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Microemulsions for Intravesical Delivery of Gemcitabine

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The objective of this work was to develop a safe and effective delivery vehicle for topical treatment of gemcitabine. The physicochemical properties, drug release rate, drug level in plasma and bladder, and histological changes of tissue after drug administration were investigated. The electrical conductivity, mean size, and viscosity of drug-loaded microemulsions were 0.8—102.0 μS/cm, 116.8—322.5 nm, and 42.9—105.0 cps$\times 10^3$, respectively. Gemcitabine loaded microemulsions showed a slower and sustained release. After intravesical administration of aqueous control and microemulsions treated, the drug concentrations in plasma were 15.11 μg/ml and 2.81—12.82 μg/ml, respectively, and the accumulation in bladder were 18.27 μg and 9.12—64.16 μg, respectively. Microemulsions slightly decreased the systemic absorption and significantly enhanced the accumulation in bladder tissue. Moreover, the preliminary toxicity studies revealed no overt adverse histological changes or tissue irritation by the microemulsion application. Therefore, the microemulsions were suggested to be a promising drug carrier for intravesical chemotherapy.

Key words gemcitabine; microemulsion; intravesical administration

Urinary bladder cancer is among the most common cancers. In 2008, an estimated 69000 new cases of bladder cancer were diagnosed in the United States (51000 in men and 18000 in women) and approximately 14000 deaths from bladder cancer were reported.[1] Its prevalence in the U.S.A. and worldwide is approximately 490000 and over one million, which makes bladder cancer a significant global public health issue.[2] Approximately 70% of all urothelial bladder cancer cases are classified as superficial bladder cancer (SBC), *i.e.*, non-muscle-invasive.[3]

The current strategy for treating superficial bladder cancer is transurethral tumor resection followed by intravesical adjuvant treatment (chemo- and/or immunotherapy) to reduce recurrence risk.[4,5] Although Bacillus Calmette-Guerin (BCG) immunotherapy is currently the most potent topical treatment, it often has local or systemic adverse effects, and 30% of high-risk patients who respond poorly to BCG still ultimately require cystectomy.[6,7] Therefore, more active chemotherapy agents are needed for patients who respond poorly to BCG.

Gemcitabine, a novel deoxycytidine analogue, is an inhibitor of DNA synthesis with a broad spectrum of antitumor activity. This agent, which has overall response rates ranging from 22.5 to 28%, is highly effective and well tolerated as a first- or second-line single-agent therapy for treating metastatic transitional cell carcinoma.[8,9] Moreover, gemcitabine induces apoptosis through Fas upregulation without activating nuclear factor-kappa B (NF-κB). Hence, it may be more effective than other anticancer drugs such as doxorubicin, mitomycin C and cisplatin for reducing undesirable side effects such as proliferation, migration, immortality, and inhibition of apoptosis. Therefore, gemcitabine is a strong candidate for intravesical therapy in SBC patients who are refractory to BCG.[10—13] However, after intravesical administration of high doses (40 mg/ml) of gemcitabine in saline solution, significant systemic absorption can still cause gastrointestinal, bladder, and bone marrow toxicity, which limits its clinical value.[14,15] Therefore, this study attempted to develop a delivery vehicle, which can increase gemcitabine accumulation in bladder tissue and/or decrease systemic exposure for effective intravesical administration with minimal side effects.

Microemuslions are dispersions of oil in water (o/w) or of water in oil (w/o) that are thermodynamically stable due to the significant reduction of interfacial tension by adsorption of surface amphiphiles. Microemulsions have been studied intensively in recent years because of their ease of production and their unique properties, including thermodynamic stability, drug solubility, and drug permeability.[16—18] Previous studies[19,20] pointed that microemulsions formulated with non-irritant components such as non-inonic surfactant with a very low topical LD_{50}[21] can be applied throughout the body, which enables significant epidermal localization of the drug. Therefore, the microemulsion system proposed in this study was designed to be an intravesical vehicle for delivering gemcitabine to the bladder. Microemulsions were prepared using a mixture of polyoxyethylene sorbitan monooleate (Tween) and sorbitan monolaurate (Span) as surfactant, ethanol as cosurfactant, isopropyl myristate (IPM) as oil phase, and distilled water as aqueous phase. The physicochemical properties of the microemulsions, including their electrical conductivity, droplet size, and viscosity as well as *in vitro* drug release properties were evaluated. *In vivo* studies including drug concentration in plasma, drug accumulation in bladder tissue, and histological changes in tissue were performed in a rat model to evaluate the effectiveness and safety of the microemulsion delivery system.

Experimental

Materials Gemcitabine hydrochloride was purchased from Scinopharm (Taiwan). Sorbitan monolaurate (Span) was from Tokyo Chemical Industry (Japan). Polyoxyethylene sorbitan monooleate (Tween) was acquired from Showa Corporation (Japan). Sodium pentanesulfonic acid was from Wako Pure Chemical (Japan). Isopropyl myristate (IPM), perchloric acid and sodium phosphate were obtained from Merck Chemicals (Germany). Acetaminophen and rhodamine B base were purchased from Sigma-Aldrich (U.S.A.). Tetrahydrouridine was obtained from Calbiochem (U.S.A.). All other chemicals and solvents were analytical reagent grade.

Preparation of Gemcitabine or Rhodamine Microemulsion Formula-

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Table 1. Composition, Particle Size, Polydispersity Index (PI), Electrical Conductivity (EC) and Viscosity of Blank and Gemcitabine Loaded Microemulsions

	IPM (%)	W (%)	S (%)	Particle size (nm)	PI	EC (μS/cm)	Viscosity (cps$\times 10^3$)
A[a]	13	35	52	299.7±22.1	0.78±0.06	28.6	42.2±0.7
B[a]	50	10	40	122.7±1.8	0.37±0.01	0.5	44.3±0.5
C[a]	20	20	60	323.8±27.7	0.79±0.09	2.3	108.8±3.4
A[b]	13	35	52	176.1±10.7	0.62±0.04	102.0	42.9±1.0
B[b]	50	10	40	322.5±9.6	0.75±0.06	0.8	45.1±1.0
C[b]	20	20	60	116.8±2.5	0.30±0.01	6.6	105.0±1.7
A[c]	13	35	52	163.7±13.7	0.68±0.11	103.6	49.8±0.4
B[c]	50	10	40	323.4±10.6	0.68±0.05	0.9	43.9±3.5
C[c]	20	20	60	120.3±3.8	0.45±0.04	6.3	102.3±2.4

Microemulsion composited of water (W) containing 40% cosurfactant, isopropyl myristate (IPM), and mixture surfactant (S) of Tween/Span=3/2. *a*) Blank microemulsions. *b*) Gemcitabine loaded microemulsions. *c*) After 2 months storage of gemcitabine loaded microemulsions.

tions The component rations of microemulsion formulations are listed in Table 1. The aqueous phase consisted of double-distilled water containing 40% of ethanol (cosurfactant) was prepared. The surfactant mixture of Tween/Sapn=3/2 and IPM was mixed well. Then the aqueous phase was added to the oily phase drop by drop. The clear and transparent microemulsions were obtained under a vortex shaken at room temperature. Gemcitabine and rhodamine were dissolved in the final microemulsion formulations to obtain concentrations of 1% and 0.5%, respectively.

Microemulsion Characterization The electrical conductivity of the microemulsions was measured by a handheld conductivity meter (WTW Cond 315i, SUNTEX, Germany) at 25±2 °C. Average particle sizes of gemcitabine microemulsions were determined by photo correlation spectroscopy by laser light scattering (Zetasizer 3000HSA, Malvern, U.K.) using a helium-neon laser with a λ of 633 nm. Samples were loaded into 1 cm^2 cylindrical cuvettes and placed in a thermostated scattering chamber. Light scattering was monitored at a fixed angle of 90° and a fixed temperature of 25 °C.

The viscosity of the microemulsions was measured using a cone-and-plate viscometer (Brookfield, Model LVDV-II, U.S.A.) maintained at 37 °C. The *x* was read 30 s after *y* was measured, at which time the level of *z* had stabilized. The sample was sheared at a rate of 20 rpm. All experiments were repeated three times, and the average results were recorded.

***In-Vitro* Gemcitabine Release** Gemcitabine release rates from the microemulsions were measured through a cellulose membrane (CelluSep® T2 with a molecular weight cutoff of 6000—8000, Sartorious, Goettingen, Germany). Franz diffusion cells with a diffusion area of 3.46 cm^2 and 20 ml of receptor volume of pH 7.4 phosphate-citrate buffer were used. One milliliter of drug-loaded microemulsion was dosed in the donor compartment. The system was kept in a temperature-controlled water bath to maintain the donor compartment temperature at 37 °C, and the receptor phase was stirred continuously at 600 rpm. At predetermined time intervals, 0.5 ml samples were taken and replaced by the same volume of fresh preheated receptor medium. Gemcitabine concentrations were determined by HPLC. Each experiment was done in triplicate.

Cumulative release of gemcitabine was plotted against square root of time:

$$Q(t)=K\times t^{(1/2)}$$

where $Q(t)$ is the cumulative amount (μg/cm^2) of gemcitabine released in time t (<60%), K (μg/(h$^{1/2}$ cm^2)) is the kinetic constant indicating gemcitabine release rate, and $t^{(1/2)}$ is square root of time.

***In Vivo* Intravesical Administration of Gemcitabine** Sprague-Dawley female rats weighing 200—250 g were used in this study according to the care and use protocol for experimental animals approved by the Institutional Review Board at this institution. Animals were housed in a temperature-controlled room with free access to food and water until use.

Each animal was anesthetized by isoflurane. The residual urine was evacuated by pressing the lower abdomen. A polyurethane catheter (25 gauge, BD Angiocath Plu®, Becton Dickinson Korea, Gyeongbuk, Korea) was inserted into the bladder through the urethra. The bladder was washed twice with 0.5 ml normal saline. An 0.8 ml quantity of gemcitabine microemulsion or saline solution (as control) was then instilled into the bladder and maintained for 1 h by ligating the urethra orifice using a cotton thread under isoflurane anesthesia. One hour after the instillation, the cotton thread was cut off and the isoflurane was moved out. The animals will promptly recover consciousness, and then evacuate the residual formulation by urine micturate. The animals were euthanized at 0.5, 1, and 2 h after drug instillation, and blood samples were collected in heparinised tubes containing 10 μl tetrahydrouridine (1 mg/ml saline) to prevent *ex vivo* degradation of the gemcitabine by cytidine deaminase in the serum. Blood samples from the jugular vein were examined for systemic exposure of gemcitabine during and after intravesical administration of gemcitabine. The heparinised tubes were centrifuged for 10 min at 4000 rpm at 4 °C. A pipette was used to transfer the top 0.2 ml plasma layer into another tube containing 0.05 ml internal standard of acetaminophen 200 μg/ml and 0.1 ml of 1 M perchloric acid. After 5 s vortex, the mixture was incubated in ice bath for 10 min then centrifuged at 16000 ***g*** for 5 min at 4 °C. The 0.02 ml of clear supernatant was analyzed by HPLC method as reported previously with some modifications.[22]

At the end of the intravesical administration experiment, the gemcitabine accumulation in the bladder was also determined by a homogenization method. After wash, the excised bladder cut to small pieces and place into a glass tube containing 2 ml saline in an ice bath. The sample was homogenized at 17800 rpm for 1 min. The homogenizer probe was washed with 2 ml of saline to recover residual adhering tissues. The two saline fractions were combined, and then shaken horizontally for 10 min. The resulting solution was centrifuged at 3000×***g*** for 5 min. The supernatant was used for the assay by HPLC.[22]

HPLC Analysis The HPLC analysis was performed using an Agilent 1200 series HPLC system. A Merck Lichrospher® C18 column (250×4 mm i.d., particle size 5 μm) was used. The mobile phase was a mixture of aqueous phase containing 3 mM pentanesulfonic acid and 50 mM sodium phosphate (adjusted to pH 3.0 by phosphoric acid) and acetonitrile at a 95 : 5 ratio and flow rate was 0.9 ml/min. The UV detection was performed at 278 nm. The detection limits for drug concentration in plasma and drug accumulation in the bladder were 25 ng/ml and 250 ng/bladder, respectively.

Penetration Depth Measurement by Confocal Laser Scanning Microscopy (CLSM) Permeation of bladder tissue by rhodamine-loaded microemulsions was investigated using CLSM (FV 500, Olympus, Tokyo, Japan). Rats were sacrificed at 1 h after drug administration, and bladders were removed intact. Connective tissue, lipoid tissue, and drug residues were removed from the bladder walls. The bladders were then sectioned into 1 mm^2 specimens to compare the penetration depths of the microemulsion formulations. Bladder wall thickness was measured by CLSM through the *z* axis at *ca.* 20 μm increments. Optical excitation was performed using a 500 nm argon laser, and fluorescence emission was detected at 540 nm. Two different sites were evaluated in each bladder. Fluorescence emissions were measured in darkness to avoid errors caused by ambient light.

Histopathological Evaluation Rats were sacrificed at 1 h after blank microemulsion instillation was performed. The bladders were removed intact, cleaned to remove connective and lipoid tissue from around the wall, and weighed to test for presence of edema. The bladders were then fixed in 10% buffered formaldehyde for 24 h. Each bladder was cut into three equal sections from the dome to the bottom. Each piece was dehydrated using ethanol and embedded in paraffin. At least three cross sections 20 μm thick were taken from each section of bladder for hematoxylin–eosin staining.

Statistical Analysis Group comparisons were performed using analysis of variance (ANOVA) tests. A p value less than 0.05 was considered statisti-

cally significant. Tukey test was then performed to analyze two groups consecutively.

Results and Discussion

Physicochemical Characterization of Microemulsions Translucent and stable microemulsions were formed by mixing different components of the oil phase with the aqueous phase containing cosurfactant and surfactant. The physicochemical parameters of blank and drug loaded microemulsions are shown in Table 1. The electrical conductivity of all blank microemulsions was 0.5 to 28.6 μS/cm, which exceeded the electrical conductivities of oil phase (0.0 μS/cm), aqueous phase with cosurfactant (0.4 μS/cm), and surfactant (0.40 μS/cm). The results were consistent with a previous report[23] that microemulsions, even w/o type, can increase the electrical conductivity of formulations. The w/o microemulsion B with higher content of oil had lower electrical conductivity than the o/w type microemulsions A and C with lower content of oil. As expected, addition of gemcitabine hydrochloride significantly increased the electrical conductivity of both type microemulsions. Electrical conductivity of the w/o type microemulsion B increased from 0.5 to 0.8 μS/cm, a 1.9 fold increase, whereas those of o/w type microemulsions A and C increased from 2.3—28.6 μS/cm to 6.6—102.0 μS/cm, a 2.9—15.5 fold increase. These data indicate that gemcitabine dissolution was fastest in aqueous phase. Moreover, electrical conductivity of both blank and drug-loaded microemulsions correlated with that of proportion of aqueous phase in formulation. The increased electrical conductivity may have been caused by increased dissociation of surfactant as a function of water content.[24]

Table 1 shows that, at 37 °C, blank and drug-loaded microemulsions exhibited viscosities of 42.2—108.8 cps$\times10^3$ and 42.9—105.0 cps$\times10^3$, respectively. Viscosity was unaffected by drug incorporation ($p>0.05$), whereas increased surfactant correlated with increased viscosity (A *vs.* C, $p<0.05$).

Mean droplet size was small in all blank and drug-loaded microemulsions (122.7—323.8 nm and 116.8—322.5 nm, respectively). Notably, the drug-loaded formulations had the smallest droplet size in o/w microemulsions and the largest droplet size in w/o microemulsions. The phenomenon might be attributed to that drug is solubilized at the interface of microemulsion droplets and shrinks the droplets by interacting with the surfactant.[25]

After 2 months storage at room temperature, none of the microemulsions in the current study revealed changes in clarity, phase behavior, or particle size (Table 1). All microemulsions exhibited gemcitabine concentrations above 98.0$\pm$1.5%, which indicated that no degradation occurred.

***In Vitro* Gemcitabine Release** It is well known that microemulsion type, internal structure, size, and viscosity might influence the drug release from microemulsions.[26] In this study, two type microemulsions with different physicochemical properties (Table 1) were investigated. Figure 1 shows their release profiles. Gemcitabine release from saline was studied as a control. Figure 1 shows that drug release from microemulsions was slower than that from saline (78% release in 1 h), which indicates the potential effectiveness of microemulsions as drug delivery vehicles for controlled release.[27] To facilitate comparison of different formulations,

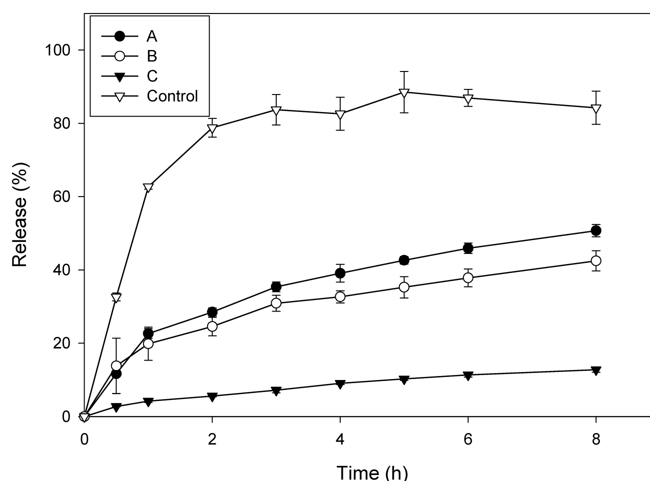

Fig. 1. *In Vitro* Release–Time Profiles of Gemcitabine from Microemulsions with Different Compositions

the release rate constant was calculated from the slope of the linear portion of the plots of cumulative drug quantity released against $t^{1/2}$ and expressed in μg/(cm^2 h$^{1/2}$). The release rates of microemulsions A, B, and C were 630.1$\pm$55.0, 390.3$\pm$96.2, and 141.1$\pm$9.6 μg/(cm^2 h$^{1/2}$), respectively, indicating that the ratio of ingredients in the microemulsions was an important factor for modulating drug release. Generally, increased emulsion viscosity tends to decrease drug release rate by increasing structural rigidity and droplet size while reducing total surface area.[28—30] The current study revealed a strong correlation ($r=0.886$) between viscosity and release rate, but a weak correlation ($r=0.291$) between droplet size and release rate. In addition, surfactant quantity corresponded positively with viscosity but negatively with release rate and droplet size (A *vs.* C, $p<0.05$). The result might be due to a decreased thermodynamic activity of the drug in microemulsion with higher concentrations of surfactant.[31] Another possibility was that drug diffusion through the double layer microemulsion might be a rate-determining step, as the viscosity plays an important role in controlling the release of the drug into the receptor.[32]

***In Vivo* Intravesical Instillation of Gemcitabine** The rats were given intravesical doses of 0.8 ml gemcitabine saline solution (control) or drug-loaded microemulsions, and the doses were maintained in the bladder for 1 h by ligating the urethral orifice. Figures 2 and 3 show the plasma concentrations and gemcitabine accumulations in the bladder, which were determined at 0.5, 1, and 2 h after drug instillation and used to evaluate the safety and efficacy of the microemulsions. Formulations were ranked by measuring the plasma drug concentration at 1 h after drug instillation as control>A>B>C. In terms of drug accumulation in the bladder, they were ranked as A>B>control>C. Strong correlations were noted between release rate and bladder accumulation ($r=0.9996$) and between release rate and plasma concentration ($r=0.9994$), which indicated that the permeation and accumulation of gemcitabine delivered by microemulsion depended on the release rate of drug from the formulation. Moreover, plasma concentrations corresponded positively with gemcitabine accumulations in the bladder. These laboratory results agree with earlier canine studies,[14] which reported significant dose-dependent systemic absorption of

gemcitabine after intravesical administration.

Compared to controls, subjects treated with microemulsion C showed lower plasma concentration ($p<0.05$) and less bladder accumulation ($p>0.05$). In subjects treated with microemulsion B, the plasma concentration and bladder accumulation were comparable to those of controls ($p>0.05$). Compared to controls, subjects treated with microemulsion A, the plasma concentration was slightly decreased ($p>0.05$) and the bladder accumulation was significantly increased ($p<0.05$). The comparison results showed that the bladder accumulation was increased without a corresponding increase in plasma drug concentration when compared to the control. The result was consistent with previous reports[20,33] which pointed that microemulsions can efficiently promote localization without concomitantly increasing systemic side effects.

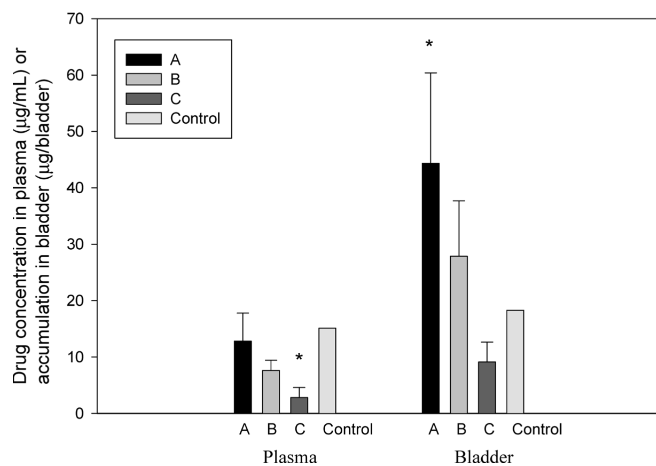

Fig. 2. Plasma Concentration and Accumulation in Bladder at 1 h after Gemcitabine Microemulsions and Aqueous Solution Intravesical Administrations in Rats ($n=6$)

$*p<0.05$ compared with saline control group.

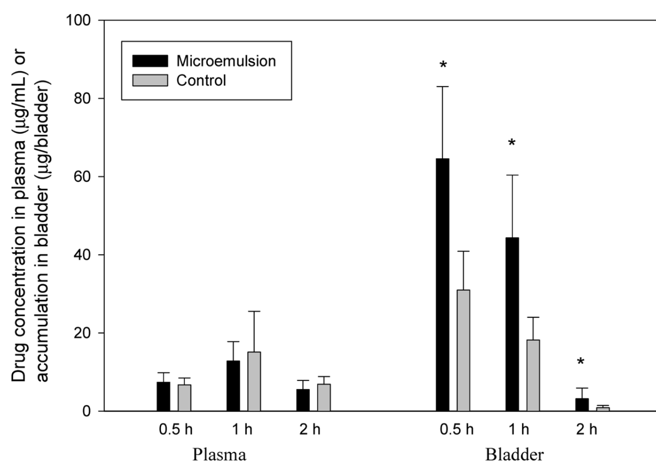

Fig. 3. Plasma Concentration and Accumulation in Bladder at 0.5, 1 and 2 h after Gemcitabine Microemulsion A and Aqueous Control Solution Intravesical Administration in Rats ($n=6$)

$*p<0.05$ compared with control group.

In addition, plasma concentration and bladder accumulation of gemcitabine were also evaluated at 0.5 and 2 h after instillation of microemulsion A and control. Figure 3 shows that, in both groups of microemulsion and control, gemcitabine was absorbed and distributed to the bladder wall by 0.5 h. After 2 h, most of the drug was eliminated, which indicated its short half-life after intravesical administration, possibly due to urine maturation and excretion. Previous dog studies[14] have also reported a short half-life after intravesical administration of 350 mg gemcitabine. However, as expected, bladder accumulation and plasma concentration at 0.5 and 2 h, respectively, were higher in subjects treated with microemulsions than in controls.

***In Vivo* CLSM Analysis** To clarify the permeation depth of microemulsion in the bladder, superficial distribution of rhodomine-loaded microemulsion was analyzed by CLSM. Figure 4 depicts the optical scanning results for the superficial layer at *ca.* 20 μm increments for 16 fragments from the surface to the muscle (left to right, top to bottom). After intravesical administration of microemulsion A, only pale signals were detected in the first image (*ca.* 20 μm) and in the 9—16th images (180—300 μm), and most signals were in the 140—160 μm range.

Histological Examination of Bladder Tissue Safety is a key factor in delivery vehicle formulation. For preliminary safety evaluation of the experimental formulation, histological photographs (Fig. 5) were compared between controls treated with saline for 1 h and the group treated with blank microemulsion A for 1 h. Mild signs of inflammatory response (subepithelial leukocyte infiltration) and epithelial cellular nuclear enlargement was seen in drug solution

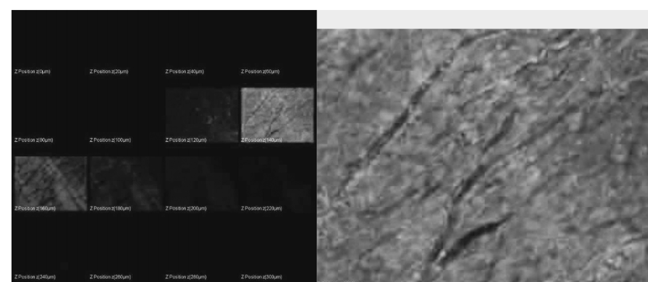

Fig. 4. Confocal Laser Scanning Microscopic (CLSM) Micrographs of the Rhodamine Intensity in Bladder after Intravesical Administration of Microemulsion A for 1 h

The full thickness was divided into 16 fragments from the surface of the bladder mucosa (left to right, top to bottom). Images below the photographs of the 16 segments are the sum of all segments.

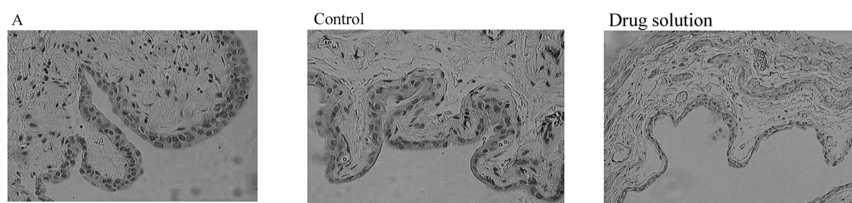

Fig. 5. Histological Examination of Rat Bladder Tissues after Intravesical Administration of Saline (Control) and Blank Microemulsion A

treated group. The photographs revealed no bladder wall damage in the microemulsion-treated groups, and their morphologies were similar to those of controls.

Conclusion

This study evaluated the potential use of microemulsions as vehicles for topical delivery of gemcitabine. The results suggest that microemulsions efficiently promote gemcitabine localization into the bladder wall. By enhancing gemcitabine accumulation in the bladder wall, the microemulsions may be useful for optimizing drug delivery without concomitantly increasing systemic side effects.

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