to intravesical perfusion with saline or 0.003% H<sub>2</sub>O<sub>2</sub> was measured.

### Results

In aged bladder urothelium, intracellular ROS and 8-OHdG levels were significantly higher than in control urothelium (fig1 A and B), while the anti-oxidant superoxide dismutase 2 (SOD2) mRNA was significantly lower (fold change=2,5) (fig1 C and D). In control urothelial cells, induction of oxidative stress using H<sub>2</sub>O<sub>2</sub> caused a significant and profound calcium response in 93% of cells. This effect was attenuated by pre-incubation with the antioxidant NAC (responding cell 58%) or the TRP channel antagonist ruthenium red (responding cell 63%). In the extracellular afferent recordings, oxidative stress induced by acute intravesical application of H<sub>2</sub>O<sub>2</sub> increased spontaneous afferent nerve discharge (from  $9.8 \pm 2.6$  vs  $15.1 \pm 2.0$ ). Bladder compliance as measured by the pressure volume relationship and the afferent response to distension of the bladder was not affected.

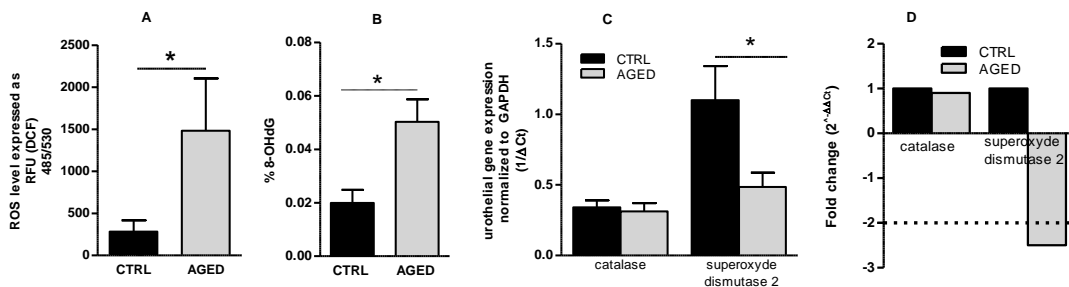


Fig1. Urothelial intracellular ROS and 8-OHdG levels (A and B). Anti-oxidants CAT and SOD2 mRNA level in the urothelium, normalized to GAPDH.

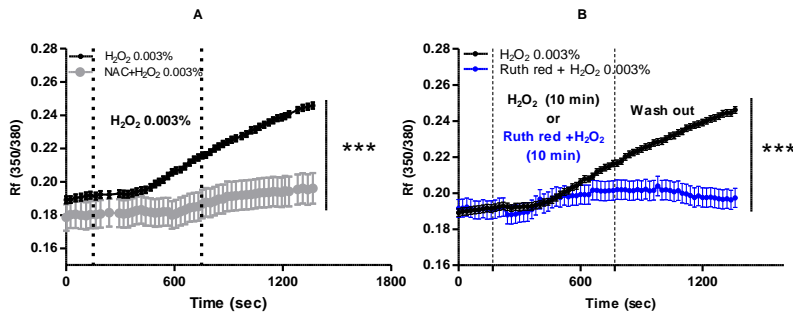


Fig2. Induction of oxidative stress in the urothelial cells by treatment with  $H_2O_2$  causes an increase in calcium influx which is significantly abolished by pre-incubation with anti-oxidant NAC and TRP channel antagonist ruthenium red

### Interpretation of results

In studies with age mice we show that ageing correlates with oxidative stress in the urothelium. This oxidative stress could result from an increase in intracellular ROS levels inducing oxidative DNA damage, as the increase of 8-OHdG level suggests, or as a consequence of the decrease in the natural anti-oxidant enzyme SOD2, which transforms toxic superoxide anion ( $O_2^{\cdot-}$ ) into hydrogen peroxide and oxygen. In control experiments, exposure of urothelial cells to the oxidant  $H_2O_2$  caused a dramatic increase in calcium responses which were significantly abolished by the anti-oxidant NAC and the TRP channel antagonist ruthenium red, suggesting oxidative stress induced by  $H_2O_2$  activates urothelial cells in a mechanisms involving the a TRP channel(s). This is in accordance with previous studies, showing that some TRP channels (such as TRPC3, TRPC4, TRPM2 and TRPM7) are regulated by oxidative stress in several types of cells<sup>(3)</sup>. Moreover in this study we also show that oxidative stress can increase bladder afferent nerve sensitivity suggesting that it may induce pain or hypersensitivity of the bladder in lower urinary tract disorders such as OAB and IC or as a result of ageing.

### Concluding message

This study demonstrates an increase in urothelial oxidative stress concurrent with increasing age. Taken together these findings suggest that in ageing there is an imbalance between oxidants and anti-oxidants leading to an increase in oxidative stress in the urothelium. This imbalance could drive increased neuronal activity leading to bladder hypersensitivity. While more studies are still required to identify the pathways involved these data suggest a possible TRP channel mechanism. Elucidating these pathways may yield novel targets for pharmacological treatment or preventative therapies for OAB and IC.

### References

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### Disclosures

**Funding:** Marie Curie TRUST Initial Training Network (ITN) **Clinical Trial:** No **Subjects:** ANIMAL **Species:** Mouse **Ethics Committee:** University of Sheffield Ethics committee

Figure 2. Treatment of urothelial cells with  $H_2O_2$  causes an increase in  $Ca^{2+}$  influx which is significantly reduced by pre-incubation with the anti-oxidant NAC (A) and with the TRP channels inhibitor ruthenium red (B).