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*Published in:*  
European Journal of Pharmacology

*DOI:*  
[10.1016/j.ejphar.2019.172703](https://doi.org/10.1016/j.ejphar.2019.172703)

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*Recommended citation(APA):*  
Kang, S. H., Chess-Williams, R., & McDermott, C. (2019). Elevated release of inflammatory but not sensory mediators from the urothelium is maintained following epirubicin treatment. *European Journal of Pharmacology*, 863, Article 172703. <https://doi.org/10.1016/j.ejphar.2019.172703>

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**Elevated release of inflammatory but not sensory mediators from the urothelium  
is maintained following epirubicin treatment.**

Sung Hyun Kang<sup>a</sup>, Russ Chess-Williams<sup>a</sup>, Catherine McDermott<sup>a</sup>

<sup>a</sup>Centre for Urology Research, Faculty of Health Sciences and Medicine, Bond  
University, Gold Coast, Queensland 4229, Australia

**Correspondence:** Dr C. McDermott, Centre for Urology Research, Faculty of Health  
Sciences and Medicine, Bond University, Gold Coast, Queensland 4229, Australia

E-mail: [camcderm@bond.edu.au](mailto:camcderm@bond.edu.au)

Tel: +61 755954777

## **Abstract**

Intravesical treatment of superficial bladder cancer with epirubicin is associated with local urological adverse effects, the causes of which are unknown. Our aim was to investigate the effects of epirubicin on the release of urothelial mediators and inflammatory cytokines. UROtsa cells were treated with epirubicin for 1 h at 37 °C. Release of urothelial transmitters and inflammatory cytokines was examined immediately, 24 h and 7 days following treatment. Release of ATP and nitric oxide were increased transiently after treatment with epirubicin (0.01mg/ml), but this was not evident one-week after treatment. Basal prostaglandin E<sub>2</sub> release was decreased at 24 h but not at 7 days. An increase in basal acetylcholine release and decrease in stretch-induced acetylcholine release were observed 24-h after treatment (0.01mg/ml). One week following epirubicin treatment (0.001 mg/ml), stretch-induced, but not basal acetylcholine release, was observed. Secretion of interleukin-6 and interleukin-8 was increased 70-fold and 5-fold respectively, at 24 h (0.01mg/ml). This was sustained and one week after epirubicin treatment (0.001mg/ml), the increase in the secretion of both inflammatory cytokines was still evident. Epirubicin treatment induces several transient changes in urothelial function. However, the increased secretion of inflammatory cytokines (IL-6 and IL-8) is sustained and these mediators may be involved in the pathophysiology of bladder toxicity following intravesical epirubicin treatment.

**Keywords:** Epirubicin; Bladder cancer; Intravesical chemotherapy; Adverse effects; Urothelium; Inflammation

## **1.0 Introduction**

Intravesical therapy is a widespread treatment approach for superficial bladder cancer and is undertaken due to a high recurrence rate (48 to 70%) of the disease after successful removal of bladder tumour (Logan et al., 2012). With intravesical treatment, high concentrations of cytotoxic agents can be administered directly into the bladder lumen to access the target cancer cells, whilst limiting systemic exposure (Dalton et al., 1991). Epirubicin, doxorubicin and mitomycin C (MMC) are commonly used agents for the treatment of superficial bladder cancer via this targeted means of delivery. Despite limited systemic side effects, there is evidence of significant local urological adverse effects such as chemical cystitis (dysuria, increased urinary frequency and urgency) following this treatment (Elmamoun et al., 2014; Koya et al., 2006).

The urothelium is in direct contact with the cytotoxic agents during intravesical treatment. While the urothelium serves as a distensible barrier to protect the underlying tissues from the potentially harmful contents of the urine, it also responds to bladder filling, by releasing a number of chemical transmitters including ATP (Burnstock, 2011a), acetylcholine (Birder, 2010), prostaglandin E2 (PGE2) (Tanaka et al., 2011) and nitric oxide (NO) (Winder et al., 2017). These transmitters are involved in regulating sensory mechanisms and bladder contraction playing essential roles in maintaining normal bladder function. Despite the urothelium acting as an effective barrier to the movement of substances from the bladder lumen to the deeper tissue layers, previous studies have shown that some intravesical chemotherapeutic agents including doxorubicin and MMC diffuse into the detrusor muscle, although

only at a fraction of the luminal concentration (Wientjes et al., 1996; Wientjes et al., 1993).

Our recent studies using RT4 cells have shown that the cytotoxic drugs doxorubicin (Kang et al., 2013) and mitomycin c (MMC) (Kang et al., 2015), alter the release of ATP, acetylcholine and PGE<sub>2</sub> from urothelial cells immediately following treatment, as well as induction of pro-inflammatory cytokines and nitric oxide. Gemcitabine treatment produced comparable transient changes in urothelial ATP and PGE<sub>2</sub> release, with sustained elevation in secretion of IL-6, IL-8 and IL-1 $\beta$  from UROtsa cells (Farr et al., 2017).

Epirubicin is another cytotoxic drug that has been used for intravesical treatment of superficial bladder cancers. Whilst systemic adverse events after intravesical epirubicin have not been reported in many trials (Elsawy et al., 2019; Hendricksen et al., 2008; Onrust et al., 1999), major local urological adverse effects associated with treatment include chemical cystitis, haematuria and contracted. Thus, the aim of this study was to investigate the effects of epirubicin on basal and stretch-induced release of urothelial transmitters from UROtsa human urothelial cells and determine whether changes in urothelial function may provide an explanation for the urological adverse effects experienced by patients treated with intravesical epirubicin. Inflammatory markers were also assessed to see if inflammation plays a role in the adverse effects associated with treatment. In addition, recovery of urothelial function was assessed 1-week post-treatment.

## **2.0 Materials and Methods**

### **2.1 Cell culture**

The UROtsa cell line was a gift from Dr. Scott Garret at the University of North Dakota. The cells were grown and maintained at 37°C in 5% CO<sub>2</sub> in Dulbecco's modified eagle medium (DMEM) (Sigma-Aldrich, St. Louis, USA). Low glucose culture medium contained 1mg/ml glucose (Sigma-Aldrich, St. Louis, USA), 1% v/v penicillin-streptomycin (Invitrogen, Victoria, Australia) and 5% v/v Foetal Bovine Serum (FBS) (Invitrogen, Victoria, Australia). Cell viability was assessed by trypan blue exclusion.

### **2.2 Epirubicin treatment of urothelial cells**

UROtsa were maintained in serum-free medium from the time of seeding for experimental treatment to the end of the recovery period (Farr et al., 2017). Twenty-four-well plates were seeded at a density of  $1.2 \times 10^5$  (for immediate effects and 24 h post-treatment effects) or  $1.0 \times 10^5$  (for 1-week post-treatment effects) UROtsa cells per well and incubated overnight to allow cells to adhere.

Cells were incubated at 37 °C for 60 mins with epirubicin (Tocris Bioscience, Bristol, UK) at concentrations up to 1 mg/ml (1.7 mM) diluted in serum-free DMEM culture medium, which is the clinical dose and duration used for intravesical therapy (Hendricksen et al., 2008). Cells were then used immediately, 24 h or 1 week later to generate samples for analysis of urothelial mediator release. During the 1-week recovery period, culture medium was changed every three days. In experiments investigating recovery over 24 h and 1 week after treatment, only 2 concentrations of epirubicin were investigated (0.01 and 0.001mg/ml). It has been reported that only

small amounts of cytotoxic drug penetrate the urothelium (Wientjes et al., 1996) and therefore lower concentrations are more likely to reflect concentrations reached in urothelial cells in vivo.

### **2.3 Mediator release from urothelial cells**

To determine the basal release of mediators, the cells were incubated for 15 min with isotonic solution (composition, mM: NaCl 130, KCl 5, CaCl<sub>2</sub> 1.5, MgCl<sub>2</sub> 1, NaHEPES 25, BSA 0.015 and glucose 5). Then, the solutions on the plate were aspirated and fresh isotonic solution was added into each well. After 10 min, the solutions in each well were collected for the measurement of basal mediator release from epirubicin-treated and control cells.

For hypo-osmotically stimulated (stretch simulation) release of urothelial mediators, hypotonic solution (isotonic solution without NaCl) was added into each well. After 10 min incubation, the solutions in each well were collected for analysis of urothelial mediators. The use of hypotonic solution causes the cells to swell, mimicking the stretch that occurs during bladder filling (Kang et al., 2013, 2015).

### **2.4 Resazurin viability assay**

Reduction of the redox dye resazurin to resorufin was used to measure the viability of cells (Farr et al., 2017). Cells were seeded in 96-well microtiter plates 24 h prior to the addition of epirubicin (0-1 mg/ml) for 1 h at 37 °C. After incubation with epirubicin, treatment medium was removed, and cells were washed twice with PBS solution prior to performing the resazurin reduction cell viability assay. While immediate effects of epirubicin on urothelial cell viability was assessed instantly after removing treatment medium and washing with PBS solution, the viability of cells 24 h or 1 week following epirubicin treatment was measured after maintaining cells with fresh serum-free culture medium at 37 °C. The resazurin reduction cell viability assay

was performed by incubating cells for 1 h with fresh medium containing 44  $\mu$ M resazurin. After 1 h incubation, reduction of resazurin to resorufin was determined by fluorescence (excitation 530 nm; emission 590 nm) using a Modulus microplate reader. Appropriate cell-free controls were also included. Under all conditions tested, the extent of resazurin reduction was directly proportional to viable cell counts (data not shown).

## **2.5 Measurement of urothelial mediators**

Release of ATP was measured using an ATP determination kit (Molecular Probes).

Luminescence was measured using a Modulus microplate reader (Promega).

Acetylcholine was measured using the Amplex® Red Acetylcholine Assay kit (Molecular Probes). Fluorescence was measured on a Modulus Microplate reader (Ex. 540/Em. 590 nm). The level of prostaglandin E<sub>2</sub> released from UROtsa cells was measured using the Prostaglandin E<sub>2</sub> EIA (Cayman Chemicals). Samples and standards were analysed using a Modulus microplate reader (420 nm). All assays were conducted according to manufacturer's instructions and ATP, Acetylcholine and PGE<sub>2</sub> concentrations were normalised to controls using corresponding resazurin reduction data.

## **2.6 Inflammatory cytokine analysis**

Cell-free incubation medium was collected from T25 culture flasks 24 h and 1 week after epirubicin pre-treatment. The presence of inflammatory cytokines (IL-8, IL-1 $\beta$ , IL-6, IL-10, IL-12p70 and tumour necrosis factor, TNF $\alpha$ ) was analysed using a BDTM Cytometric Bead Array Human Inflammatory Cytokines Kit. Standard and sample fluorescence were determined on a BD™ FACS Calibur flow cytometer. Concentrations of inflammatory cytokines were normalised to controls using corresponding resazurin reduction data.

## **2.7 Nitrate/nitrite analysis**

Cell-free incubation medium was collected from T25 culture flasks 24 h and 1 week after epirubicin pre-treatment. Release of urothelial nitric oxide into the incubation medium was assessed by measuring its metabolites nitrate and nitrite using a Nitrate/Nitrite Fluorometric Assay Kit (Cayman Chemicals). Fluorescence was measured on a Modulus Microplate reader. Nitrate/nitrite concentrations measured were normalised to controls using corresponding resazurin reduction data.

## **2.8 Statistical analysis**

Results are expressed as mean  $\pm$  standard error of the mean (S.E.M.). Data were analysed using a paired Student's t test or one-way ANOVA with Dunnett multiple comparisons test, using GraphPad InStat3 software (San Diego, CA). Significance levels were defined as  $P < 0.05$  (\*),  $P < 0.01$  (\*\*) and  $P < 0.001$  (\*\*\*)

### **3.0 Results**

#### **3.1 Effects of epirubicin on urothelial viability and release of mediators**

Urothelial cell survival following treatment with epirubicin at concentrations  $\geq 0.01$  mg/ml was significantly reduced all time points tested post-treatment with complete cell death observed in cells treated with 1 mg/ml epirubicin compared to control cells at all time points (Fig. 1).

The urothelial cells released ATP under basal conditions and cell stretch induced with hypotonic solution significantly increased release by approximately 8-fold (controls in Fig. 2A-B). The cells also released acetylcholine with basal concentrations reaching levels 100-fold greater than those observed with ATP. Stimulation with hypotonic solution again further increased levels by approximately 5-fold (controls in Fig. 2C-D). PGE<sub>2</sub> release was also observed, but at much lower levels (only 10% of that to ATP), but again stimulation of cells increased release in this case by about 10-fold (controls in Fig. 2E-F).

Immediately following treatment at the clinical dose (1 mg/ml), complete cell death was observed and urothelial mediator release could not be measured in cells treated with this concentration. However, treatment with lower concentrations of epirubicin were examined and the release data corrected for changes in cell number. Epirubicin had no effect on basal or stimulated release of mediators at low concentration but did enhance both basal and stimulated release of ATP at 0.1mg/ml (Fig. 2 A&B).

Acetylcholine and PGE<sub>2</sub> were unaffected by treatment with epirubicin at any concentration (Fig. 2 C-F).

Since urothelial ATP release was affected immediately after epirubicin treatment, the ability of the urothelium to recover was assessed. Release of urothelial mediators was measured 24 h after epirubicin treatment with low epirubicin concentrations ( $\leq 0.01$

mg/ml) as these concentrations are likely to be more relevant since only a small fraction of intravesical chemotherapeutic agents penetrates the urothelium (Wientjes et al., 1996; Wientjes et al., 1993). Both basal and stimulated release of ATP from urothelial cells treated with 0.01 mg/ml epirubicin increased significantly compared to vehicle-treated controls 24 h following treatment, with basal levels increasing 7-fold and stimulated levels increasing 2-fold (Fig. 3A-B). While basal release of acetylcholine was significantly increased in cells treated with 0.01 mg/ml epirubicin, the increase was only 30%, and the opposite occurred with stimulated levels of acetylcholine where the same concentration of epirubicin depressed release by almost 70% (Fig. 3C-D). Basal PGE<sub>2</sub> release was significantly reduced in cells treated with 0.01 mg/ml epirubicin at 24 h (Fig. 3E). However, stimulated release of PGE<sub>2</sub> remained unchanged compared to control cells 24 h following epirubicin treatment (Fig. 3F).

One week after treatment with 0.001 mg/ml epirubicin, basal levels of ATP and acetylcholine were unaffected, but stimulated release for these two mediators was changed by epirubicin treatment but with opposite effects: stimulated ATP levels were reduced whilst those to acetylcholine were increased (Fig. 4). As previously, basal PGE<sub>2</sub> release was unaffected by epirubicin treatment. It should be noted however that hypotonic stimulation prostaglandin E<sub>2</sub> response over basal was no longer present at this timepoint (Fig. 4E and F).

### **Effects of epirubicin on inflammatory cytokine and nitric oxide release**

As a measure of urothelial inflammation, the presence of inflammatory cytokines IL-8, IL-1 $\beta$ , IL-6, IL-10, IL-12p70 and TNF $\alpha$  in urothelial cell incubation medium was tested 24 h and 1 week after epirubicin treatment. Neither IL-1 $\beta$ , IL-6, IL-10, IL-

12p70 nor TNF $\alpha$  were detected in control or treated samples at any timepoint. However, IL-6 was secreted by untreated urothelial cells at 24 h, while urothelial cells treated with epirubicin (0.01 mg/ml) showed a 70-fold increase in release of this pro-inflammatory cytokine ( $53.24 \pm 5.02$  pg/ml control vs.  $3511 \pm 494$  pg/ml treated,  $P < 0.01$ ) (Fig. 5A). IL-8 was also secreted by untreated urothelial cells at 24 h, and treatment with epirubicin (0.01 mg/ml) resulted in a 5-fold increase in release ( $292 \pm 23$  pg/ml control vs.  $1519 \pm 89$  pg/ml treated,  $P < 0.01$ ) (Fig. 5C). One week after epirubicin treatment the release of IL-6 and IL-8 continued to be elevated compared to controls (IL-6, 8-fold increase,  $P < 0.001$ ; IL-8, 3-fold increase,  $P < 0.001$ ) (Fig. 5B). To determine the effects of epirubicin on urothelial nitric oxide release, nitrate and nitrite (metabolites of nitric oxide) were measured in incubation medium. Nitric oxide was released by untreated UROtsa cells at 24 h, with a significant increase observed from UROtsa cells treated with epirubicin (0.01 mg/ml, Fig. 5E), but 1 week after treatment no significant differences could be detected compared to control (Fig. 5F).

#### 4.0 Discussion

The present study investigated the effects of the intravesical chemotherapeutic agent, epirubicin on urothelial function, markers of inflammation and the ability of urothelial function to recover following epirubicin treatment.

It is well established that in normal bladder, the release of ATP from the urothelium acts on purinoreceptors (P2X<sub>2/3</sub>) present on sensory nerves to initiate the micturition reflex and to give rise to perception of pain through low threshold A $\delta$ -fibres and high threshold C-fibres respectively (Burnstock, 2011b). In addition, ATP can act directly on the detrusor muscle causing bladder contraction (West et al., 2018). Intravesical epirubicin treatment causes urological adverse effects such as painful urination and urgency, so it was notable to find elevation of both basal and stimulated ATP release immediately after epirubicin treatment (0.1 mg/ml), which was still evident 24 h following treatment. An increase in stretch-induced urothelial ATP release has been reported in a number of disorders of the bladder including idiopathic and neurogenic bladder overactivity and painful bladder syndrome (Kumar et al., 2007; Takezawa et al., 2017). Thus, the initial enhancement of ATP release suggests that this mechanism could directly contribute to the initial pain and bladder overactivity reported in patients treated with intravesical epirubicin. However, in our study, elevated ATP levels were not maintained and one week after treatment, levels were actually depressed, suggesting that the changes in ATP are not responsible for these symptoms in the longer term. The decrease in stretch induced ATP release, 1 week following treatment may reflect changes in adenosine, which has previously been reported to inhibit distension-induced ATP release via activation of the A<sub>1</sub> receptor (Dunning-Davies et al., 2013).

Urothelial acetylcholine can exert effects on detrusor and sensory nerves, although its role in regulating bladder function is not as clear as that for ATP. Acetylcholine can contract detrusor smooth muscle, but whether concentrations reaching the muscle from the urothelium are great enough to influence contraction is not known. The urothelium itself expresses muscarinic receptors and the acetylcholine released during stretch has been shown to stimulate pacemakers in the lamina propria to increase the spontaneous rate of phasic contractions (Moro et al., 2011), which may ultimately lead to increased detrusor activity and hence overactivity. Acetylcholine also influences sensory nerve activity but again there are conflicting reports with both stimulation and inhibition of nerve activity being reported (Daly et al., 2010). Thus the role of acetylcholine in bladder function has not been fully elucidated but overall it increases bladder contractile activity and this underlies the use of muscarinic antagonists in the treatment of bladder overactivity (Kumar et al., 2003). Following urothelial cell treatment with epirubicin, there was a transient decrease in acetylcholine release at 24 hours post-treatment, which was reversed to a more than 2-fold elevation of release one week after treatment, which could possibly contribute to symptoms of overactivity in patients.

The role of urothelial-derived PGE<sub>2</sub> in bladder function is not well understood, but inhibition of cyclooxygenase improves storage function in rats with detrusor overactivity, indicating potential stimulatory effects of PGE<sub>2</sub> on the micturition reflex (Yokoyama, 2010). This is supported by a report that over-expression of PGE<sub>2</sub> in mice results in bladder overactivity via PGE<sub>2</sub> activation of C-fibres (Aoki et al., 2009; Maggi et al., 1988). In our study, epirubicin had no effect on either basal or stimulated release of PGE<sub>2</sub> from UROtsa cells immediately, or 1-week post-treatment; however, a minor decrease in basal release was observed at 24 h. Therefore, like other

intravesical chemotherapeutic agents tested previously doxorubicin, gemcitabine and MMC; epirubicin also results in alterations in PGE<sub>2</sub> release from urothelial cells, although transiently (Farr et al., 2017; Kang et al., 2013, 2015). The changes in PGE<sub>2</sub> release following epirubicin treatment may be reflective of concurrent changes in ATP release, as regulation of these urothelial mediators are thought to be correlated (Tanaka et al., 2011).

The link between release of inflammatory mediators and intravesical cytotoxic agents such as Bacillus Calmette-Guerin (de Boer et al., 1997) and doxorubicin (Abou El Hassan et al., 2003) has been reported previously and studies have also shown that mitomycin C induces release of IL-8 in human fibroblasts (Chou et al., 2007). In the present study, increases in IL-8 and IL-6 were observed 24 hours following epirubicin treatment, and both inflammatory cytokines remained elevated 1 week after treatment. Inflammation has been shown to increase bladder afferent excitability and is believed to contribute to bladder overactivity and pain (Hayashi et al., 2009). IL-8 and IL-6 play important roles in the generation and propagation of chronic inflammation: IL-8 attracts neutrophils to the site of inflammation (Petering et al., 1999), whilst IL-6 increases the expression of endothelial leukocyte adhesion molecules thus promoting leukocyte accumulation (Barnes et al., 2011; Rose-John, 2012) and rescues T cells from apoptosis, which promotes a chronic inflammatory cell infiltrate (Curnow et al., 2004; Narimatsu et al., 2001). A recent study has suggested that inflammation may be a key factor in the development of bladder overactivity, where increased levels of pro-inflammatory cytokines including IL-6 and IL-8 were detected in a chronic bladder ischemia model of overactivity (Nomiya et al., 2012).

Inflammatory cytokines have been also demonstrated to be elevated in our previous cytotoxic drug studies, where urothelial cells were treated with either mitomycin C or

doxorubicin. An increase in IL-8 from RT4 urothelial cells 24 h and 1 week after initial mitomycin C treatment (Kang et al., 2015). In addition, doxorubicin treatment induced the release of IL-8 and IL-1 $\beta$  from RT4 cells 24 h following treatment (Kang et al., 2013). Thus, our data suggests that the induction of inflammatory cytokines and their persistent release are common to chemotherapeutic agents such as doxorubicin, MMC and epirubicin and therefore may play a significant role in development of the bladder overactivity and pain experienced by patients following intravesical chemotherapeutic treatment. In addition, the decrease in stimulated release of ATP one week after the treatment reported here may be due to the effects of inflammatory cytokine release by epirubicin treatment, since a study by Mansfield & Hughes (2014) demonstrated that exposing human urothelial cells to inflammatory mediators (histamine and serotonin) resulted in reduced stretch-induced release of ATP.

Nitric oxide along with acetylcholine is co-released from parasympathetic nerves and exerts an inhibitory effect on smooth muscle contraction (Dokita et al., 1991), but the urothelium is another source of NO in the bladder (Birder et al., 1998; Giglio et al., 2005). The role of urothelial-derived NO in bladder function is uncertain. However, some indirect evidence suggests its potential function in modulation sensory nerve activity (Giglio et al., 2005) and nitric oxide donors have been shown to have inhibitory effects on afferent nerve activity (Aizawa et al., 2011), urothelial contraction (Moro et al., 2012) and pacemaker activity (Moro et al., 2012). Increased levels of nitric oxide have been reported with inflammatory diseases of the bladder and after intravesical immunotherapy for bladder cancer (Ehren et al., 1999; Logadottir et al., 2004). A study involving bladder cancer patients treated with Bacillus Calmette-Guerin has shown localisation of inducible nitric oxide synthase (iNOS) expression on the urothelium (Koskela et al., 2008). Also, increased NOS

expression and enhanced nitric oxide release from the bladder are associated with cyclophosphamide-induced cystitis (Andersson et al., 2008; Aronsson et al., 2014). Increased nitric oxide production in the bladder wall has been reported in patients with interstitial cystitis, with a concurrent increase in IL-6 mRNA (Logadottir et al., 2014).

Several studies have assessed the impact of anthracyclines (including doxorubicin and epirubicin) on NO signalling pathways, in particular in relation to the cardiotoxicity associated with these agents. In the heart, exposure to these drugs increases myocardial iNOS expression resulting in enhanced NO production (Pacher et al., 2003; Weinstein et al., 2000). The present study has demonstrated an increase in NO release from UROtsa cells 24 h after epirubicin treatment. This is consistent with the effects of MMC on RT4 urothelial cells, however the levels returned to normal 1-week following epirubicin treatment unlike in MMC treated cells where the changes in NO release were still evident 1 week later (Kang et al., 2015). These findings suggest that an initial increase in NO is common to both MMC and epirubicin treatment, but recovery to normal levels may be time dependent. These also reflect why side-effects are more commonly reported following MMC treatment compared to other intravesical drugs (Shelley et al., 2012).

Hence, the increase in NO release 24 h following epirubicin treatment may initially contribute to alterations in bladder function experienced in patients treated with intravesical epirubicin, but as there is recovery after 1 week following the treatment, it is likely that the direct effects of changes in nitric oxide in patients treated with intravesical epirubicin is short- rather than long-term.

## **5.0 Conclusion**

In conclusion, the effects of intravesical agents on tumours are well understood, but their safety profiles have received less attention. Due to the targeted delivery method, systemic side effects are rare following intravesical treatments, however, local urinary adverse effects are frequently reported. The results of this study suggest that while alterations in urothelial mediator release (ATP, acetylcholine and NO) could contribute both transiently and long-term to local adverse effects experienced by patients treated with epirubicin, the most likely mechanism causing adverse effects is the induction of urothelial inflammatory cytokine release which is sustained and has been reported to be strongly associated with development of bladder dysfunction such as bladder pain and overactivity.

**Funding Source:** This study was funded by a project grant from Cancer Council Queensland.

**Fig. 1:** Effect of 1 h epirubicin treatment on urothelial cell viability assessed by resazurin reduction immediately [A], 24 h [B] and 1 week [C] after treatment. Data are expressed as means  $\pm$  S.E.M. (N=6). \*\*P<0.01 and \*\*\*P<0.001 compared to vehicle control cultures.

**Fig. 2:** Immediate effects of 1 h epirubicin treatment on basal extracellular ATP [A], acetylcholine [C] and prostaglandin E<sub>2</sub> [E] concentrations, and ATP [B], acetylcholine [D] and prostaglandin E<sub>2</sub> [F] responses to hypo-osmotic stimulation. Data are expressed as means  $\pm$  S.E.M. (N=6). \*\*P<0.01 compared to vehicle control.

**Fig. 3:** Twenty-four-hour post-treatment effects of epirubicin on basal extracellular ATP [A], acetylcholine [C], PGE<sub>2</sub> [E] concentrations, and ATP [B], acetylcholine [D] and PGE<sub>2</sub> [F] responses to cell stretch. Data are expressed as means  $\pm$  S.E.M. (N=6). \*P<0.05 and \*\*P<0.01 compared to vehicle control.

**Fig. 4:** One-week post-treatment effects of epirubicin on basal extracellular ATP [A], acetylcholine [C], PGE<sub>2</sub> [E] concentrations, and ATP [B], acetylcholine [D] and PGE<sub>2</sub> [F] response to cell stretch. Data are expressed as means  $\pm$  S.E.M.. \*P<0.05 compared to vehicle control.

**Fig. 5:** IL-6 secreted from control and epirubicin treated urothelial cells 24 h [A] and 1 week [B] after treatment. IL-8 secreted from control and epirubicin treated urothelial cells 24 h [C] and 1 week [D] after treatment. Nitrite/nitrate released from cells 24 h [E] and 1 week [F] following epirubicin treatment. Data are expressed as means  $\pm$  S.E.M. (N=6). \*\*P<0.01 and \*\*\*P<0.001 compared to vehicle control.

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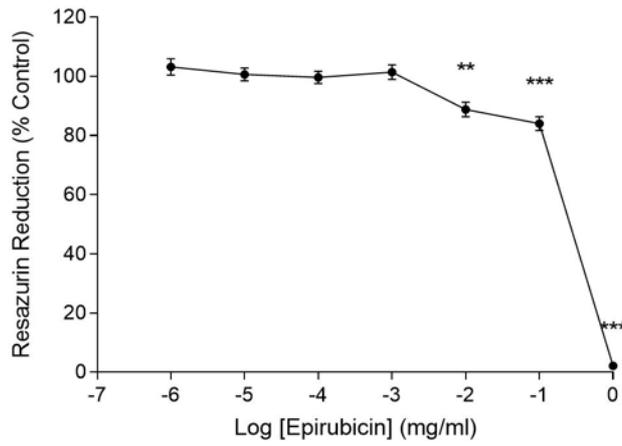
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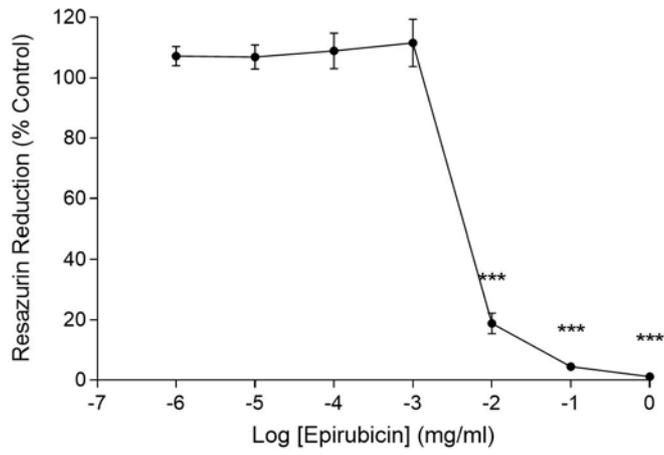
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**A (Immediately)**



**B (24 hour)**



**C (1 week)**

