In-vitro cytocidal effect of water on bladder cancer cells: The potential role for intraperitoneal lavage during radical cystectomy

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Abstract

Introduction: We investigate the cytocidal effect of water on bladder cancer cells. Intraperitoneal lavage with sterile water is sometimes used during radical cystectomy to lyse cancer cells that might have escaped the surgical specimen. The efficacy of this approach at the cellular level is unknown.
Methods: Three bladder cancer cell lines of varying grade, RT4, TCCSUP and T24 were exposed to sterile water, and morphological changes were closely observed under microscopy. Changes of cell membrane integrity, cell viability, and cell number of re-incubated cells after water exposure were measured to determine water induced hypotonic shock.
Results: The low-grade RT4 cells started swelling immediately upon exposure to water followed by rupture within 3 minutes. The higher grade TCCSUP and T24 cells demonstrated limited hypotonic swelling with significantly less cell rupture after 10 minutes. The damage to cell membrane of RT4 cells was evident at 1 minute; only 10.0% of cells were intact at 10 minutes. On the other hand, 41.9% and 77.8% of TCCSUP and T24 cells were intact at 10 minutes, respectively. Percentage of viable cells at 10 minutes was 2.1 ± 2.3%, 2.3 ± 0.4%, and 16.1 ± 0.6% for RT4, TCCSUP, and T24, respectively.
Conclusions: Cytocidal effect of hypotonic shock can be achieved, to varying degrees, by exposing bladder cancer cells to water for at least 10 minutes. This in vitro study may have bearing on the effects seen with intraperitoneal lavage using sterile water during radical cystectomy.

Introduction

Radical cystectomy is the main stay of therapy for muscle-invasive bladder cancer. Despite well-performed surgeries, the cure rates with surgery alone are 52% to 72.9% for organ-confined disease.[1,2] In patients who develop a pelvic recurrence after surgery, cure rates are extremely poor at 3%.[3] While some pelvic recurrences are explained by positive margins, many are not. It has been hypothesized by some that a non-insignificant number of these recurrences might be due to viable tumour cells that escape the surgical specimen during radical cystectomy, despite best practices to prevent this from occurring.

It is common practice among oncologic surgeons to irrigate the surgical field (in this case the pelvis and lower retroperitoneum) after surgical extirpation. While there are several reasons for doing so (i.e., evaluating hemostasis, diluting microbe counts), one goal is also to potentially dilute and lyse viable tumour cells that might be free floating. This practice is driven by the theoretical potential for sterile water to initiate water-induced hypotonic shock resulting in cell lysis and thus decreased tumour cell burden, seeding potential, and local recurrence.[4-6] There are conflicting data regarding the utility of this approach; others have even considered intraperitoneal chemotherapy to lyse potential tumour cells.[7,8]

While studies have attempted to explain the exact molecular mechanism in support of intraperitoneal lavage with sterile water, its benefit is controversial. In one study analyzing cultures of bladder cancer cells, sterile water led to significant cytolysis.[9] The effect of sterile water was comparable to mitomycin C in bladder cancer cells.[9] In addition, it was reported that the bladder irrigation with sterile water after transurethral resection reduced recurrence rates of non-muscle-invasive bladder cancer.[10,11] While the effect of intraperitoneal lavage with sterile water has been studied in colorectal[12] and ovarian cancer,[13] data are lacking in bladder cancer literature in regards to optimal duration of sterile water exposure. We investigated the effect of water on bladder cancer cells by analyzing changes in cellular morphology, viability, membrane integrity, and DNA fragmentation at varying time points of water exposure.

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Methods

Cell culture and preparation

Human bladder cancer cell lines RT4 (low grade), T24 (high grade) and TCC-SUP (high grade) were obtained from the American Type Culture Collection (ATCC) and fingerprinted by the Specimen Core of the MD Anderson Specialized Program of Research Excellence in Bladder Cancer. These cell lines were maintained as adherent cells at 37°C, 5% CO_2 in a humidified atmosphere in MEM supplemented with 10% FBS, penicillin, streptomycin, vitamins, L-glutamine, non-essential amino acids, and pyruvate supplements.

After culturing bladder cancer cells in T75 flasks, medium was completely removed from the flasks and sterile water was added. The flasks were mounted on the stage of Olympus IX71 live cell imaging microscope (Olympus, Tokyo, Japan), and serial changes in bladder cancer cells were recorded at 0, 1, 3, 5, and 10 minutes using a 20× magnification.

A total of 5×10^3 cells/well were plated in 96-well plates. After 24 hours of incubation, these cells were exposed to sterile water for 1, 3, 5, or 10 minutes after which the water was exchanged for medium. After 24 hours, cell viability was assessed by pulsing the cells for 2 hours with dimethyl thiazolyl diphenyl tetrazolium salt (MTT) (5 mg/mL in PBS) followed by solubilization of Formazan crystals in 100 μL of lysis buffer containing 20% sodium dodecyl sulfate and 50% dimethylformamide. Colour development was quantified by measuring the optical densities at 570 nm. All measurements were repeated in quadruples and the results were shown by mean ± standard error of the mean (SEM).

Bladder cancer cells were detached from culture flasks in a trypsin-EDTA solution and centrifuged. The cells were suspended in medium and 2×10^5 cells were placed in each eppendorf tube. The cell suspension was centrifuged and the pelleted cells were re-suspended in sterile water and incubated for 10, 30 or 60 minutes. Thereafter, the suspension was centrifuged, and the cells in pellets were suspended in medium and seeded. At 48 hours after plating, the cells were detached from the plates in a trypsin-EDTA solution and counted on a hemocytometer. All measurements were repeated in triplicates and the results were shown by mean ± SEM.

Bladder cancer cells were detached from culture flasks in a trypsin-EDTA solution and centrifuged. The pellets were suspended in 5 mL medium and the cell suspension was divided in 5 tubes. After centrifuge, the pelleted cells were re-suspended in sterile water and incubated for 1, 3, 5 or 10 minutes. The suspending solution was displaced into a Vi-CELL Sample Cup (Beckman Coulter, Fullerton, CA) and the intact cell membranes of bladder cancer cells were calculated using the trypan blue-exclusion method. All analyses were performed in triplicates and the results were shown by mean ± SEM.

Results

Morphological changes were seen in all three bladder cancer cell lines after exposure to sterile water (Fig. 1). RT4 cells started swelling immediately upon exposure to sterile water followed by rupture within 3 minutes. T24 and TCCSUP cells demonstrated limited hypotonic swelling with few cell ruptures within 10 minutes.

The viability of bladder cancer cells were observed after exposure to sterile water by MTT assay (Fig. 2). The viability of RT4 cells started to decrease to 23.5 ± 1.2% after 1-minute exposure time to sterile water. TCCSUP and T24 cells, on the other hand, required 5 and 10 minutes, respectively, to achieve similar decreases in cell viability. Percentage of viable cells at 10 minutes was 2.1 ± 2.3%, 2.3 ± 0.4%, and 16.1 ± 0.6% for RT4, TCCSUP, and T24, respectively.

In the trypan blue-exclusion assay (Fig. 3), live cells with intact cell membrane are not stained. We determined the percentage of live cells with intact cell membranes measuring non-stained cells after exposure to sterile water. Similar to results from the MTT assay, percentage of intact membranes in RT4 cells decreased more than twofold after a 1-minute exposure time to water and decreased to 10.0 ± 0.1% after 10 minutes. In both the TCCSUP and T24 cells, percentage of intact membranes was greater than 40% even after a 10-minute exposure time.

We also evaluated the cell number of re-incubated cells after exposure to sterile water (Fig. 4). At 10 minutes exposure to sterile water, cell numbers were 2.2 ± 0.4% of control in the RT4 cell line. On the other hand, T24 and TCCSUP cells needed more than 30 minutes of exposure to sterile water to reach a similar decrease in cell number.

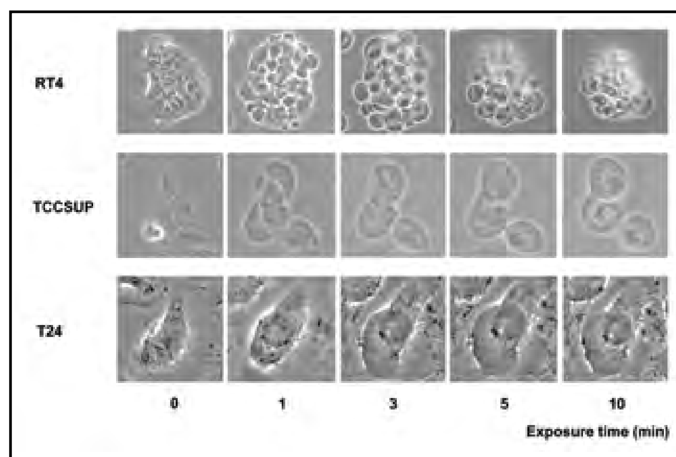

Fig. 1. Representative images of RT4, TCCSUP and T24 cells before and after exposure to sterile water. RT4 cells started to swell immediately, then followed by cell rupture. On the other hand, T24 cells kept its form up to 10 minutes.

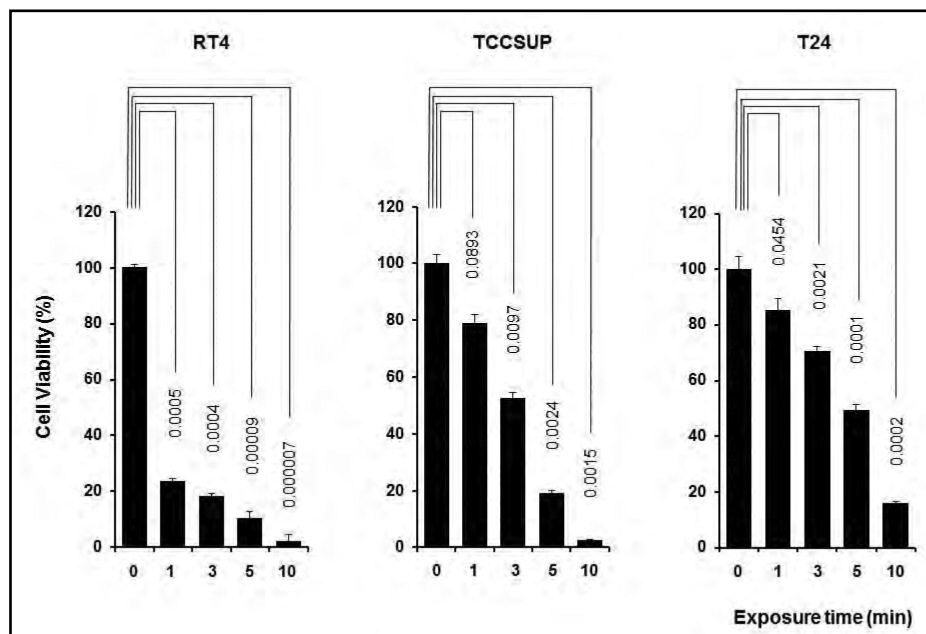

Fig. 2. Cell viability of bladder cancer cells after exposure to sterile water. The viability of RT4 cells started to decrease within short exposure to sterile water, while T24 cells showed resistance to sterile water treatment.

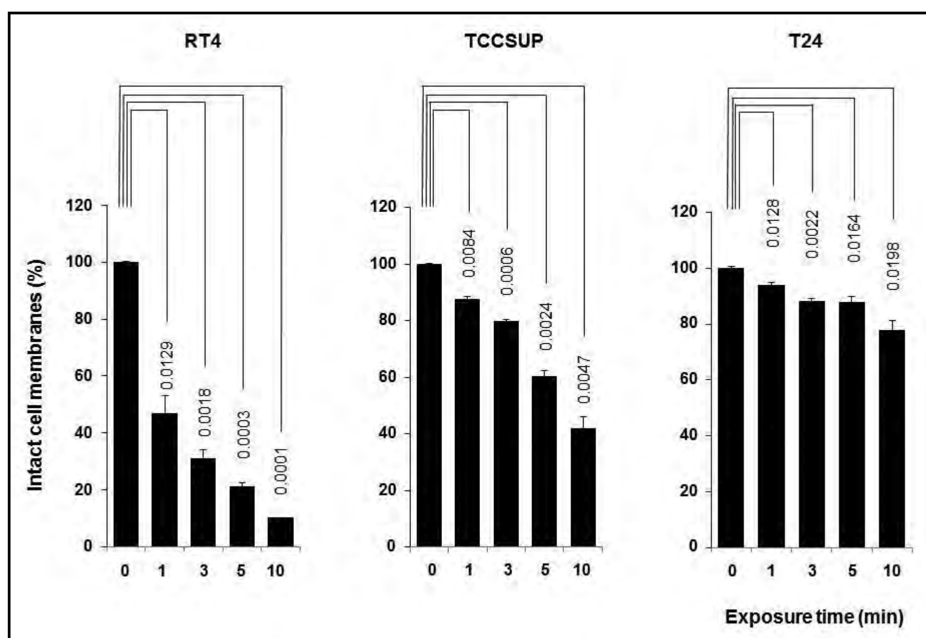

Fig. 3. Intact cell membranes of bladder cancer cells after exposure to sterile water. The damage of cell membranes in RT4 cells was evident at 1 minute exposure to sterile water. In both the TCCSUP and T24 cells, percentage of intact membranes was not reduced to below 40% even after a 10 minute exposure time.

Discussion

Radical cystectomy with extended pelvic lymph node dissection is the gold standard for patients with muscle-invasive bladder cancer. With advances in surgical technique and an improved understanding of the disease process, oncologic outcomes have improved. However, many patients still recur despite these improvements.[1,14]

It is commonly believed that pelvic recurrence is infrequent and is due to nodal metastasis and positive surgical margins, however this is not entirely accurate. The Vanderbilt group reviewed their experience and found that

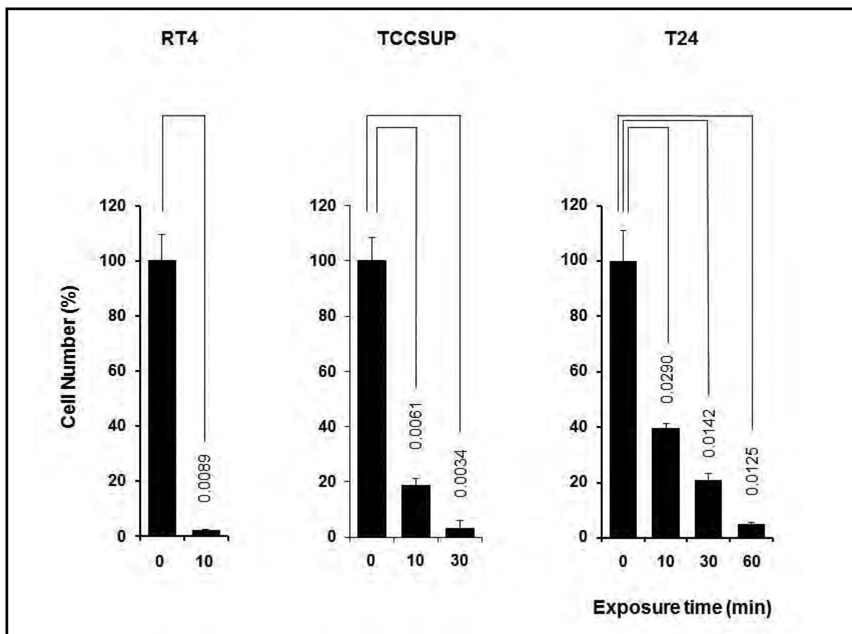

Fig. 4. The cell number of re-incubated bladder cancer cells after exposure to sterile water. At 10 minutes exposure to sterile water, there were few attached RT4 cells. TCCSUP and T24 cells needed more than 30 minutes exposure to be killed completely.

initial recurrence was most common in the pelvis, and was statistically related to lymphovascular invasion but not stage, positive margins or positive nodes.[15] Reports such as these have prompted experts to suggest that a major cause of pelvic recurrences might be seeding which occurs at the time of radical cystectomy, when viable tumour cells escape the surgical specimen.

A common practice among oncologic surgeons is to irrigate the surgical field after surgical extirpation to potentially dilute and lyse viable tumour cells that might be free floating. While this practice is not unique to our institution or urology, there is little evidence to suggest the utility of this practice or appropriate duration of this treatment to have an effect on bladder cancer cell death.[7] Prior research studies in colorectal and ovarian cancer show conflicting results in regards to the cytolytic efficacy of intraperitoneal lavage with sterile water.[12,13] Furthermore, prior bladder cancer research has demonstrated conflicting evidence regarding the role of intraperitoneal lavage with sterile water.[7-9]

Our study was specifically intended to study the time course of events in bladder cancer cells after exposure to water. First, we confirmed the cytocidal effect of hypotonic shock induced by sterile water on bladder cancer cells. In particular, RT4 cells, derived from a low-grade tumour, clearly showed cell swelling followed by cell rupture after exposure to sterile water. T24 cells demonstrated limited hypotonic swelling with limited cell ruptures. Potentially, the strength of cytoskeleton, cell membrane or the expression of chloride channels may differ among bladder cancer cell lines of different grades as seen in other malignancies.[16] While the concept of osmotic cell lysis is not novel, we were interestingly able to discern varying susceptibilities of different bladder cancer cell lines to water induced-hypotonic shock effect.

Second, we calculated the duration of exposure to sterile water required to lyse bladder cancer cells of varying cell lines. This is the first study, to our knowledge, which was able to quantify the exposure time required to achieve cell lysis and decrease cell viability. The lytic effect of sterile water on tumour cells has been well-studied in a variety of cancer settings,[7-9,13] however, the dwell time required to achieve cancer cell lysis has not been reported. We found the required duration of exposure to sterile water to severely limit the number of cancer cells in each bladder cell line by a re-incubation experiment.

In the present study, RT4 bladder cancer cells appeared to be the most sensitive to sterile water among the cell lines. Most of re-incubated RT4 cells could not survive after 10 minutes exposure to sterile water. These findings indicate the cytolytic efficacy of intraperitoneal lavage with sterile water for 10 minutes to some extent. On the other hand, T24 cells needed more than 30 minutes of exposure to sterile water to have a cytocidal effect. In the human setting, intraperitoneal lavage for 30 minutes or more is difficult at radical cystectomy. Sterile water lysis does not compensate for a low quality oncologic procedure.

Our findings must be interpreted in the context of the study design: our data were generated from cultured bladder

cancer cell lines of different grades in an in vitro setting. In the clinical setting, inherit characteristics of patients' cancer cells and immunology likely play a significant role not only in the survival of cancer cells. Even with these caveats, sterile water is readily available and relatively inexpensive. In addition, the simplicity of performing intraperitoneal lavage with sterile water does not add unnecessary complications to surgery other than the additional time required. While we also use (and encourage others to use, methods such as ligation of the distal ureteral stump, ligation of the urethra during surgery), the use of lavage is a simple measure that can aid in the lysis of cells that might escape the surgical specimen. Although there may be incremental benefits of increased cell lysis with additional exposure time of cancer cells to water, this needs to be individually weighed by the risk of prolonging the time of surgery for the patient.

Conclusion

Bladder cancer cell lines differ in their sensitivity to hypotonic shock of sterile water, with low-grade cells undergoing the fastest and most significant decrease in viability. The exposure to water is time dependent with increased tumour cell lysis observed with prolonged exposure.

Competing interests: Rikiya Taoka, Stephen B. Williams, Philip L. Ho, and Ashish M. Kamat all declare no competing financial or personal interests.

This paper has been peer-reviewed.

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