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Short communication

THE EFFECT OF HYPEROSMOLAR STIMULI AND CYCLOPHOSPHAMIDE ON THE CULTURE OF NORMAL RAT UROTHELIAL CELLS *in vitro*

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Abstract: Highly concentrated urine may induce a harmful effect on the urinary bladder. Therefore, we considered osmolarity of the urine as a basic pathomechanism of mucosal damage. The influence of both cyclophosphamide (CYP) and hyperosmolar stimuli (HS) on the urothelium are not well described. The purpose was to evaluate the effect of CYP and HS on rat urothelial cultured cells (RUCC). 15 Wistar rats were used for RUCC preparation. RUCC were exposed to HS (2080 and 3222 mOsm/l NaCl) for 15 min and CYP (1 mg/ml) for 4 hrs. APC-labelled annexin V was used to quantitatively determine the percentage of apoptotic cells and propidium iodide (PI) as a standard flow cytometric viability probe to distinguish necrotic cells from viable ones. Annexin V-APC (+), annexin V-APC and PI (+), and PI (+) cells were analysed as apoptotic, dead, and necrotic cells, respectively. The results were presented in percentage values. The flow cytometric analysis was done on a FACSCalibur Flow Cytometer using Cell-Quest software. Treatment with 2080 and 3222 mOsm/l HS resulted in

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Abbreviations used: CYP – cyclophosphamide; CGRP – calcitonin gene-related peptide; DO – detrusor overactivity; FBS – fetal bovine serum; GAG – glycosaminoglycans; HEPES – 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid; HS – hyperosmolar stimuli; LUTS – lower urinary tract symptoms; MEM – minimal essential medium; RUCC – rat urothelial cultured cells; TRPV1 – transient receptor potential vanilloid subtype 1; TRPV4 – transient receptor potential vanilloid subtype 4; TRPM8 – transient receptor potential vanilloid subfamily M member 8; TRPA1 – transient receptor potential ankyrin subtype 4; SP – substance P

23.7 ± 3.9% and 26.0 ± 1.5% apoptotic cells, respectively, 14.3 ± 1.4% and 19.4 ± 2.7% necrotic cells, respectively and 60.5 ± 1.4% and 48.6 ± 5.3% dead cells, respectively. The effect of CYP on RUCC was similar to the effect of HS. After CYP the apoptotic and necrotic cells were 23.1 ± 0.3% and 17.9 ± 7.4%, respectively. The percentage of dead cells was 57.7 ± 10.8%. CYP and HS induced apoptosis and necrosis in RUCC. 3222 mOsm/l HS had the most harmful effect based on the percentage of necrotic and apoptotic cells.

Key words: Urothelial cell, Culture, Cyclophosphamide, Hyperosmolarity, Bladder, Rat, Apoptosis, Necrosis, Overactive bladder

INTRODUCTION

Cells die due to necrosis or apoptosis. Apoptosis plays a complementary and opposing role to cell proliferation in regulating the number of cells in tissues [1]. Apoptosis is the process of programmed cell death. During apoptosis cell changes occur (such as blebbing, loss of cell membrane feature, cell shrinkage, nuclear and chromosomal fragmentation, chromatin condensation) followed by cell death. The main difference between necrosis and apoptosis is that apoptotic cells do not stimulate the immune system. In the case of phagocytes they locate, engulf and quickly remove dead cells before the contents of the cell can spill out onto adjacent cells [2].

The urothelium used to be perceived as a passive barrier in the bladder. Nowadays, the urothelium is known to exhibit dynamic sensory properties that convey information regarding the local intravesical environment to bladder afferent fibres [3, 4]. Urothelial cells transduce mechanical, chemical and thermal stimuli via different receptors, e.g. transient receptor potential vanilloid subtype 1 (TRPV1) [5]. Stimulation of these receptors triggers release of neurotransmitters from the urothelial cells, which may activate and/or sensitize the afferent fibres in a paracrine manner [6]. Emerging evidence implies that TRPV1 plays probably a pivotal role in urinary bladder detrusor overactivity (DO) development [7].

Urothelial cell culture provides an *in vitro* model for better understanding of the role of urothelium in the pathogenesis and pathophysiology of urinary tract reconstruction and regeneration, bladder cancer, cystitis, and functional disorders (e.g. overactive bladder) [8]. The most commonly used is a rat model, although the use of rat urothelial cells has been limited due to the difficulty of their isolation and maintenance in a long-term culture [9].

Garland *et al*. [10] revealed that osmotic changes may influence TRPV1 receptor activity. It is believed that cyclophosphamide and hyperosmolar urine may activate afferent fibres directly and/or indirectly via urothelium, leading to DO. Additionally, previous in vivo studies showed that hyperosmolar intravesical stimulation and cyclophosphamide treatment (in acute and chronic models of cystitis) induce detrusor overactivity [11, 12]. Kulick *et al*. reported that the

mean, the lowest and the highest values of rat's urine osmolarity were 2080, 1553 and 3222 mOsm/l, respectively [13]. In the acute model of cyclophosphamide-induced cystitis, DO developed 4 hours after cyclophosphamide administration [11]. Unfortunately, the influence of cyclophosphamide and hyperosmolar stimuli on urothelium is not well described. Therefore, the purpose of the present study was to evaluate the effect of cyclophosphamide and hyperosmolar stimuli on the culture of normal rat urothelial cells (RUCC) *in vitro*.

MATERIAL AND METHODS

Animals

Urothelial cells were isolated from 15 adult female Wistar rats (weight: 200-250 g). Rats were housed individually per cage. The animal room was maintained at a constant temperature (23^{o}C) and humidity with a 12:12 h alternating light-dark cycle. The rats were fed with animal food (Labofeed; Kcynia, Poland) without water restraint. The study was approved by the Regional Animals Ethical Committee (No. 19/2011 - Jagiellonian University, Cracow, Poland).

Urothelial cell isolation and culture

Rat urothelial cell cultures were prepared according to the procedure described by Birder *et al.* [14], with slight modification of the experimental protocol. 15 female Wistar rats were anaesthetized with intraperitoneal injection of 1.2 g/kg urethane (Sigma-Aldrich, St. Louis, USA), and the urinary bladders were removed and placed in cold minimal essential medium (MEM; Invitrogen, Carlsbad, CA) supplemented with 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid (HEPES; 2.5 g/l; Sigma, Germany) and containing penicillin/streptomycin/fungizone (PSF; 1%; Sigma, Germany). The bladder was cut open to expose the urothelium and incubated in dispase (2.5 mg/ml; Sigma, Germany) overnight at 4°C. Urothelial cells were gently scraped from the underlying tissue, placed in trypsin (0.25% wt/vol; Sigma, Germany) for 10-15 min at 37°C, and dissociated by trituration. Cells were suspended in MEM containing 10% fetal bovine serum (FBS; Gibco BRL; Invitrogen, Grand Island, NY) and centrifuged at 416 g for 10 min. The supernatant was removed and cells were suspended in keratinocyte basal medium (Invitrogen) with 1% PSF, centrifuged again, and resuspended in fresh media. Cells were plated in triplicate on temperature-responsive cell culture surface plates (ThermoScientific, NY) at density 1x10^{6} cells/ml and cultured at 37^{o}C in a 5% CO_2 incubator of 90% humidity. Media were changed every other day. Cells were used for experiments after 96 hours of culture.

Urothelial cell culture exposure to hyperosmolar stimuli

Urothelial cell cultures (seeded in triplicate) were exposed to two different hyperosmolar solutions of final NaCl concentration, 2080 mOsm/l and 3222 mOsm/l, for 15 minutes each. Sodium chloride was added to the medium to achieve the final milliosmolarity. Supernatants were discarded, cells washed three times and medium replaced with fresh keratinocyte medium.

Urothelial cell culture exposure to cyclophosphamide
Four-day culture of rat urothelial cells (seeded in triplicate) was exposed to cyclophosphamide at a concentration of 1 mg/ml (Endoxan, BaxterOncology, Germany) for 4 hours. After incubation, culture supernatants were discarded, cells washed three times and medium replaced with fresh keratinocyte medium.

In vitro animal model of rat urothelial cell culture (control group)
Rat urothelial cells (seeded in triplicate) not treated with hyperosmolar solutions of saline (solution osmolarity was 308 mOsm/l) or cyclophosphamide served as controls.

Cell death evaluation by flow cytometry analysis
24 hours after hyperosmolar saline and cyclophosphamide administration the urothelial cells were detached from culture plates by placement of cell cultures at room temperature under sterile conditions for 20 min, strictly according to the manufacturer's instructions. APC-labelled annexin V (BD, Pharmingen^{TM}, USA) was used to quantitatively determine the percentage of cells within the population that were undergoing apoptosis. Propidium iodide (PI; BD, Pharmingen^{TM}, USA) was used as a standard flow cytometric viability probe to distinguish necrotic cells from viable ones. Annexin V-APC positive cells were analysed as apoptotic; annexin V-APC and PI positive cells were either in the end stage of apoptosis or undergoing necrosis and were analysed as already dead; and PI positive cells were necrotic. For staining, urothelial cells were washed twice with cold phosphate-buffered saline (PBS) and resuspended in 1x binding buffer (BD, Pharmingen ^{TM}, USA) at concentration $1x10^6$ cells/ml. Then 100 µl of solution was transferred to a 5 ml culture tube and 5 µl of annexin V-APC and 5 µl of PI were added. Cells were gently vortexed and incubated in the dark for 15 minutes at RT. Prior to flow cytometric analysis 400 µl of 1x binding buffer was added and cells were analysed on a FACSCalibur Flow Cytometer (Becton Dickinson, San Jose, CA) using Cell-Quest software. The Cell-Quest software provided the percentage calculation of the cell types in RUCC. Controls to set up compensation and quadrants included unstained cells, cells stained with annexin V-APC alone (for FL-4 fluorescence) and cells stained with PI alone (detected in FL-3). A minimum of 10.000 events were collected on each sample.

Statistical analysis
All data were expressed as mean and (±) standard deviation (SD) and compared using the Student *t*-test with $p < 0.05$ defined as significantly different.

RESULTS

In vitro animal model of rat urothelial cell culture
The density of rat urothelial cell culture achieved the level of $1x10^6$ cells/ml. The *in vitro* animal model of rat urothelial cell culture was characterized by 18.2% ± 0.5%

apoptotic cells (AnV+ cells) and 6.1% ± 1.8% necrotic cells (PI+ cells). The percentage of dead cells (apoptotic and/or necrotic; AnV+/PI+) was 71.1% ± 4.0% (Tab. 1, Fig. 1).

Tab. 1. Percentages of rat urothelial cell types in normal rat urothelial cell culture (RUCC), and in RUCC after hyperosmolar (2080 mOsm/l and 3222 mOsm/l) and cyclophosphamide (CYP; 1 mg/ml) stimuli.

Percentage of rat urothelial cells in RUCC	Control (normal) rat urothelial cell culture (RUCC)	RUCC with hyperosmolar stimuli HS (2080 mOsm/l)	RUCC with hyperosmolar stimuli HS (3222 mOsm/l)	RUCC with CYP	p
Annexin V positive cells (AnV+) [%]	18.2 ± 0.5	23.7 ± 3.9*	26.0 ± 1.5**, &	23.1 ± 0.3#	*0.066 **0.034 #0.0005 &0.21
Annexin V and PI positive cells (AnV+PI+) [%]	71.1 ± 4.0	60.5 ± 1.4*	48.6 ± 5.3**, &	57.7 ± 10.8#	*0.016 **0.002 #0.07 &0.02
PI positive cells (PI+) [%]	6.1 ± 1.8	14.3 ± 1.4*	19.4 ± 2.7**, &	17.9 ± 7.4#	*0.016 **0.003 #0.05 &0.03

*statistically significant differences between RUCC after 2080 mOsm/l HS stimuli and normal RUCC ($p < 0.05$).
**statistically significant differences between RUCC after 3222 mOsm/l HS stimuli and normal RUCC ($p < 0.05$).
#statistically significant differences between RUCC after CYP stimuli and normal RUCC ($p < 0.05$).
&statistically significant differences between RUCC after 2080 mOsm/l HS stimuli and RUCC after 3222 mOsm/l HS stimuli ($p < 0.05$).

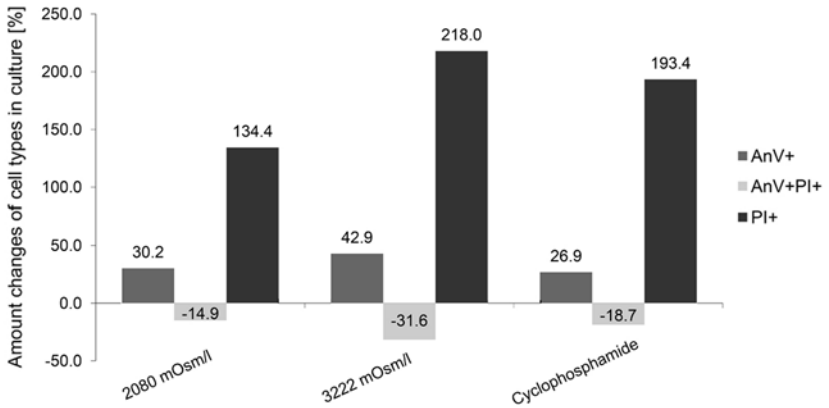

Fig. 1. Percentage changes of rat urothelial cells in RUCC after hyperosmolar (2080 mOsm/l and 3222 mOsm/l) and cyclophosphamide (1 mg/ml) stimuli as compared to control RUCC. The percentage increment (positive values) or decrement (negative values) of the amount of different types of rat urothelial cells was compared to the values of normal RUCC (set at 100%). For example, in RUCC after 2080 mOsm/l HS stimuli there is an increased amount of AnV+ cells, as compared to control (normal) RUCC. The increase is about 30% of the value of normal RUCC.

Effect of hyperosmolar stimuli on rat urothelial cell culture

The hyperosmolar stimulation significantly induced apoptosis and necrosis of urothelial cells of rat urothelial cell culture (RUCC), as compared to control RUCC. The percentage of apoptotic cells (AnV+) in 2080 mOsm/l and 3222 mOsm/l hyperosmolar saline stimulated RUCC was 23.7% ± 3.9% and 26.0% ± 1.5%, respectively. Moreover, the percentage of necrotic cells (PI+) in 2080 mOsm/l and 3222 mOsm/l hyperosmolar saline stimulated RUCC was 14.3% ± 1.4% and 19.4% ± 2.7%, respectively. As a result of increased quantity of apoptotic and necrotic cells separately, the average of dead cells (AnV+/PI+) was significantly lower in 2080 mOsm/l and 3222 mOsm/l hyperosmolar saline stimulated RUCC (60.5% ± 1.4% and 48.6% ± 5.3%, respectively), as compared to control RUCC (71.1% ± 4.0%). The 3222 mOsm/l hyperosmolar saline had the most harmful effect on the RUCC (severity of apoptosis and necrosis). Additionally, the amount of AnV+ cells and PI+ cells increased with the increase of the hyperosmolarity from 2080 mOsm/l to 3222 mOsm/l (Tab. 1).

Effect of cyclophosphamide on rat urothelial cell culture

The effect of cyclophosphamide on RUCC was similar to the effect of hyperosmolar stimulation of RUCC. After CYP treatment the percentage of apoptotic cells (AnV+) and necrotic cells (PI+) was 23.1% ± 0.3% and 17.9% ± 7.4%, respectively. In comparison to control RUCC, the average of dead cells (AnV+/PI+) was significantly lower (71.1% ± 4.0% vs. 57.7% ± 10.8%), as a result of increased incidence of AnV+ and PI+ urothelial cells (Tab. 1, Fig. 1).

DISCUSSION

This study demonstrates that cultured urothelial cells from rat were affected by cyclophosphamide and hyperosmolar stimuli. The hyperosmolar stimulation and cyclophosphamide significantly induced apoptosis and necrosis of urothelial cells, as compared to control.

Urothelial cells play an important role in physiology and in the pathophysiology of functional urinary tract diseases (e.g. overactive bladder, interstitial cystitis, painful bladder syndrome, etc.). Despite previous theories that the urothelium is only a passive membrane between urine and the urinary tract, nowadays the urothelium is known to be an active barrier which regulates the urinary bladder activity, as well as presenting significant changes in case of bladder disorders. Urothelial cells exhibit neuron-like properties that contribute to afferent activity [15]. Additionally, urothelial cells express a wide range of receptors (e.g. vanilloids, purinergic) and ion channels, and also release neurotransmitters (adenosine triphosphate, nitric oxide, acetylcholine, etc.) in response to different stimuli (mechanical, thermal, chemical, etc.). Evidence shows that all above-mentioned components are implicated in the interaction between nerves fibres and urothelial cells. The substances released from urothelial cells can alter the excitability of bladder afferent fibres, influencing bladder activity [14, 16, 17].

Such a pathomechanism appears to be of particular importance in the development of functional bladder disorders, especially in the case of detrusor overactivity.

Other scientists have described in vivo experiments on detrusor overactivity in animal models using cyclophosphamide and hyperosmolar stimuli [11, 12]. However, still there are no data regarding the influence of cyclophosphamide and hyperosmolar stimuli on the culture of normal rat urothelial cells *in vitro*. The mode of action of hyperosmolar saline and cyclophosphamide is distinctive. Protein-protein interactions and different proteases are crucial for most cellular processes, cell motility and apoptosis [18, 19]. In the case of hyperosmolar solution, the urothelial cells may die as a consequence of dehydration, caused by changes in molecular protein-protein interaction and nucleic acid structure. The development of the detection of circulating nucleic acids provides screening for several disorders [20]. Therefore, such a diagnostic tool may be applied in the evaluation of bladder disorders; however, large-scale studies are strongly required. In contrast, cyclophosphamide as a cytostatic agent affects the cell cycle via changes of DNA structure. Differences in the percentages of apoptotic and necrotic cells obtained after cyclophosphamide and hyperosmolarity may result from different cell death pathways triggered upon chemical and stress stimuli.

Previous studies revealed that a single dose of cyclophosphamide administered intraperitoneally leads to widespread necrosis of the urothelium in rats. After 24 hours only a few urothelial cells survive and retain their ability to proliferate and re-epithelialise damaged areas [21, 22]. In the normal state, the apoptosis of urothelial cells of the urinary bladder takes place at low rates. CYP treatment increases the number of apoptotic urothelial cells during the regeneration processes [23]. Also, different mechanisms of apoptotic cell removal from the tissue have been described: the cells can be exfoliated into the lumen of an organ; they can be phagocytosed and digested, mostly by macrophages, or even by adjacent cells in tumours [23-26].

Moreover, Kullmann *et al*. [27] showed that stimulation of different receptors (TRPV1, TRPV4, TRPM8, TRPA1, and ASIC-like channels) in primary urothelial cell culture induces ionic currents and changes in intracellular calcium concentration. It was postulated that TRP channels might be involved in the mechanisms underlying the release of transmitters from the urothelium and might contribute to the putative sensory function of the urothelium, as well as playing a role in homeostatic mechanisms related to cell proliferation and differentiation.

Recent evidence and the present investigation showed that the way urothelial cells die might influence the urinary bladder and detrusor activity [28-30]. The stimulation of vanilloid-sensitive nerve terminals through vanilloid receptors (TRPV1) by vanilloids or other agents (bradykinin, protons, etc.) may release different neuropeptides such as substance P (SP), calcitonin gene-related peptide (CGRP) and interleukins, generating local responses in blood vessels, mastocytes and lymphocytes, causing neurogenic inflammation and in consequence leading to detrusor overactivity [31]. Contrary to apoptosis, the necrosis of urothelial cells provides an extensive release of neurotransmitters

from dead cells and consequently activates bladder afferent C fibres and induces neurogenic inflammation, leading to detrusor overactivity or deterioration of existing chronic bladder illness. Therefore, it is important to prevent necrosis of urothelial cells for better control and/or reduction of lower urinary tract symptoms (LUTS) in the case of urinary bladder disorders (especially detrusor overactivity). Instillations of 0.2% sodium chondroitin sulphate resulted in a more sustained improvement or cure of the symptoms of bladder overactivity due to development of a glycosaminoglycan layer [32]. Also Yeh *et al*. [33] revealed that hyaluronic acid treatment can ameliorate H_2O_2-induced bladder hyperactivity, probably via antioxidant activity and the inhibition of purinergic and muscarinic pathways. Intravesical glycosaminoglycan (GAG) instillations may be crucial for reducing urothelial cell necrosis in the urinary bladder, because the GAG may develop the layer covering the bladder urothelium and protect against intravesical agents.

CONCLUSION

In conclusion, this study demonstrated that cultured urothelial cells from rat were affected by cyclophosphamide and hyperosmolar stimuli leading to apoptosis and necrosis of urothelial cells of rat urothelial cell culture. Additionally, osmotic damage (by the highest concentration of urine) to urothelial cells is comparable to chemical cyclophosphamide-induced apoptosis and necrosis of rat urothelial cell culture.

Conflict of interests - None declared.

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