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Studies on the preparation, characterization and pharmacological evaluation of tolterodine PLGA microspheres

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ABSTRACT

In this study, poly(D,L-lactide-co-glycolide) (PLGA) microspheres of tolterodine depot formulation were prepared using oil in water (o/w) method to investigate their potential pharmacokinetic and pharmacodynamic advantages over tolterodine L-tartrate tablets. Morphological studies of the microspheres showed a spherical shape and smooth surface with mean size of 50.69–83.01 μm, and the encapsulation efficiency was improved from 62.55 to 79.10% when the polymer concentration increased from 180 to 230 mg/ml. The addition of stearic or palmitic acids could significantly raise the drug entrapment efficiency but only slightly affected the *in vitro* release. A low initial burst followed by a proximately constant release of tolterodine was noticed in the *in vitro* release profiles. The *in vivo* study was carried out by intramuscular (i.m.) administration of tolterodine-loaded microspheres on beagle dogs, and a sustained release of drug from the PLGA microspheres was achieved until the 18th day with a low initial burst. Since the absence of hepatic first pass metabolism, only a single active compound—tolterodine was detected in the plasma. This avoided the coexistence of two active compounds in plasma in the case of oral administration of tolterodine, which may lead to a difficulty in dose control due to the different metabolic capacity of patients. In the pharmacodynamic study, the influence of tolterodine PLGA microspheres on the inhibition of carbachol-induced rat urinary bladder contraction was more significant than that of tolterodine L-tartrate tablets. There were invisible changes in rat bladder slices between tolterodine-loaded PLGA microspheres group and tolterodine L-tartrate tablets group. These results indicate that the continuous inhibition of muscarinic receptor may offer an alternative therapy of urge incontinence.

1. Introduction

Muscarinic receptors mediate the bladder contraction of normal voiding and bladder overactivity associated with urge. Consequently, muscarinic antagonists are most widely used to treat for urge urinary incontinence (Modiri et al., 2002). However, most drugs lack functional selectivity for the bladder and their usefulness may be limited by adverse effects except tolterodine. Tolterodine is a potent and competitive muscarinic receptor antagonist with more specificity for M_2 receptor in clinical development for the treatment of urge urinary incontinence and other symptoms of unstable bladder (Nilvebrant et al., 1997) but less affinity for M_3 receptor with a direct correlate to dry mouth (Dmochowski and Appell, 2000). After oral administration, it is rapidly absorbed and the maximum plasma concentration (C_{max}) typically occurs within 1–2 h. The metabolic pathways of tolterodine are mainly mediated by liver cytochrome P450 2D6 creating an active metabolite of 5-hydroxymethyl derivative (DD01). Though DD01 has a similar pharmacological profile with tolterodine, the plasma concentration of tolterodine and DD01 depends on the metabolic capacity of individuals, which leads to a difficulty in the dose control for different patients (Nilvebrant et al., 1997). Thus, to develop a novel formulation which prevents tolterodine from first pass effect may profit the patient because of lower variation in efficacy and less adverse effect as well as lower risk of drug–drug interaction.

Microspheres technology has been utilized extensively to develop formulations with a sustained release of one therapeutic agent to maintain targeted concentration in the body for a sustained period of time (Edlund and Albertsson, 2002). This drug delivery system has already been applied to improve the therapeutic response and to reduce adverse effects (Zolnik and Burgess, 2008). Microspheres are usually administered by intramuscular or subcutaneous route. The drugs in microspheres are absorbed by the capillaries of the injection site and lymph organs, enter the systemic circulation, and then tend to be distributed to the target organ to play a pharmacodynamics (Morita et al., 2001), which can bypass the first pass effect and avoid pre-systemic elimination in the GI tract or liver. The absolute bioavailability of tolterodine following oral administration is highly variable ranging from 10

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to 70%, because the CYP2D6 activity depending on genetic differences varies among different individuals (Dmochowski and Appell, 2000). Liver impairment can significantly affect pharmacokinetics of tolterodine. Tolterodine PLGA microspheres may offer a possibility to avoid the first pass effect and produce less DD01 causing reductions in individual difference, drug–drug interactions and greatly increase the bioavailability of tolterodine. The sustained delivery of tolterodine from the microspheres formulation can continuously inhibit muscarinic receptor, which may offer an alternative therapeutic method in the treatment of overactive bladder (OAB) patient with less adverse effect such as thirst and constipation.

Previously, the conscious rat cystometry model was used to study the effects of various drugs on bladder function without compromising micturition reflexes with anesthetics. Several parameters, including micturition frequency, micturition pressure, bladder capacity, micturition volume and residual volume have been used to quantify bladder overactivity (Modiri et al., 2002). It has been reported that the mean frequency of micturition was reduced after tolterodine treatment (Dmochowski and Appell, 2000). So the mean number of micturition within 3 h is considered as an indicator for evaluating the efficacy of tolterodine formulations.

In this study, preparation, characterization, *in vitro* and *in vivo* release and pharmacokinetics of tolterodine-loaded PLGA microspheres were investigated. An animal model was also used to compare the pharmacological effects of tolterodine microspheres (TMSs) and tolterodine L-tartrate tablets (TTs) in conscious rats.

2. Materials and methods

2.1. Materials

Poly(D,L-lactide-co-glycolide, PLGA) RG 502H (lactide/glycolide ratio, 50/50; Mw, 8300) and RG 503H (lactide/glycolide ratio, 50/50; Mw, 28,000) were supplied by Boehringer–Ingelheim AG (Ingelheim, Germany). Stearic acid and palmitic acid were supplied by Beijing Yili Chemicals Co., Ltd. Carbachol (carbamylcholine chloride) and polyvinyl alcohol (PVA) (Mw 13,000–23,000) was supplied by Sigma Chemical Company. Tolterodine L-tartrate tablet was supplied by Lunan Pharmaceutical Better Limited Corporation. Tolterodine tartrate was obtained from Beijing Gaobo Pharm-Chemicals Tech. Co., Ltd. Tolterodine free base was prepared in our laboratory.

The experiments were performed on female Sprague–Dawley (SD) rats weighing between 200 ± 20 g and beagle dogs weighing between 12.0 ± 1.0 kg. The animals were kept in plastic cages in a room at a temperature of 20 ± 4 °C, with a 12:12 light-dark cycle. Food and water were freely available. All experiments were performed in strict accordance with the Guide for the Care and Use of Laboratory Animals as adopted by the China National Institutes of Health.

2.2. Preparation of the microspheres

An oil in water (O/W) emulsion solvent evaporation method was used to prepare tolterodine-loaded PLGA microspheres. Briefly, 15 ml of dichloromethane (DCM) solution containing tolterodine and PLGA was slowly added to 1.5 l of 0.5% (w/v) aqueous PVA solution (saturated with 1 ml DCM) and homogenized at various rates for 3 min. Stirring was continued at 25 °C for 4 h until the microspheres were solidified. The microspheres were collected by filtration through a 10 μm sieve and a 154 μm sieve, and then washed three times with cold water. After 1 ml of aqueous Mannitol (20%, w/v) was added to prevent the aggregation of microparticles, the microspheres were freeze dried.

2.3. Morphological characterization

The surface morphology of microspheres was observed by scanning electron microscope (SEM) (JXA-840, JEOL, Japan). Dried microspheres were mounted on metal stubs using double-sided adhesive tape with conductive effect, sputter coated with a thin layer of gold and analyzed with SEM at an accelerating voltage of 20 kV.

2.4. Particle sizing

LS13 320 laser particle size analyzer (Beckman Coulter, Inc., USA) was used to determine the mean particle diameter and particle size distribution. The particle size was expressed as the volume-weighted mean particle diameter in micrometer.

2.5. Drug loading and encapsulation efficiency

To determine loading percentage of tolterodine in the microspheres, 10 mg of the dried microspheres were dissolved in 1 ml of acetone. The resulting solution was then diluted with 0.01 M hydrochloric acid to 25 ml, filtered though a 0.45 μm type membrane filter (Fisher Scientific) and injected into a high pressure liquid chromatography (HPLC) system for determination of the concentration as well as the amount of tolterodine. The HPLC system consisted of a Waters 600 pump and a Waters 2487 Dual Absorbance Detector (Waters Corp., USA) set at 283 nm. Agilent XDB C18 column (4.6 mm × 250 mm, 5 μm, Agilent Technologies, Inc., Santa Clara, USA) was utilized for drug separation, while a methanol–20 mM of pH 3.0 acetate buffer system (65:35, v/v) was used as the mobile phase. The flow rate was set at 1 ml/min. The chromatography was carried out at 40 °C and the injection volume was 20 μl.

The drug loading percentage and encapsulation efficiency were calculated.

$$\text{Encapsulation efficiency}\,(\%) = 100 \times \frac{\text{drug loading determined by HPLC}}{\text{theoretical drug loading}}$$

2.6. In vitro drug release studies

The *in vitro* release of microspheres was measured in phosphate buffered saline (PBS, pH7.4) containing 0.02% sodium azide and 0.01% Tween 80 at temperature of 37 °C. In a 15 ml centrifuge tube, approximately 10 mg of microspheres were suspended in 6 ml of PBS and shaken horizontally at 100 rpm in a shaking bath maintained at 37 °C. Samples of 4 ml were removed from the tubes at sampling times of 3 h, 1, 2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, 24, 26 and 28 days after centrifugation at 4000 rpm for 5 min. The medium removed from the tubes was replaced with the same amount of fresh buffer solution. The collected samples were filtered through a 0.45 μm filter and subjected to further HPLC analysis described above.

2.7. In vivo pharmacokinetics evaluation

Pharmacokinetic studies of TMS and TT were carried out with beagle dogs weighting (12.0 ± 1.0) kg ($n = 3$). For TT, the animals were intragastric (i.g.) administered at a dose of 160 μg/kg. Blood samples were drawn at 0, 0.5, 1, 2, 4, 6, 8, 10, 12 and 24 h following.

The microspheres, which were suspended in the solution containing 0.5% sterile carboxymethyl cellulose sodium, 0.9% NaCl and 0.1% Tween 80, were intramuscular (i.m.) injected to the dogs at a dose (tolterodine) of 720 μg/kg dog. The dose of TMS was calculated from 160 μg/kg (the daily dose of oral administration) × 325.5 (Mw of tolterodine)/475.6 (Mw of tolterodine L-tartrate) × 10 days × 66% (absolute bioavailability of oral administration) = 720 μg/kg.

Before and during the sampling time, the animals had free access to food and water. Blood samples were collected pre-dose (0 min) and at 0.125, 1, 2, 4, 6, 8, 10, 12, 14, 16, 18 days post-dose into heparinized tubes. Plasma was separated by centrifugation at 15,000 rpm for 5 min and stored at −80 °C until analysis. The contents of tolterodine and DD01 in plasma samples were determined by a high pressure liquid chromatography–mass spectrometer (HPLC–MS) (SCIEX API 4000 triple-quadrupole mass spectrometer) and expressed as concentrations of tolterodine and DD01 obtained in ng/ml and cumulative release percentage of plasma at various time points.

2.8. Pharmacodynamic studies

Thirty-two female SD rats were divided into four groups: saline/saline, carbachol (0.05 mg/kg, subcutaneous (s.c.) injection)/saline, carbachol (0.05 mg/kg, s.c.)/TMS (3 mg/kg, i.m.), carbachol (0.05 mg/kg, s.c.)/TT (0.2 mg/kg, i.g.). Each group was housed in a well ventilized rearing cage in an animal room for at lease 3 days before the study. Gentle manipulation was maintained all through the study. TT group was administered once daily for 7 consecutive days. The number of micturition and voided volume were recorded for 3 h at 1, 3, 5 and 7 days after administration of carbachol in rats. TMS group was tested at the same time of 1, 3, 5, 7, 11 and 15 days. The total voided volume of each rat for one episode was recorded in a metabolic cage during the experimental period, and the Na^+ and K^+ contents in urine were determined using a semi-automatic biochemistry analyzer (ShanDong Gaomi Caihong Analytical Instruments Co. Ltd., China). After the completion of testing, the bladder sections were prepared ($n = 4$ per group) for the histological evaluation.

2.9. Statistical analysis

Values were expressed as mean ± standard deviation (S.D.) for each group. Statistical evaluation of the experimental data was performed using Student's t-test. A $p < 0.05$ was considered statistically significant.

3. Results and discussion

3.1. Microsphere morphology and drug loading

The surface morphology of tolterodine PLGA microspheres was observed by SEM. The microspheres were spherical in shape with a smooth surface and the size was uniform and appropriate for administration to rat via intramuscular injection.

Previous reports have shown that several formulation parameters may affect the drug encapsulation, such as drug/polymer ratio, polymer concentration, solvent evaporation time and stirring speed (Yen et al., 2001). Table 1 shows the actual drug loading, encapsulation efficiency and particle size distribution of the tolterodine microspheres prepared with different polymers, homogenization speed, and addition of saturated fatty acids. The mean particle size of tolterodine-loaded PLGA microspheres ranged from 50.69 to 83.01 μm, and increased when polymer concentration increased as well as the drug encapsulation from 62.55% (batch 2) to 79.10% (batch 1) due to the higher viscosity in a droplet of the internal phase (Chun et al., 2005; Su et al., 2009).

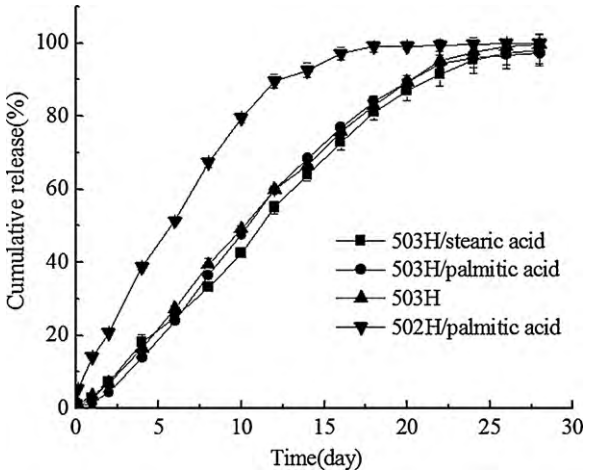

Fig. 1. *In vitro* cumulative releases of tolterodine-loaded microspheres prepared from 503H/stearic acid (batch 5), 503H/palimitic acid (batch 6), 503H (batch 8) and 502H/palmitic acid (batch 9). Each point represents mean ± S.D. ($n = 3$).

Raising the homogenization speed did not significantly affect the encapsulation efficiency. While the addition of lipophilic carboxylic acid, including stearic and palmitic acid, in tolterodine PLGA microspheres formation significantly improved the encapsulation efficiency. It has been reported that the interaction between the amine moiety of tolterodine and the carboxylic acid can enhance the compatibility of the excipients and drug (Fahmy et al., 2005; O'Donnell and McGinity, 1997). The higher molecular weight of RG 503H led to a higher viscosity of polymer solution, which also increased the encapsulation efficiency of tolterodine (Chu et al., 2007; Thomasin et al., 1998).

3.2. In vitro drug release studies

The cumulative *in vitro* release profiles of tolterodine from the microspheres in phosphate buffer (0.05 M, pH 7.4) were shown in Fig. 1. All the formulations showed a low initial burst release. From the first day to the 20th day, the tolterodine release rates were relatively constant, suggesting that tolterodine was well entrapped (Zolnik and Burgess, 2008). The fattic acids, either stearic or palmitic acids, significantly improved the drug encapsulation (Table 1), but they revealed limited effects on the *in vitro* release of tolterodine from microspheres.

Fig. 1 also showed the impacts of polymer on the *in vitro* release of tolterodine from PLGA microspheres. The degradation of PLGA with a lower molecular weight produced more small fragments, which made polymer matrix more hydrophilic and higher water uptake, thereby enhancing tolterodine release (Duvvuri et al., 2006; Blanco and Alonso, 1998). The lower molecular weight RG 502H led to a significant higher initial drug release and faster sustained release than RG 503H.

3.3. Pharmacokinetic studies

Pharmacokinetic studies were carried out in beagle dogs (12.0 ± 1.0 kg) using the microspheres prepared from RG 503H (batch 5). The time course of the plasma concentrations of tolterodine and DD01 after the administration of TT was summarized in Fig. 2A. The pharmacokinetic parameters calculated from the plasma drug concentration vs. time profiles were listed in Table 2. After i.g. administration of TT, the C_{max} of tolterodine, DD01 were 1.63 ± 0.43 and 0.99 ± 0.31 ng/ml, respectively. The drugs were exhausted after 12 h.

Table 1
Formulations processing conditions and characteristic of tolterodine PLGA microspheres.

Batch	PLGA	PLGA/dichloromethane (w/v)	Homogenization speed (rpm)	Stearic acid (mg)	Palmitic acid (mg)	Theoretical drug loading (%)	Actual drug loading (%)	Encapsulation efficiency (%)	Particle size (μm)
1	503H	23%	1500	100	–	8	6.33 ± 0.14	79.10 ± 1.76	58.61 ± 0.31
2	503H	18%	1500	100	–	8	5.00 ± 0.59	62.55 ± 7.40	53.01 ± 0.36
3	503H	18%	1000	100	–	8	5.17 ± 0.15	64.59 ± 1.50	74.58 ± 0.20
4	503H	18%	1000	80	–	8	4.19 ± 0.40	52.36 ± 5.04	69.78 ± 0.29
5[a]	503H	18%	1000	100	–	10	6.14 ± 0.06	61.39 ± 0.64	62.24 ± 0.46
6	503H	18%	1000	–	100	10	6.36 ± 0.26	63.60 ± 2.57	81.30 ± 0.36
7	503H	18%	1000	–	80	10	6.24 ± 0.35	62.41 ± 3.54	83.01 ± 0.22
8	503H	18%	1000	–	–	10	3.90 ± 0.20	39.02 ± 1.96	81.04 ± 0.29
9	502H	18%	1000	–	100	10	5.76 ± 0.14	57.63 ± 1.36	53.45 ± 0.68
10	502H	18%	1500	–	100	10	5.25 ± 0.20	52.52 ± 1.95	50.69 ± 0.19

[a] It was used in pharmacokinetic and pharmacodynamic studies.

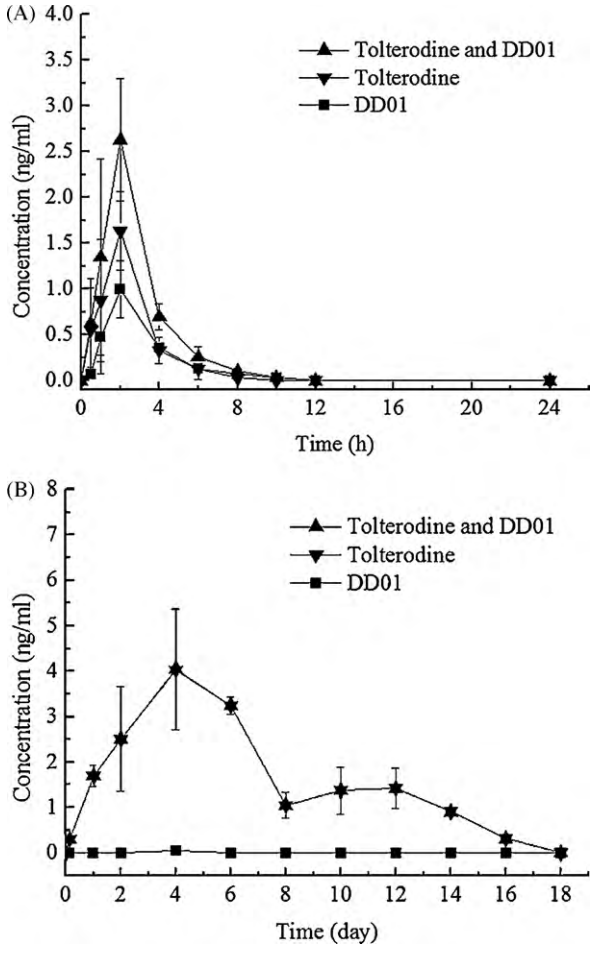

Fig. 2. Plasma concentration–time profiles for tolterodine after administration of (A) tolterodine tartrate tablets (160 μg/kg, i.g.) and (B) tolterodine microspheres (batch 5) suspension (720 μg/kg, i.m.). Each point represents mean ± S.D. (n = 3).

Table 2
Pharmacokinetic evaluation of the beagle dog plasma data (mean ± S.D., n = 3).

Parameters	TMS		TT	
	Tolterodine	DD01	Tolterodine	DD01
Dose (mg/kg)	0.72		0.16	
C_{max} (ng/ml)	4.04 ± 1.33	0.05 ± 0.03	1.63 ± 0.43	0.99 ± 0.31
T_{max}[a]	4.00 ± 0.00	4.00 ± 0.00	2.00 ± 0.00	2.00 ± 0.00
$AUC_{0-\infty}$ (ng h/ml)	29.45 ± 2.01	0.02 ± 0.03	4.32 ± 0.27	3.02 ± 0.63

[a] The unit is day for TMS and hour for TT.

In Fig. 2B, the initial release was low after i.m. administration of TMS, which was similar with the *in vitro* release. Only a trace of the active metabolite DD01 was found on the 4th day at 0.05 ± 0.03 ng/ml and no detectable DD01 (<0.05 ng/ml) was found on the other days. On the 4th day, the total plasma concentration of tolterodine and DD01 reached a C_{max} of 4.04 ± 1.33 ng/ml, and then slowly fell until the drug was exhausted on the 18th day. As has been reported previously, after oral administration, about half of the tolterodine is metabolized to DD01 in the liver by cytochrome P450 2D6 during first pass in human (Brynne et al., 1998). In this study, due to the absence of hepatic first pass metabolism, after i.m. injection of TMS, tolterodine was the only active molecules existed almost all the time (Fig. 2B) and it may profit the patient in the dose control.

It has been reported that tolterodine was metabolized along two different pathways in mouse, rat, dog and human. Mice and dogs produce the active metabolite of tolterodine, DD01, as do humans while rats produce very little DD01 due to a different metabolic profile (Andersson et al., 1998). Previous study showed that there was no dose-dependent clinical efficacy and the absolute bioavailability was highly variable, ranging from 10 to 70% after oral tolterodine due to the differences in metabolic capacity. Moreover, Brynne et al. have reported that a significant inhibition of the metabolism of tolterodine resulted in an approximate 2.5-fold increase in area under curve (AUC) (Brynne et al., 1999). From the above analysis it is quite evident that tolterodine microsphere delivery system, compared with oral administration, could increase the bioavailability and reduce bypass first pass metabolism by the liver significantly, resulting a single active compound of tolterodine in plasma. It indicated that the microspheres formulation offers not only a different release behavior but also a different metabolism of the drug, which may profit the patient in the dose control and the reduction of potential adverse effect from two active compounds in body (Jori et al., 1971).

The cumulative *in vivo* and *in vitro* releases of tolterodine from microspheres prepared from RG 503H were showed in Fig. 3. The *in vivo* release is significantly faster than that *in vitro* in the 0.05 M PBS buffer. In order to get a good *in vivo*/*in vitro* correlation, the ionic strength of the buffer was adjusted. The results showed that the reduction of the ionic strength accelerated the release of drug *in vitro* (D'Souza et al., 2005). When a 0.02 M PBS was used as the medium of *in vitro* experiment, the cumulative released curves of *in vitro* and *in vivo* were almost identical, which indicated a good correlation (Fig. 3).

3.4. Pharmacodynamic studies

The *in vivo* pharmacological response versus time profiles in rats after i.m. injection of TMS and oral administration of TT were showed in Fig. 4. In the case of the carbachol group, the mean number of micturition within 3 h was 7.85 ± 1.44. It was signif-

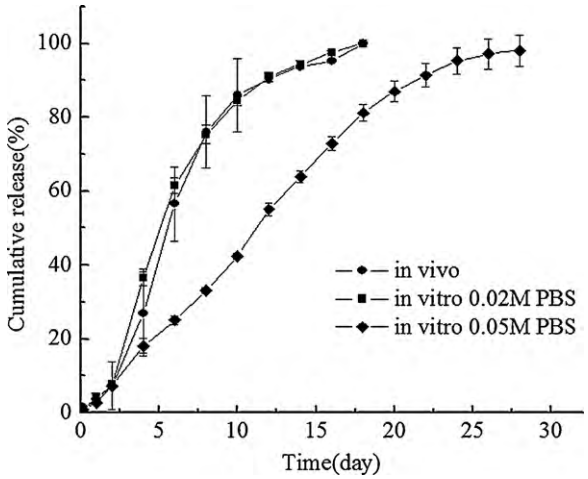

Fig. 3. Cumulative released curves of the *in vitro* release and *in vivo* release of tolterodine PLGA microspheres (Table 1, batch 5). Each point represents mean ± S.D. ($n = 3$).

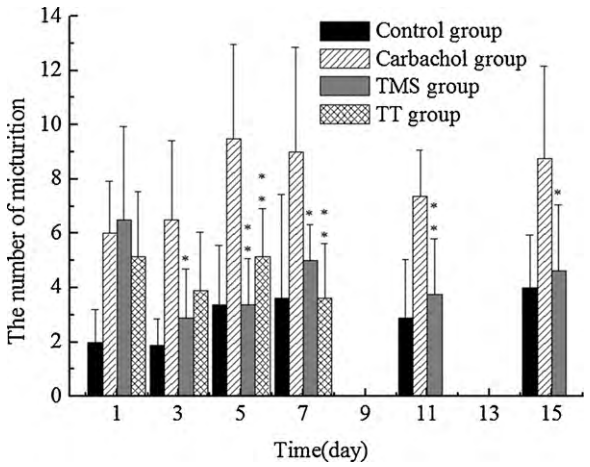

Fig. 4. Effects of tolterodine-loaded PLGA microspheres (i.m.) and tolterodine tartrate tablets (i.g.) on the increasing number of micturition in 3 h induced by carbachol in rats. Each point represents mean ± S.D. ($n = 8$). * and ** represent $p < 0.05$ and $p < 0.01$ vs. carbachol group.

icantly higher than that in the control group (mean number of micturition was 2.95 ± 0.80). The results indicate that carbachol could significantly increase the contractions of the rat urinary bladder.

After i.g. administration of TT (0.2 mg/kg day), the contractions of the rat urinary bladder were improved and the mean number of micturition was reduced to 5.13 ± 2.42 from 6.00 ± 1.93 on the first day, to 3.88 ± 2.17 from 6.50 ± 2.93 on the third day and to 5.13 ± 1.81 from 9.00 ± 3.85 on the fifth day ($p < 0.01$).

After a single i.m. injection of microspheres (Table 1, batch 5) at a dose of 3 mg/kg (0.2 mg/kg day), the mean number of micturition was reduced to 2.88 ± 1.81 ($p < 0.05$) on the third day and 3.38 ± 1.69 ($p < 0.01$) on the fifth day. The significant effects were

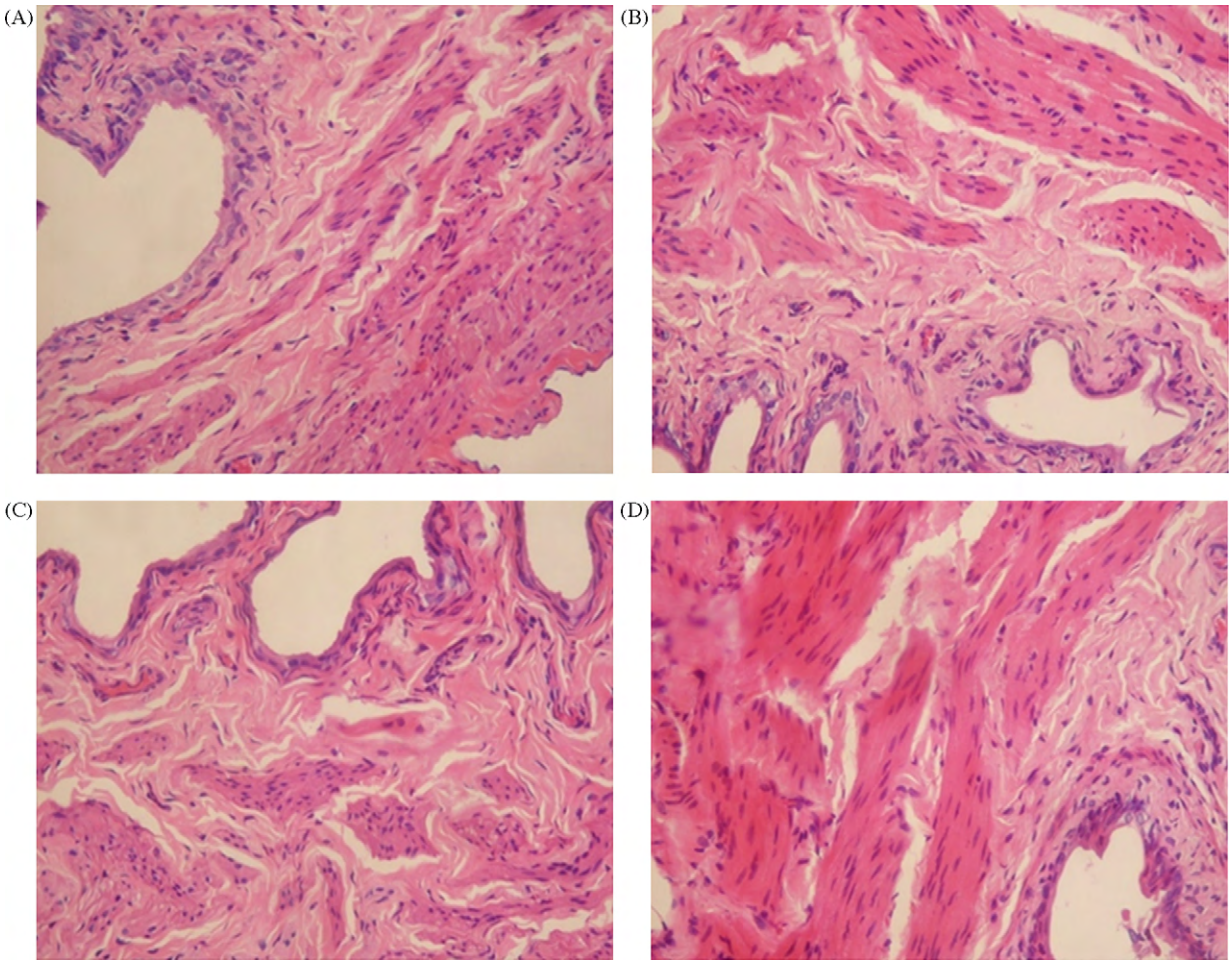

Fig. 5. Rat bladder biopsies from (A) control group, (B) carbachol group, (C) tolterodine-loaded microspheres group and (D) tolterodine tartrate tablets group.

last to the 15th day, indicating that TMS could produce prolonged pharmacological responses. These results indicate that TMS may offer a better therapeutic effect than oral administration of tolterodine.

Fig. 5 showed that the control group bladder had an urothelium of 4–5 layers of cells, a lamina propria and a smooth muscle layer with longitudinal and transversal fibers. The structure of the control bladder tissue revealed no abnormalities. There was no obvious pathological difference in rat bladder slices between the drug-treated and control groups. In summary, no histological change occurred to the rat bladder after the administration of tolterodine-loaded PLGA microspheres.

Before and after the experiment, the contents of urinary sodium and potassium were measured. The results suggested that the contents decreased, but did not reach a statistically significant level ($p > 0.05$). Meanwhile, there was no significant difference in the contents of urinary sodium and potassium between the drug-administered group and the control group, either ($p > 0.05$).

The application of PLGA microspheres to anticholinergic/antimuscarinis agents was reported in several patents or patent applications. However, no product was launched in the market so far. We are still carrying on further evaluating of this methodology on other model drugs in this area.

4. Conclusions

We prepared tolterodine-loaded microspheres with two biodegradable polymers (PLGA) using O/W emulsion technique. Results showed that tolterodine PLGA microspheres had spherical shape and smooth surface without pores. Encapsulation efficiency of tolterodine PLGA microspheres increased with raising polymer concentration and the addition of fattic acids. The *in vitro* release tests showed that all the formulations had a low initial burst followed by a prolonged release. From 1 to 20 days, the tolterodine release rates were relatively constant. Pharmacokinetic studies indicated that the release profile of tolterodine microspheres prepared form RG 503H lasted for 18 days in beagle dogs. Tolterodine microspheres could reduce individual differences and drug–drug interactions through bypassing the first pass in the liver and producing very little DD01. Preliminary pharmacological experiments showed that an i.m. administration of TMS (3 mg/kg) significantly decreased the number of carbachol-induced micturition for 15 days, indicating that TMS could provide prolonged pharmacological efficacy. The continued inhibition of muscarinic receptor of the microspheres formulation might provide a more effective treatment of OAB patient than that of the oral formulation which inhibits the receptor impulsively. There was no significant difference in bladder morphology and the contents of urinary sodium and potassium between the control and drug-administered groups.

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