Chloride Ion Transport Blockade Enhances Cytocidal Effects of Sterile Water on Bladder Cancer Cells

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Abstract

Background:

Bladder cancer is a major health concern worldwide. The conventional intravesical Bacillus Calmette–Guérin therapy has certain shortcomings; thereby, demanding novel alternatives. Although sterile water is a probable agent for such novel intravesical therapies, bladder cancer cell lines differ in their sensitivity to hypotonic shock due to sterile water. Therefore, we aimed to investigate whether Cl^- channel blockers enhance the cytocidal effect of hypotonic shock on bladder cancer cells resistant to sterile water.

Methods:

Bladder cancer cell lines of varying grades (RT112, T24, and J82) were exposed to sterile water, and morphological changes were closely observed using microscopy. Sterile water-induced changes in cell membrane integrity and cell viability were analyzed to determine the effects of hypotonic shock. These effects were further analyzed using a Cl^- channel blocker.

Results:

T24 and J82 cells started swelling immediately upon exposure to sterile water and ruptured within 10 min. RT112 cells demonstrated limited hypotonic swelling with few cell ruptures. After treatment with the Cl^- channel blocker, RT112 cells ruptured faster as compared to that in cells treated with sterile water. The percentages of viable dimethylsulfoxide and 5-nitro-2-(3-phenylpropylamino) benzoic acid -treated (50, 100, 200, and 300 μM) RT112 cells after 10 min of exposure to sterile water were 13.6 % ± 3.4 %, 6.3 % ± 1.2 %, 2.0 % ± 1.1 %, 0.7 % ± 0.7 %, and 0 %, respectively.

Conclusions:

Taken together, the Cl^- channel blockers enhanced the cytocidal effects of hypotonic shock in bladder cancer cells. Intravesical therapy with sterile water after treatment with a Cl^- channel blocker represents a potential new adjuvant therapy after TURBT with high efficacy.

Background

Bladder cancer is the ninth most common cancer worldwide with 390,000 new cases diagnosed and 150,000 deaths each year [1]. Nearly three out of four diagnosed patients have non-muscle-invasive bladder cancer (NMIBC). During the progression of NMIBC, 50 % of patients exhibit recurrence among which 9 % of cases invade the *muscularis propria* [2]. Radical cystectomy is the mainstay of therapy for muscle invasive bladder cancer. Intravesical Bacillus Calmette–Guérin (BCG) after transurethral resection of bladder tumor (TURBT) reduced the risk of intravesical recurrence in patients with high-risk NMIBC [3, 4, 5]. Intravesical BCG is more effective than intravesical chemotherapy [6, 7]. Therefore, the European

Association of Urology guidelines recommend performing intravesical BCG after TURBT in patients with high-risk NMIBC [8].

However, intravesical BCG is associated with more side effects compared to those associated with intravesical chemotherapy. Notably, 19 % of patients who underwent intravesical BCG were made to terminate therapy owing to the associated side effects in the European Organization for the Research and Treatment of Cancer Genito-Urinary Group trial 30911 [7]. Moreover, the current shortage of BCG is a global problem that has resulted in increasing demand and supply constraints. Therefore, it is imperative to develop novel intravesical therapy.

We have previously revealed the cytocidal effect of sterile water on bladder cancer cells [9, 10]. Although sterile water may be a new agent for intravesical therapy, bladder cancer cell lines differ in their sensitivity to hypotonic shock with sterile water. Therefore, future studies should focus on detailed analyses of the cytocidal effects of hypotonic shock for successful clinical application.

In this study, we treated bladder cancer cells with 5-nitro-2-(3-phenylpropylamino) benzoic acid (NPPB), a Cl^- channel blocker since Cl^- outflow via Cl^- channels is key in regulating cell volume during hypotonic shock. The study findings demonstrate the efficacy of intravesical therapy using sterile water after TURBT.

Methods

Cell culture, observation of morphological changes, cell proliferation assay and cell membrane analysis were performed with reference to previous studies [9, 10, 15].

Cell culture

Human bladder cancer cell lines RT112, T24, and J82 were obtained from the American Type Culture Collection. We selected one low-grade cell line (RT112) and two high-grade cell lines (T24 and J82) for this study. These adherent cell lines were maintained at 37 °C and 5 % CO_2 in a humidified atmosphere in RPMI-1640 (Wako, Osaka, Japan) supplemented with 10 % fetal bovine serum (Sigma, St. Louis, MO, USA), HEPES (Sigma), and penicillin-streptomycin (Thermo Fisher Scientific, Waltham, MA, USA).

Morphological changes in bladder cancer cells after exposure to sterile water

After culturing bladder cancer cells in T75 flasks, the medium was completely removed and sterile water was added. For the NPPB experiments, the cells were pre-incubated with culture medium containing 50, 100, 200, and 300 μM NPPB for 30 min at 37 °C in a 5 % CO_2 containing humidified atmosphere. The flasks were mounted on the stage of a KEYENCE BZ-9000 All-in-One Fluorescence Microscope (Osaka, Japan), and changes in the cells were recorded at 0, 1, 3, 5, and 10 min.

Cell viability

Cells were plated in 96-well plates at a density of 5×10^3 cells/well. After 24 h of incubation, cells were exposed to sterile water for 1, 3, 5, or 10 min after which the water was replaced with medium. In the control group, the medium was replaced at the same time. For the NPPB experiments, cells were pre-incubated with culture medium containing 50, 100, 200, and 300 μM NPPB for 30 min at 37 °C in a 5 % CO_2 containing humidified atmosphere. After 24 h, cell viability was assessed by pulsing the cells for 2 h with dimethyl thiazolyl diphenyl tetrazolium (MTT; 5 mg/mL in phosphate-buffered saline) followed by solubilization of formazan crystals in 100 μL of lysis buffer containing 20 % sodium dodecyl sulfate and 50 % dimethylformamide and colorimetry at 570 nm. All measurements were in quadruplicates, and data were represented as mean ± standard error of mean.

Analysis of cell membranes

Bladder cancer cells were detached from the culture flasks using trypsin-EDTA and centrifuged. The pellets were resuspended in 5 mL of medium and the cell suspension was divided into five tubes. After centrifugation, the pelleted cells were resuspended in sterile water and incubated for 1, 3, 5, or 10 min. For the NPPB experiments, cells were pre-incubated with culture medium containing 50, 100, 200, and 300 μM NPPB for 30 min at 37 °C in a 5 % CO_2 containing humidified atmosphere. The suspensions were added to LUNA-II™ (Logos Biosystems, Gyeonggi-do, South Korea), and the percentage of intact cell membranes was determined using the trypan blue-exclusion method. All analyses were performed in triplicates, and the data have been represented as mean ± standard error of mean.

Results

Morphological changes were observed in the bladder cancer cell lines after exposure to sterile water (Fig. 1). T24 and J82 cells started swelling immediately upon exposure to sterile water and ruptured within 10 min. RT112 cells demonstrated limited hypotonic swelling with few cell ruptures. High concentrations of NPPB enabled RT112 cells to rupture faster as compared to dimethyl sulfoxide (DMSO; Fig. 2).

Next, we used the MTT assay to analyze bladder cancer cell viability after exposure to sterile water (Fig. 3). In each of the bladder cancer cell lines, the decrease in cell viability was dependent on the duration of exposure to sterile water. We also found differences in the cytocidal effects of hypotonic shock on RT112, T24, and J82 cells induced by sterile water. RT112 cells were more resistant to hypotonic shock, even when exposed to sterile water for 10 min. We analyzed cell viability in NPPB (50, 100, 200, and 300 μM) and DMSO-treated cells after exposure to sterile water. NPPB treatment decreased the viability of the bladder cancer cells upon exposure to sterile water than the viabilities observed in DMSO-treated cells (percentage of viable DMSO and NPPB-treated [50, 100, 200, and 300 μM] RT112 cells after 10 min of exposure to water: 9.1 % ± 2.3 %, 2.3 % ± 0.7 %, 1.7 % ± 0.5 %, 0.8 % ± 0.5 %, and 1.2 % ± 1.2 %, respectively).

In the trypan blue-exclusion assay (Fig. 4), live cells with intact cell membranes were not stained. We determined the percentage of live cells with intact cell membranes by counting non-stained cells after

exposure to sterile water. The trypan blue-exclusion assay showed results similar to those obtained using the MTT assay. The percentages of viable DMSO and NPPB-treated (50, 100, 200, 300 μM) RT112 cells after 10 min of sterile water exposure were 13.6 % ± 3.4 %, 6.3 % ± 1.2 %, 2.0 % ± 1.1 %, 0.7 % ± 0.7 %, and 0 %, respectively.

Discussion

We confirmed that Cl^- channel blockers enhanced the cytocidal effects of hypotonic shock using a range of bladder cancer cells. Thus, intravesical therapy using sterile water with a Cl^- channel blocker may be a feasible treatment option for NMIBC patients, thereby avoiding radical surgery.

We have previously revealed cytocidal effects in bladder cancer cells [9, 10]. However, the phenotypes were different in each bladder cancer cell line. Moreover, clinical research has demonstrated no significant differences in the recurrence-free rates between patients undergoing sterile water irrigation and control patients [11]. Thus, simply irrigating with sterile water may not be effective in clinical application.

Recent reports have shown the roles of ion transporters in cancer cells; numerous types of ion transporters affect various organs in cancer patients [12, 13, 14]. Cl^- channels/transporters are important in cancer cells. Iitaka et al. reported that NPPB increases cell volume by inhibiting regulatory volume and enhancing the cytocidal effects of the hypotonic solution in gastric cancer cells [15]. The activation of Cl^- channels is key in regulating the cell volume of several gastric cancer cells. The inhibition of Cl^- channels during hypotonic shock enhances cell swelling, thereby enabling its cytocidal effects.

Mechanisms underlying the regulation of cell volume have been studied upon exposing cells to abrupt changes in extracellular osmolarity. Cells exposed to hypotonic extracellular fluids initially swell as more or less perfect osmometers but approach the original cell volume based on regulatory cell volume decrease (RVD). Cells exposed to hypertonic extracellular fluids initially shrink like almost perfect osmometers but approach original cell volume by regulatory cell volume increase. Ion transport across the cell membrane is the most efficient and rapid way of altering cellular osmolarity [16].

Several ion transport systems are activated by cell swelling during RVD. RVD is caused by the outflow of water accompanying the extracellular discharge of KCl. K^+ channel, Cl^- channel, or K^+/Cl^- co-transporter are the most frequently utilized transport systems for the release of KCl. Intracellular Cl^- activity is important for the regulation of intracellular osmolarity. Intracellular Cl^- activity decreases during osmotic cell swelling. However, osmotic cell shrinkage is expected to increase intracellular Cl^- activity. Thus, Cl^- transport plays an important role in human cells.

Therefore, this study investigated whether Cl^- channel blockers enhance the effect of sterile water on bladder cancer cells. As in previous studies, bladder cancer cells differed in their sensitivity to sterile

water-induced hypotonic shock. RT112 cells demonstrated limited hypotonic swelling with less cell rupture. However, the cytocidal effect of sterile water was enhanced in cells after treatment with the Cl^- channel blocker. RT112 cells treated with the Cl^- channel blocker showed faster swelling and rupture upon exposure to sterile water as compared to the phenotypes observed in control cells. Moreover, the percentage of live cells after exposure to sterile water with Cl^- channel blocker decreased as compared to the control cells. These findings provide experimental evidence that the combination of sterile water with Cl^- channel blocker was more effective in imparting hypotonic shock-induced cytocidal effects on cultured bladder cancer cells otherwise resistant to sterile water.

The high recurrence rate in NMIBC is attributed to the adhesion of free-floating tumor cells during TURBT. The most significant potential use of this treatment is in early post-operative instillation since floating tumor cells have the greatest contact area with sterile water. We believe that single bladder irrigation directly after TURBT is the most efficient. Thus, we plan on conducting a pilot study for single bladder irrigation with Cl^- channel blocker and sterile water for 30 min each to achieve successful clinical application.

The major limitation of the present study is that the effect of sterile water and Cl^- channel blocker on normal urothelium was not investigated. Nonetheless, our observations of the novel cytocidal effect of sterile water with the Cl^- channel blocker against bladder cancer cell indicate that this is a potentially effective therapy useful for reducing the risk of intravesical recurrence in patients with NMIBC.

Conclusions

In summary, the blockade of the Cl^- channel enhanced the cytocidal effects of hypotonic shock in bladder cancer cells. However, this study has some limitations: these findings must be validated using a patient cohort. This is because specific characteristics of patient cancer cells and immune system are important for cancer cell survival and other factors. Nonetheless, intravesical therapy with sterile water after treatment with a Cl^- channel blocker may be a novel adjuvant therapy after TURBT that is associated with high efficacy and low cost.

Abbreviations

NMIBC: non-muscle-invasive bladder cancer; BCG: Bacillus Calmette–Guérin; TURBT: transurethral resection of bladder tumor; NPPB: 5-nitro-2-(3-phenylpropylamino) benzoic acid; MTT: dimethyl thiazolyl diphenyl tetrazolium; DMSO: dimethyl sulfoxide; RVD: regulatory cell volume decrease.

Declarations

1. Ethics approval and consent to participate:

Not applicable

2. Consent for publication:

Not applicable

3. Availability of data and materials:

All data are available from the authors on request.

4. Competing interests:

The authors declare that they have no competing interests.

5. Funding:

This research received no external funding.

6. Authors' contributions:

RT and MS conceptualized and designed the study. YM, YT, and ZX were involved in data acquisition. YM analyzed and interpreted the data. YM drafted the initial manuscript. RT critically revised the manuscript. YM conducted statistical analysis. RT and MS supervised the study overall. All authors have read and approved the final manuscript.

7. Acknowledgements:

Not applicable

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Figures

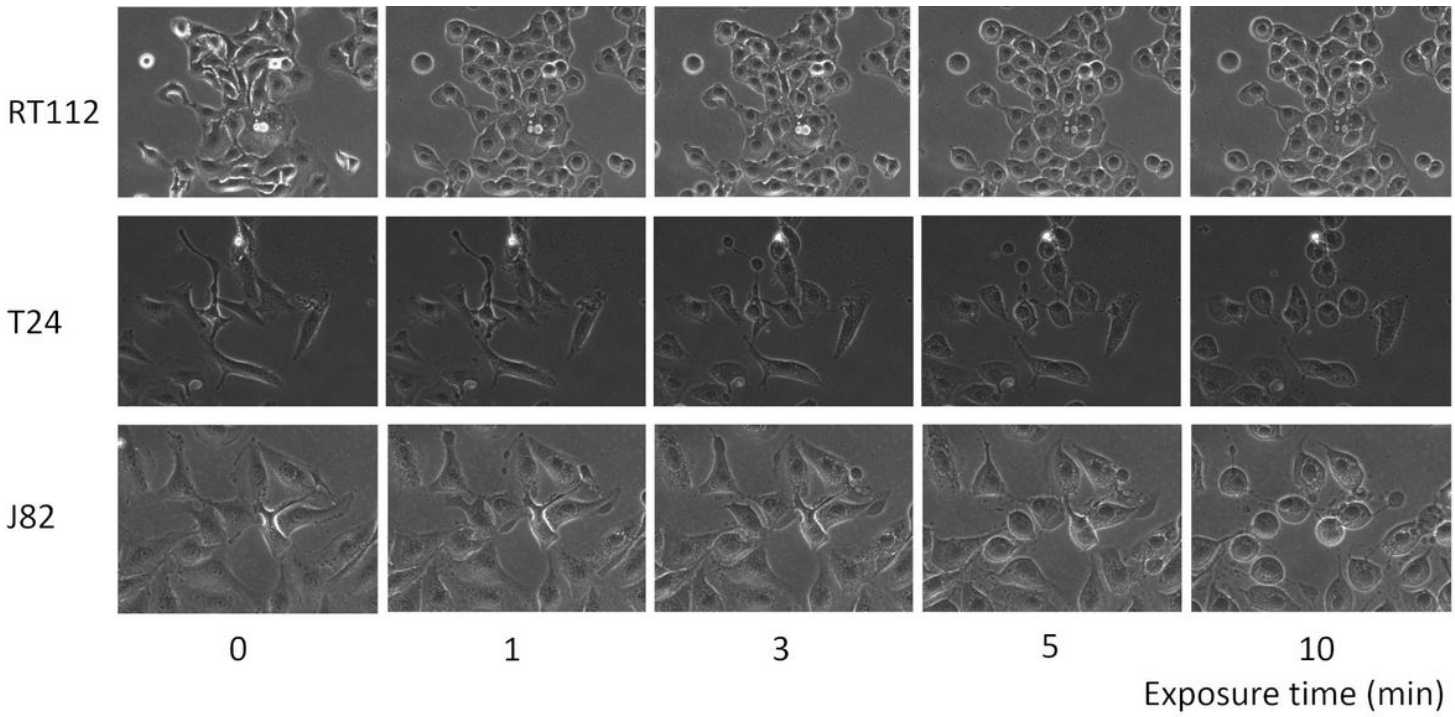

Figure 1

Representative images of RT112, T24, and J82 cells before and after exposure to sterile water. T24 and J82 cells started swelling immediately upon exposure to sterile water and ruptured within 5 min. RT112 cells demonstrated limited hypotonic swelling with few cell ruptures within 10 min of exposure to water.

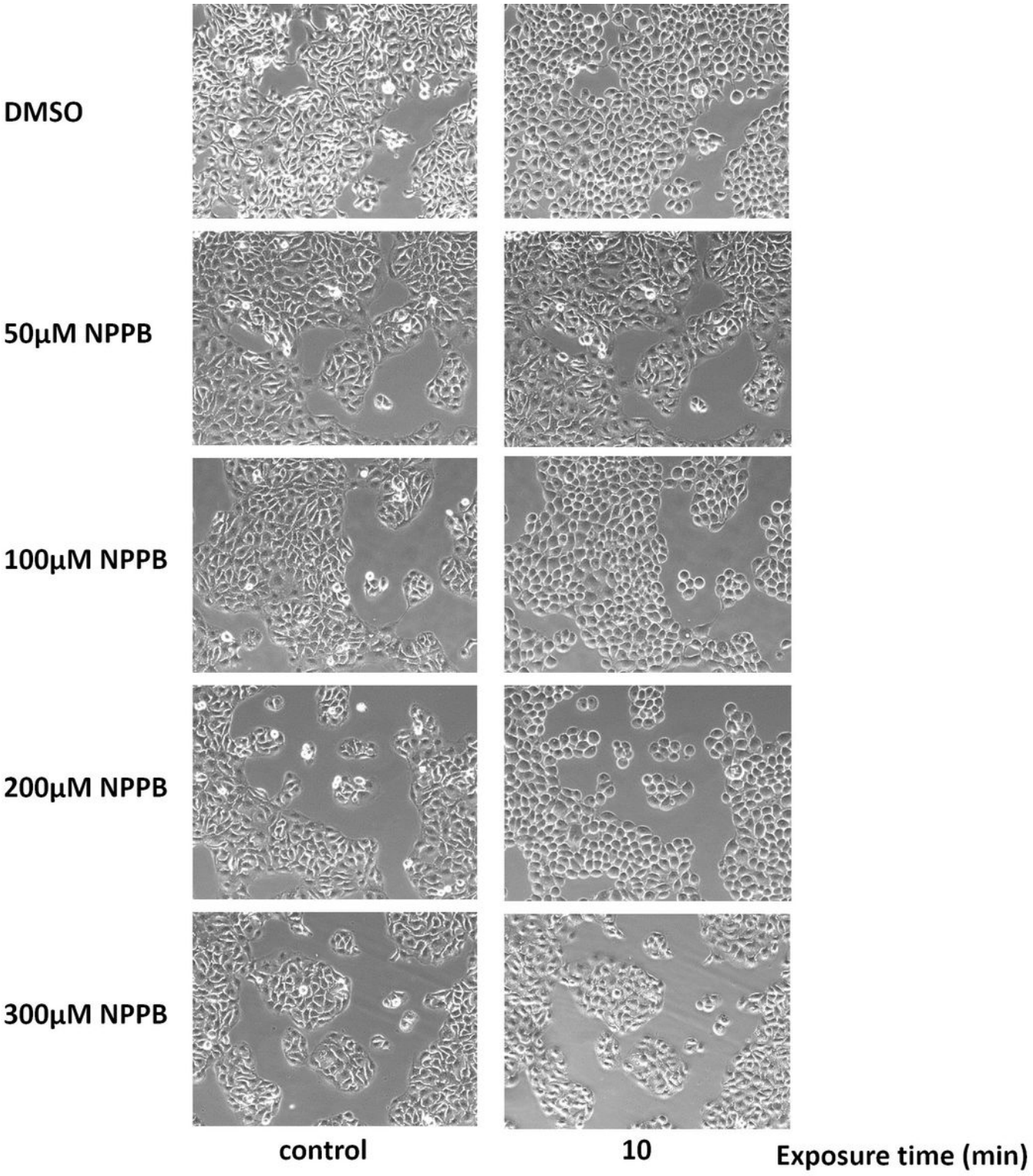

Figure 2

Representative images of dimethylsulfoxide (DMSO) and NPPB-treated RT112 cells before and after exposure to sterile water. NPPB-cells ruptured faster after exposure to water as compared to the rupture in DMSO-treated cells. (DMSO – dimethylsulfoxide; NPPB – 5-nitro-2-(3-phenylpropylamino) benzoic acid)

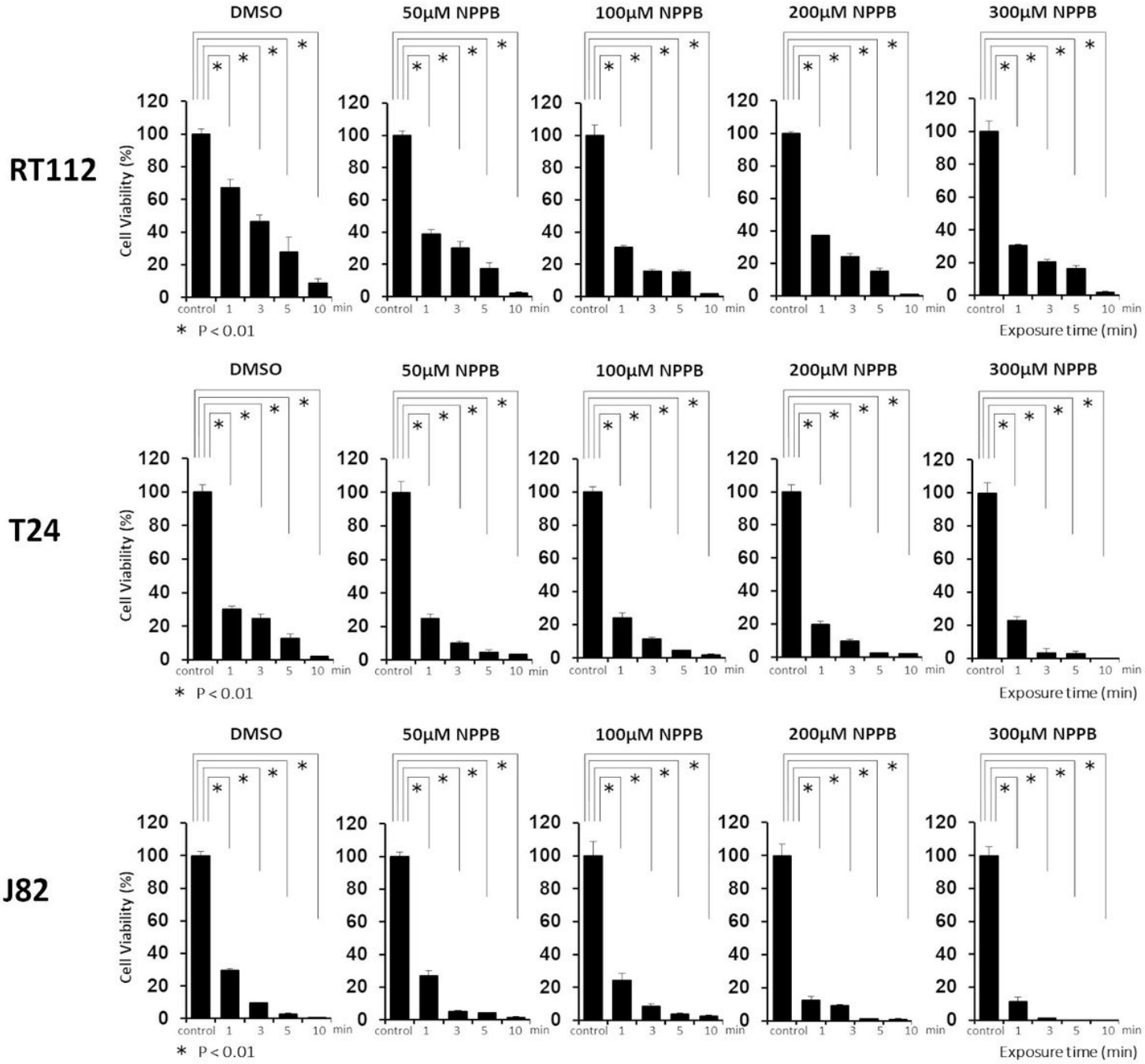

Figure 3

Viability of NPPB-treated (50, 100, 200, or 300 μM) bladder cancer cells after exposure to sterile water. The viabilities of NPPB-treated cells decreased faster upon exposure to sterile water than of those without NPPB. (DMSO – dimethylsulfoxide; NPPB – 5-nitro-2-(3-phenylpropylamino) benzoic acid)

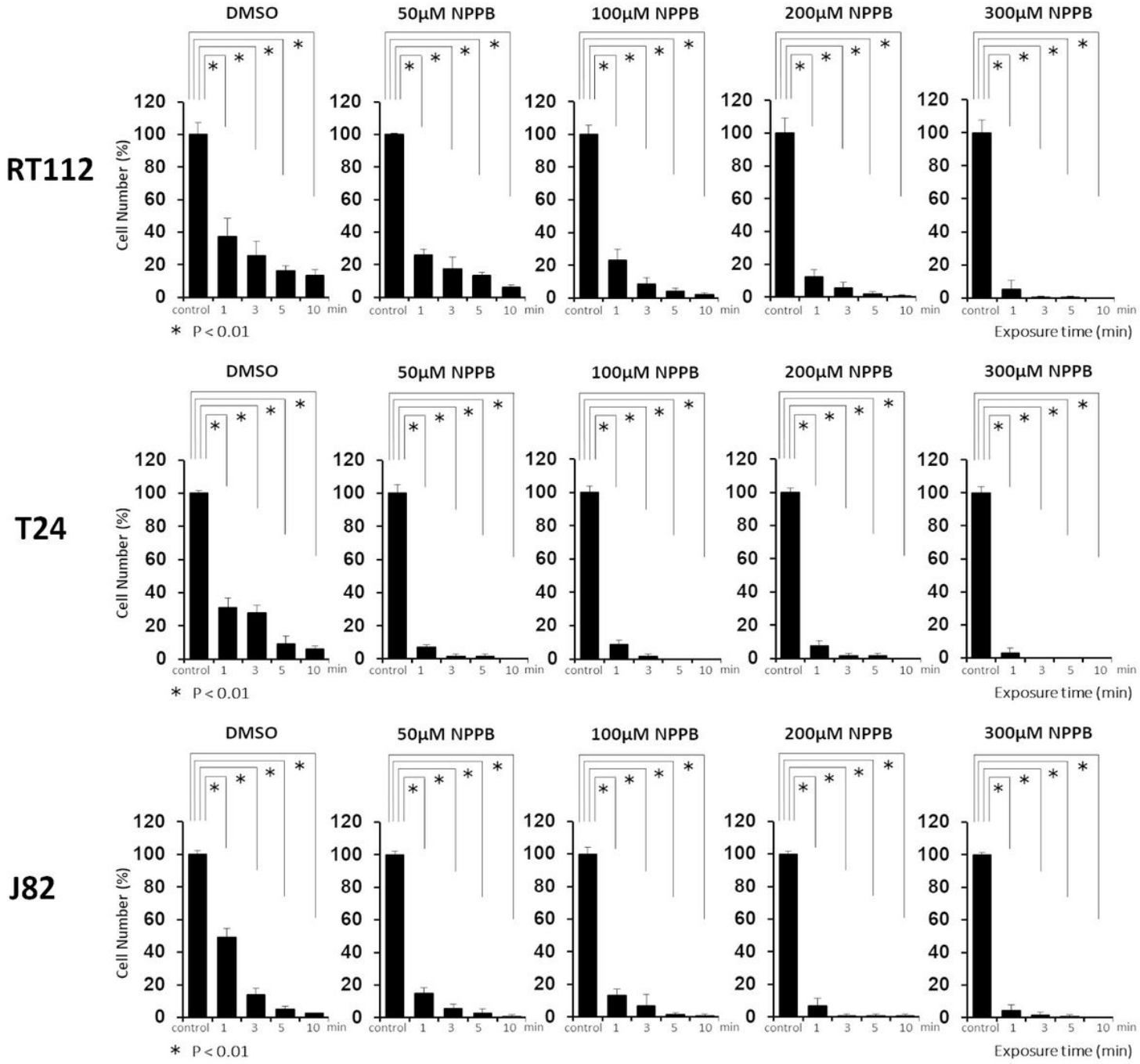

Figure 4

Intactness of cell membranes of NPPB-treated bladder cancer cells after exposure to sterile water. The number of intact membranes of the NPPB-treated bladder cancer cell lines decreased more rapidly upon exposure to sterile water than those without NPPB.(DMSO – dimethylsulfoxide; NPPB – 5-nitro-2-(3-phenylpropylamino) benzoic acid)