

Preparation of Polyethylene Glycol Monomethyl Ether Chitosan-diethylenetriamine Pentaacetic Acid and Its Effect on $^{89}\text{SrCl}_2$ Excretion and Radiation Protection in the Digestive Tract of Rats

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Abstract

In this study, hydrophilic polymer polyethylene glycol monomethyl ether chitosan-diethylenetriamine pentaacetic acid (mPEGCS-DTPA) was synthesized via chemical synthesis and cross-linking, and its cytotoxicity and radiation protective effect *in vivo* were studied. The results revealed that mPEGCS-DTPA exhibits good cytocompatibility and increases the excretion of radionuclides through the digestive and urinary tracts. The pathological results of the small intestine revealed that mPEGCS-DTPA exerts a good radiation-protective effect and could reduce the α and β -emitting radiation-induced damage of the intestinal mucosa. MPEGCS-DTPA can increase the excretion of radionuclides in the digestive tract and exhibits effective protection against radiation.

1. Introduction

After the severe nuclear power plant accidents at Chernobyl on 26 April 1986, Fukushima in March, 2011, and atomic bombs at Hiroshima and Nagasaki, high levels (large amount) of diverse fission products have been discharged into the environment [1–3]. The environment of nuclear exposure sites is highly complex, and the radionuclides may be ingested into the blood through intestinal mucosa leading to cumulative damage to the human body, causing serious diseases and death [4–6, 3]. Importantly, the tissue and organ damage can be avoided by preventing the absorption of radionuclides through the digestive tract when nuclear accidents happened. Moreover, the broad-spectrum contaminative radionuclide chelating pharmaceuticals such as DTPA and EDTA have revealed side effects [7], and there are no fixed dosages for the digestive tract. Hence, early nuclear decontamination of the digestive tract is not suitable during nuclear exposure.

Diethylenetriamine pentaacetic acid (DTPA) is a broad-spectrum chelating agent with certain side effects [7]. However, the dosage form of DTPA or its structure can be modified to reduce its side effects, improve chelating ability, reduce dosage, increase usage, and extend its treatment time [8, 9].

Chitosan (CS) has nontoxicity, biodegradability, good biocompatibility, and can be absorbed by organisms [10–12]. Furthermore, some experiments have shown that chitosan has radioprotective effects [13]. Additionally, it may scavenge oxygen free radicals and exhibit anti-inflammatory properties [14].

Polyethylene glycol (PEG) is a type of linear, noncharged, nontoxic, nonantigenic, nonimmunogenic, biocompatible, highly soluble, and biodegradable polymer material, which can be dissolved in aqueous solution as well as in organic solvents [15]. PEG is a commonly used drug for clinical application in the gastrointestinal tract, and is approved by the FDA for use in food and medicine [16]. Polyethylene glycol monomethyl ether (mPEG), exhibiting both hydrophilicity and flexibility, is used to increase the water solubility of macromolecules and in grafting polymers [17].

In the present study, mPEGCS-DTPA was synthesized from diethylenetriamine pentaacetic acid, chitosan, and polyethylene glycol by organic synthesis and chemical cross-linking, and an oral solution was

prepared. We assessed the effect of mPEGCS-DTPA on the excretion and radioprotective effects of radionuclide ^{89}Sr in the digestive tract of rats.

2. Materials And Methods

2.1. Materials

Chitosan (Chitosan, molecular weight 5000, degree of deacetylation = 92.3%, Nantong Xingcheng Biological products Factory, Jiangsu, China), mPEG (molecular weight 1900, Aladdin, Shanghai, China), DTPA (X-Y Biotechnology, Shanghai, China), dialysis bag (molecular weight interception is 8000–14000, Sangon Biotech Co. Ltd. Shanghai, China). ICE-6 cells were obtained from iCell Bioscience Inc. Ltd. (Shanghai, China), and were incubated in 1640 medium containing 10% fetal bovine Serum and 1% penicillin-streptomycin solution (Sigma Chemical Co.) in a humidified atmosphere of 5% CO_2 at 37 °C. The Cell Counting Kit-8 was purchased from Do Jindo Laboratories (CCK-8, Tokyo, Japan).

2.2. Preparation of mPEGCS-DTPA

2.2.1 Preparation of iodinated polyethylene glycol monomethyl ether (mPEGI)

In this experiment, 20 g (10 mmol) MPEG was added into a 250 mL three-neck flask, thereafter, 8 mL of triphenylphosphine (30 mmol) and 4.3 mL (30 mmol) of methyl iodide were added and reacted at 130 °C for 6 h under nitrogen protection. The solution was allowed to cool at 20~30°C, and then toluene was added. Next, the solution was stirred to dissolve the crude product, and then poured into anhydrous ether to obtain a white precipitate. The filter residue was washed thrice with anhydrous ether and the light yellow mPEGI solid powder was obtained by vacuum drying.

2.2.2 Preparation of phthalic anhydride chitosan

Here, 4.967 g (31 mmol- NH_2) chitosan, 13.797 g phthalic anhydride (93 mmol) and 200 mL N-dimethylformamide (DMF) were added into a 250 mL flask, protected by nitrogen, and reacted at 130 °C for 7 h. The reaction was cooled at 20~30°C, filtered, and the filtrate was poured into ice water to precipitate a dark-yellow solid, which was washed with ethanol and dried under vacuum to obtain dark-yellow phthalic anhydride chitosan solid.

2.2.3 Preparation of mPEG chitosan (mPEGCS)

In general, 2.967 g AgNO_3 was dissolved in 10 mL water. Next, 1.032 g KOH was dissolved in 5 mL water, then dripped into the dissolved AgNO_3 , and the gray precipitate was obtained via filtration. Thereafter, 0.77 g (3.3 mmol) gray precipitate was added into a 250 mL flask, and 4.571 g (2 mmol) mPEGI, 0.582 g (2 mmol) phthaloyl chitosan, and 40 mL DMF were added and refluxed at 60 °C for 16 h. Later, 30 mL 85% hydrazine hydrate and 60 mL water were added, after which the temperature was increased to 90 °C, and then reacted for 15 h. Furthermore, the solution was cooled, filtered, and then 50 mL double-deionized

(DD) water was added. Next, the water and unreacted hydrazine hydrate were removed by rotary evaporation. The process was repeated thrice. The filtrate was decompressed and evaporated to remove the solvent to stickiness, and then transferred to a dialysis bag, dialyzed with DD water for 3 days, and freeze-dried; later, 2.3 g mPEGCS white solid was obtained.

2.2.4 Preparation of mPEGCS diethylenetriamine pentaacetic acid (mPEGCS-DTPA)

Herein, 100 mg polyethylene glycol monomethyl ether chitosan (mPEGCS) was added to 5 mL water, and then added with 196.7 mg (0.5 mmol) diethylenetriamine pentaacetic acid (DTPA), 115.0 mg (0.6 mmol) EDC, 69.05 mg (0.6 mmol) NHS and then reacted for 24 h at 30 °C. The solution was then transferred to a dialysis bag, dialyzed with DD water, and freeze-dried, leaving 163 mg white mPEGCS-DTPA solids[8, 9].

2.3 Characterization of mPEGCS-DTPA

2.3.1 Fourier transform infrared spectroscopy (FT-IR)

The appropriate amounts of samples were mixed with KBr powder at a mass ratio of 1:200, and then tested after fine pressing; the scanning range of approximately 4000–400 cm^{-1} (FT-IR800) was tested with mPEGCS-DTPA.

2.3.2 Nuclear magnetic hydrogen spectroscopy analysis (^1H NMR)

The superconducting nuclear magnetic resonance instrument was used to scan the nuclear magnetic hydrogen spectrum (BrukerAV-400). Chitosan, mPEGCS and mPEGCS-DTPA polymer was dissolved in D_2O solution. Scanning condition: frequency, 400 MHz, range, approximately 0–14 ppm.

2.4 Cytotoxicity test of mPEGCS-DTPA

The cytotoxicity of mPEGCS-DTPA on cells was evaluated via CCK-8 assay, according to the manufacturer's instructions. Briefly, ICE-6 cells were cultured in 96-well plates at a density of 5×10^3 cells per well and treated with mPEGCS-DTPA at different concentrations (0, 0.01, 0.05, 0.1, and 0.5 mg/mL) for 2 days at 37°C. The absorbance was measured using a Thermo Scientific microplate reader at 450 nm.

2.5 Rat gastrointestinal (GI) model

Eighteen female Sprague-Dawley rats (6–8 weeks of age) were purchased from the Experimental Animal Center of Army military Medical University (Chongqing, China). Rats were housed under a room temperature of 20–25 °C and relative humidity of 40–70% with free movement and access to drinking water and were fed ordinary rat food. The whole study was completed in the State Key Laboratory of Trauma, Burns and Combined Injury, Institute of Combined Injury, Chongqing Engineering Research Center for Nanomedicine, College of Preventive Medicine, Third Military Medical University. The experimental

protocols were approved by the Laboratory Animal Welfare and Ethics Committee of Third Military Medical University [certificate number: SYXK(Yu)20170002]. After a 7-days adaptation period, the rats (180–220 g) with good health were selected for use.

The rats were administered by oral gavage with $^{89}\text{SrCl}_2$ (48.8×10^4 Bq/mL, 1 mL). Briefly, the rats were anesthetized with 1% pentobarbital sodium and intragastrically instilled using a gastric perfusion needle. Thereafter, they were randomly divided into three groups ($n = 6$ per group): NS ($^{89}\text{SrCl}_2 + \text{NS}$, 1 mL of normal saline was administered by oral gavage 30 min after $^{89}\text{SrCl}_2$), DTPA (60 mg/kg of DTPA dissolved in 1 mL of normal saline was administered via oral gavage 30 min after $^{89}\text{SrCl}_2$), and mPEGCS-DTPA (60 mg/kg of mPEGCS-DTPA dissolved in 1 mL of normal saline was administered via oral gavage 30 min after $^{89}\text{SrCl}_2$). Rats in the experimental groups were placed in metabolic cages individually, thereafter, feces and urine were collected daily for future measurement. The venous blood (100 μL) of rats was collected by cutting their tail before and 2, 8, and 24 h after oral administration of $^{89}\text{SrCl}_2$; the blood sample was then added to the known amount of nitric acid, and the β -radioactivity was detected. At 24 h after oral gavage of $^{89}\text{SrCl}_2$, 1% pentobarbital sodium was administered to anesthetize the rats. The metacarpal bones were removed aseptically and stored in a refrigerator at 4 °C for follow-up and β -radioactivity detection. At 48 h, all rats were sacrificed, and then left femur tissues were collected and stored at 4 °C for further radiochemical analysis, the small intestine tissues were collected and fixed in 4% paraformaldehyde for 48 h for further evaluation. The metacarpal bones and femoral bones were ashed at 750 °C for 4 h, dissolved in HNO_3 , and neutralized to PH 4–6 with 1 mmol/L NaOH. Thereafter, the small intestinal tissue was fixed with paraformaldehyde for 48 h, followed by routine paraffin embedding. Sectioning and HE staining were used to observe the degree of histopathological injury, and the images of pathological sections were further captured. Feces were homogenized and subjected to the same procedure as for the bones. Urine was collected at the bottom of the metabolic cage with a known volume of HNO_3 . Radioactivity of these samples was measured in a scintillation cocktail (AQUASOL-2, NEW England Nuclear) by a liquid scintillation spectrometer (LKB, Ractbeta 1219). The measurements were performed for 1 min with corrections for chemiluminescence and chemical quenching [18, 19].

2.6. Statistical analysis

All data are presented as the mean \pm standard deviation (SD). Statistical significance was determined by one-way ANOVA followed by LSD post hoc analysis and repeated-measurement data were analyzed by repeated-measurement analysis of variance (pairwise comparison using the least significant difference test) (SPSS statistics 25, IBM Inc.). P -values < 0.05 were considered statistically significant.

3. Results

3.1. Preparation and characterization of mPEGCS-DTPA

To synthesize mPEGCS-DTPA, mPEG was initially grafted with chitosan 6-OH, thereafter, the hydrophilic compound mPEGCS was synthesized [20, 21]. Then, mPEGCS-DTPA was constructed by a chemical bond between $-NH_2$ and $-COOH$ under EDC/NHS condition. MPEGCS-DTPA showed good solubility in water, which is convenient for the follow-up preparation of the oral dosage form for the digestive tract [22, 23].

The absorption peak of the carboxyl group $-COOH$ appeared near 1639.38 cm^{-1} (Fig. 1B), that of secondary amide appeared at 1395.34 cm^{-1} (Fig. 1B), and that of the ether bond appeared at 1108.99 cm^{-1} (Fig. 1B). The results indicated that MPEG and DTPA were grafted on chitosan.

The 1H NMR of chitosan, mPEGCS, mPEGCS and mPEGCS-DTPA are illustrated in Figure 1C. The single peaks near the PEGCS δ 2.5, 3.6, and 3.7–4.0 ppm are the proton peak on the mPEG side chain $-OCH_3$, $-OCH_2CH_2$ and the protons on the chitosan C (3–6) and chitosan C (1) acetal, respectively, indicating that the polyethylene glycol monomethyl ether branch is attached to the chitosan. Moreover, the proton peak of chitosan- NH_2 in mPEGCS around δ 2.6–3.0 ppm (Fig. 1C) disappeared in mPEGCS-DTPA. Thus, DTPA was successfully grafted onto mPEGCS.

3.2 *In vitro* biocompatibility

To estimate the biocompatibility of mPEGCS-DTPA, CCK-8 assay was used to quantitatively evaluate the viability of ICE-6 cells post mPEGCS-DTPA treatment (Fig.2). MPEGCS-DTPA exhibited excellent biocompatibility, with cell viability being $>99\%$, even at PEGCS-DTPA concentrations up to 0.5 mg/mL.

3.3. Effect of mPEGCS-DTPA on the excretion of radioactive strontium in rats

3.3.1 Radioactive level of strontium in rat bones after oral gavage of drugs

The rats were administered by oral gavage of $^{89}SrCl_2$ for 30 min. The β -radioactivity count in the metacarpal bone of the mPEGCS-DTPA group was lower than that of the NS group at 24 h (Fig. 3A; $P < 0.01$). The β -radioactivity count in the femur measured at 48 h in the mPEGCS-DTPA group was lower than that in the NS group (Fig. 3B; $P < 0.01$).

3.3.2 The β -radioactivity counting in blood of rats at different time after oral gavage of $^{89}SrCl_2$.

The β -radioactivity counting of each group was measured before, 2, 8, and 24 h after oral administration of $^{89}SrCl_2$ (Fig. 4). The changing trend of blood radioactivity count in the DTPA group and mPEGCS-DTPA group was similar as that in the NS group, and β -radioactivity counting in rats indicates rapid absorption with a radioactive blood peak observed 2h after ingestion[24]. However, the radioactivity count in the mPEGCS-DTPA group and the DTPA group was lower than that in the NS group at 2 h ($P < 0.05$), that in the mPEGCS-DTPA group was lower than that in the NS group at 8 h ($P < 0.01$), and that in the DTPA

group was lower than that in the NS group at 8 h ($P < 0.05$). No significant difference was observed in the blood radioactivity between mPEGCS-DTPA and DTPA groups at 2 and 8 h phase point.

3.3.3. Comparison of fecal excretion after oral gavage of drugs

Feces were collected daily before and after oral gavage of $^{89}\text{SrCl}_2$ in rats for measuring the daily radioactivity count. Under normal feeding conditions, the total fecal radioactivity count of rats in each group was at the basal level, at 24h after oral gavage of $^{89}\text{SrCl}_2$ (Fig. 5A), the β -radioactivity counting in mPEGCS-DTPA group was higher than that in the NS group on Day 1 ($P < 0.01$), whereas that (Fig. 5B) in the DTPA group was higher than that in the NS group on Day 2 ($P < 0.01$).

3.3.4. Comparison of urine excretion after oral gavage of drugs

Urine was collected to measure the daily radioactivity count of urine before and after oral gavage of $^{89}\text{SrCl}_2$ in rats. Under normal feeding conditions, the urinary radioactivity count of rats in each group was at the basal level, and the urine was collected daily after oral gavage of $^{89}\text{SrCl}_2$. The total urine radioactivity count of rats after oral gavage of $^{89}\text{SrCl}_2$ at 24 h (Fig. 6A) in the mPEGCS-DTPA group was higher than that in the NS group ($P < 0.05$) and the DTPA group ($P < 0.01$) on Day 1, whereas the count in the mPEGCS-DTPA group (Fig. 6B) was higher than that in the NS and DTPA groups ($P < 0.001$).

3.4. Pathological changes of small intestine injury induced by radiation in rats

The intestinal tissue structure was abnormal and there was pathological damage of the small intestine of rats, including defect of mucous membranes, loss of mucous layer, accumulation and infiltration of inflammatory cells, exfoliation and necrosis of villi, and loss of glands, in the NS and DTPA groups, there were less observed in the mPEGCS-DTPA group.

4. Discussion

The development of effective methods and drugs to mitigate the nuclides absorption through intestinal mucus is an important area when nuclear accidents or terrorism happens.

PEG is commonly used in hospitals when colonoscopy is needed, due to its cathartic effect[16, 25]. DTPA is used as chelating agent, and chitosan has a protective effect on radiation [13]. mPEGCS-DTPA was synthesized by chemical synthesis and cross-linking; it is a hydrophilic compound and cannot pass through the phospholipid bilayer of the cell membrane. mPEGCS-DTPA chelates heavy metal ion Sr, and can be quickly excreted from the body through the gastrointestinal tract[16], which can effectively reduce the absorption of heavy metal ions into the blood through the intestinal tract and prevent serious secondary damage to the body [26].

In this study, rats were orally gavaged with $^{89}\text{SrCl}_2$ (48.8×10^4 Bq/mL, 1 mL). At 48 h after oral gavage of $^{89}\text{SrCl}_2$, the rats were sacrificed. The radioactivity count of the heart, liver, spleen, kidney and small intestine were close to the basal level, and a high amount of the radionuclide strontium was deposited in the bone tissue [4, 27, 5, 6].

The newly synthesized mPEGCS-DTPA was co-cultured with rat ICE-6 cells. CCK8 assay revealed that mPEGCS-DTPA was compatible with rat ICE-6 cells at different concentrations [28]. The deposition of radionuclides in metacarpus and femur in mPEGCS-DTPA group was lower than that in NS group, indicating that mPEGCS-DTPA has certain chelating and promoting excretion ability. Previous studies have reported that the peak of strontium absorption into the blood through the digestive tract occurs at 60–120 min, which is consistent with our experimental results. The radioactivity count in blood at 24 h was close to the basal level, and we speculated that strontium was absorbed through the digestive tract mainly within 24 h after intake [5, 29]. The count of blood radioactivity in the mPEGCS-DTPA group and DTPA group was lower than that in the NS group at 2 and 8 h, and the lowest was observed in the mPEGCS-DTPA group at 8 h, which may be related to the massive excretion from the digestive tract. To find out the reasons for the differences in radioactivity count between different groups of bone tissue and blood, pathways of drug metabolism were also exposed. The results of fecal excretion revealed that the radioactive count of feces in the mPEGCS-DTPA group was higher than that in the other two groups on the first day after ingestion of $^{89}\text{SrCl}_2$, and the total radioactive deposition in feces in the DTPA group was higher than that in the NS group on the second day after intake of strontium, which may be related to the slow excretion of DTPA chelating metal ions through the digestive tract [24, 15, 16]. However, no significant difference was observed in the chelation inhibition effect in the digestive tract compared with the traditional drug DTPA. Chitosan ingested through the digestive tract can be detected in the kidney in a short time [30, 10, 12]. In this study, the total radioactivity count in urine from the mPEGCS-DTPA group was significantly higher than that of the other two groups on the 1st and 2nd days after ingestion of $^{89}\text{SrCl}_2$. We speculated that few $^{89}\text{SrCl}_2$ was absorbed into the blood and chelated by mPEGCS-DTPA, and which might be excreted through urine [31, 30, 32].

The small intestinal mucosal injury caused by $^{89}\text{SrCl}_2$ represents radiation injury; in the mPEGCS-DTPA group, this injury was less severe than that in the other two groups as observed by HE staining after ingestion of $^{89}\text{SrCl}_2$ for 48 h. Moreover, chitosan has a certain radiation-protective effect on α and β rays, which may cause ionizing radiation damage [13]. Combined with the previous research results of HE staining of rat small intestine, we speculate that the newly synthesized mPEGCS-DTPA has a certain radiation-protective effect on α and β rays, which may cause electric radiation damage to the digestive tract mucosa.

Thus, the results indicate that mPEGCS-DTPA exhibits good cytocompatibility, increases the excretion of the radionuclide strontium, and exerts radiation-protective effect against same in the digestive tract. Although the effect of the excretion of mPEGCS-DTPA still needs to be further explored and improved, it

increases the oral dosage form of DTPA used in the digestive tract, has good ionizing radiation-protective effect, and can be used for clinical application.

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Figures

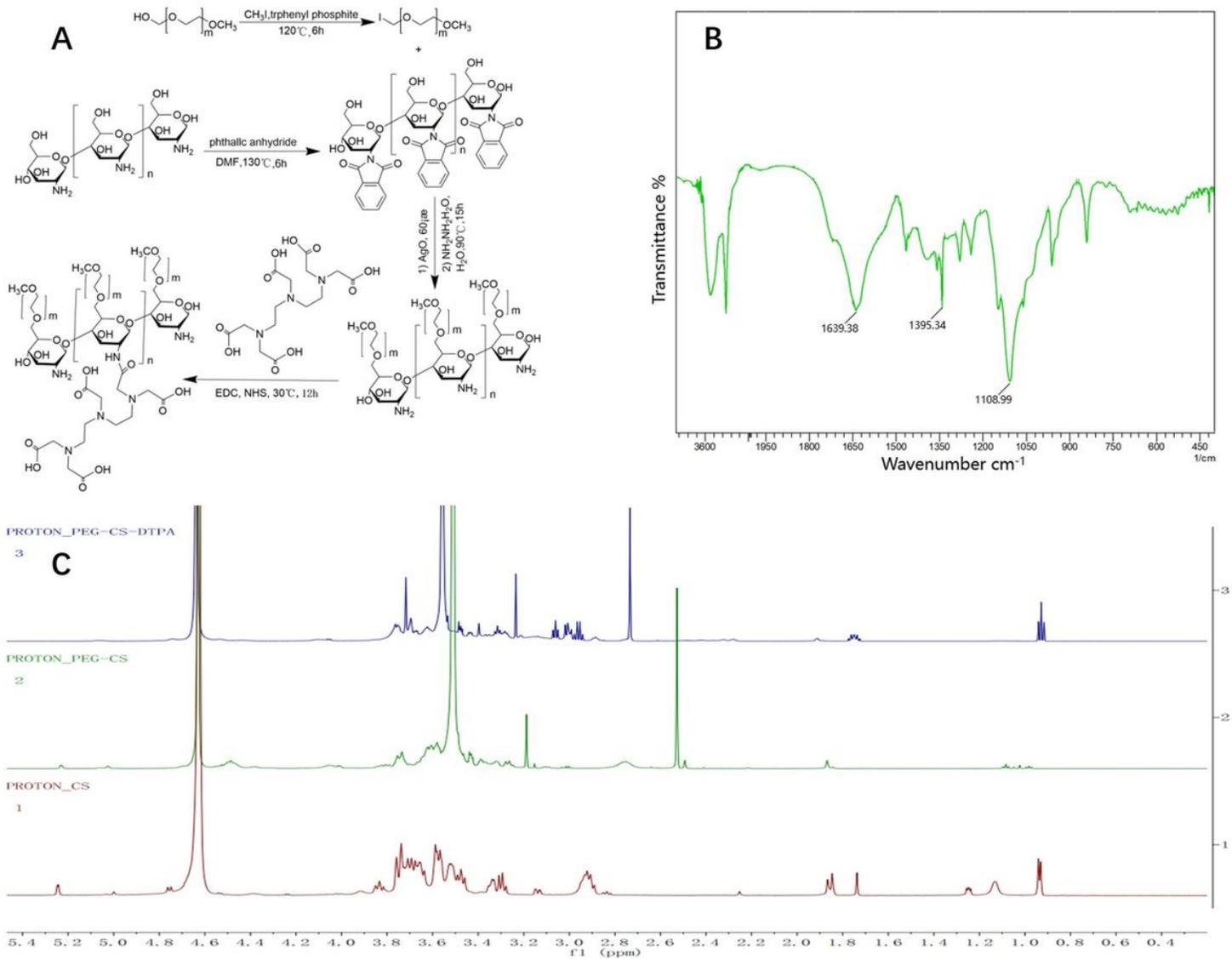


Figure 1

Preparation and characterization of mPEGCS-DTPA. A. Synthesis of mPEGCS-DTPA. B. FT-IR of mPEGCS-DTPA. C. ^1H NMR of chitosan, mPEGCS and mPEGCS-DTPA

mPEGCS-DTPA-48h

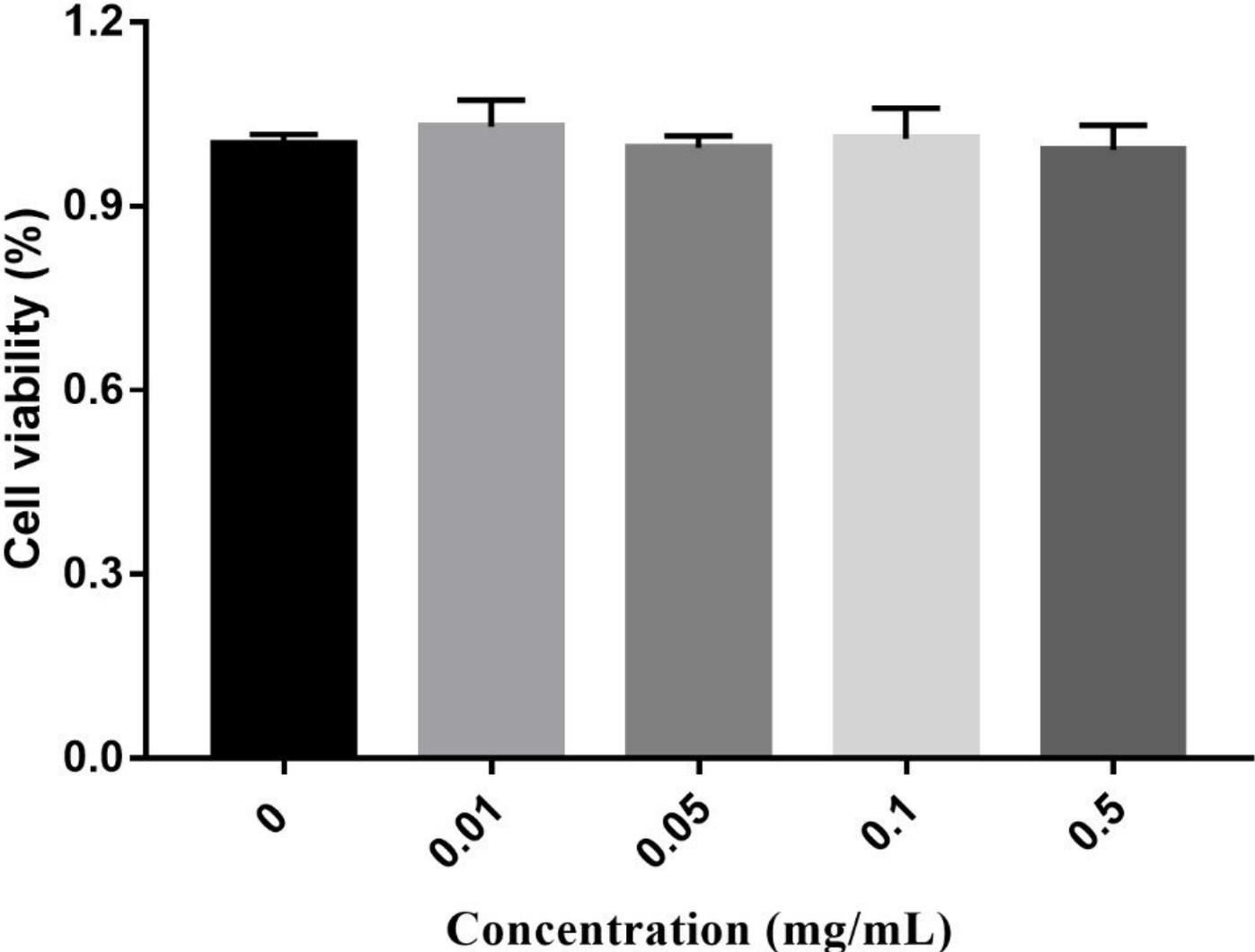


Figure 2

Cellular viability of mPEGCS-DTPA. The cell viability was determined by CCK-8 assay.

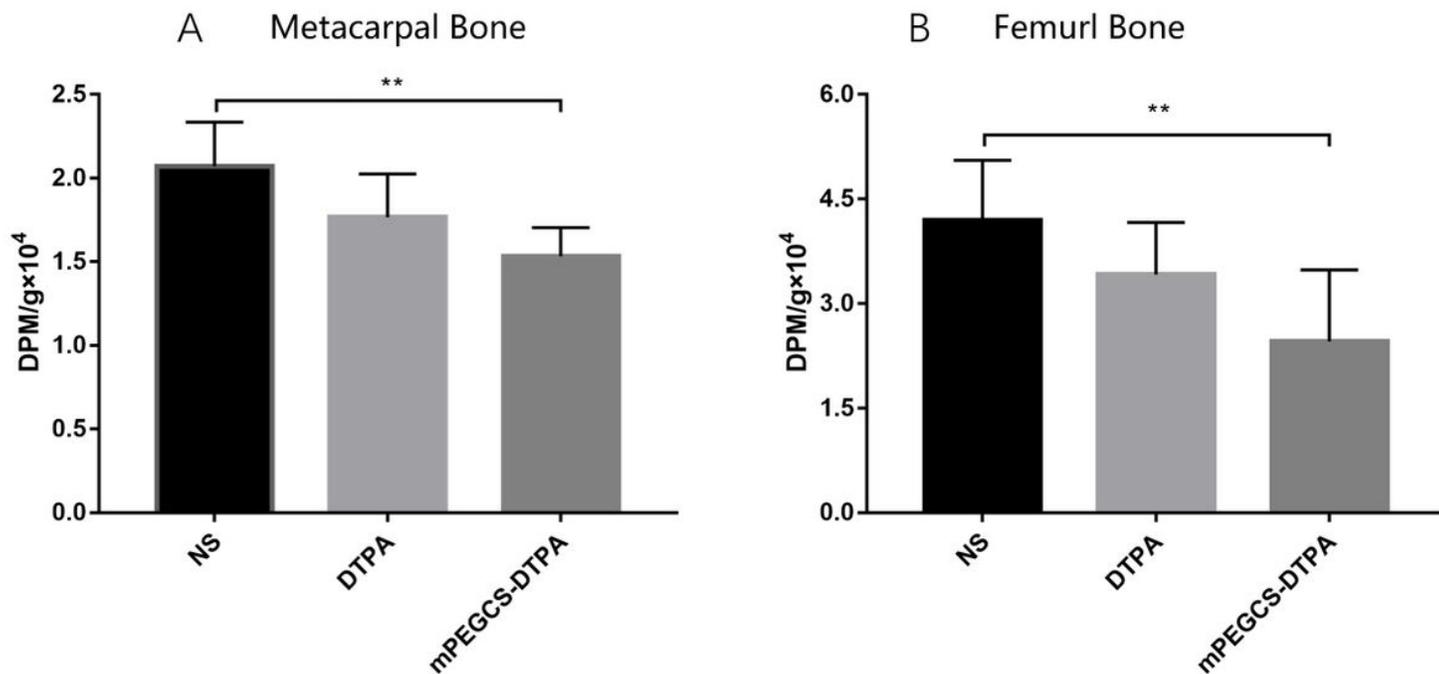


Figure 3

Measurement of radioactivity in bone after oral gavage of radioactive strontium in rats. A. Measurement of radioactivity in rat metacarpal bone at 24-h phase point. B. Measurement of radioactivity in rat femur at 48 h phase point. (*p < 0.05, **p < 0.01, and ***p < 0.001)

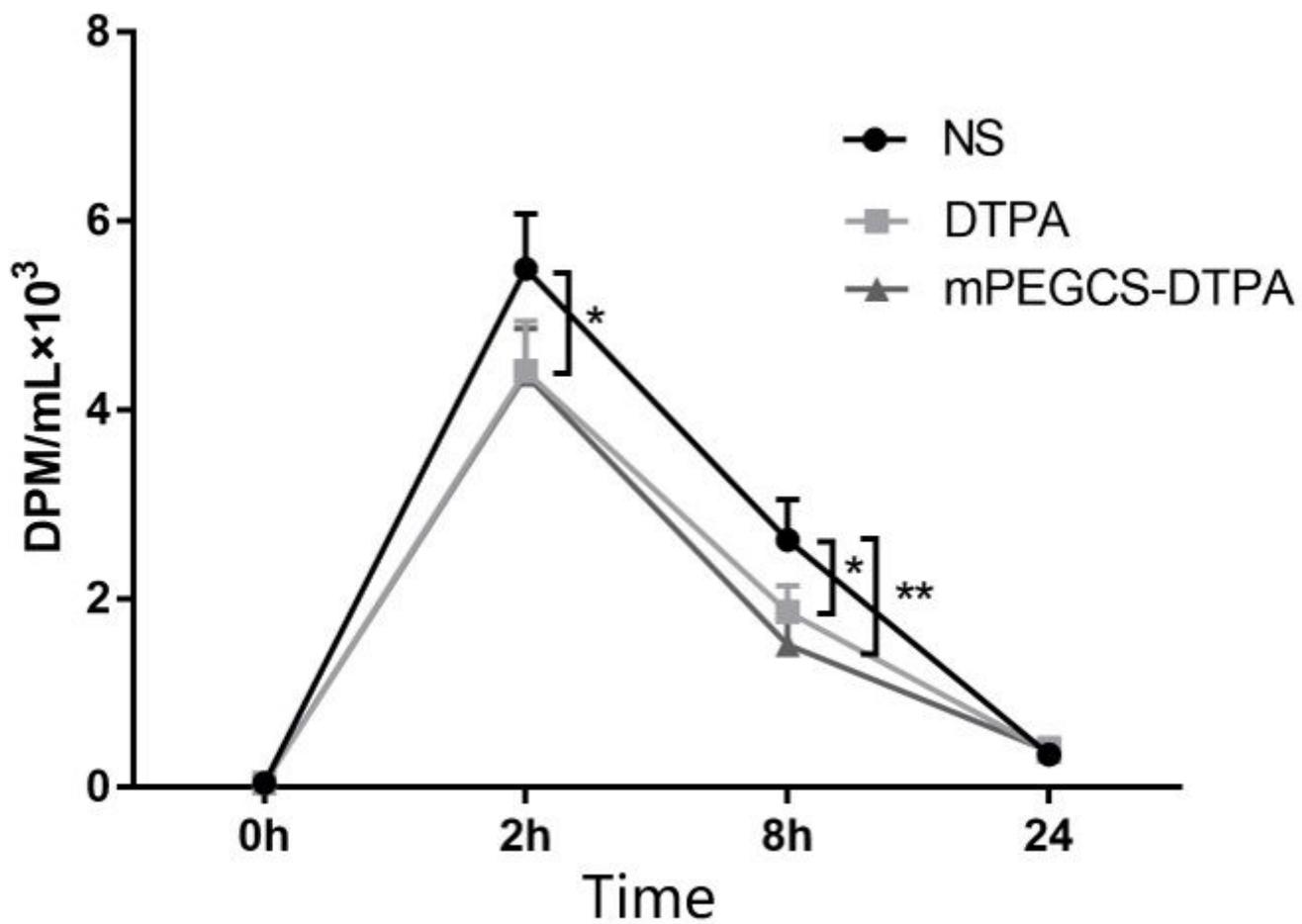


Figure 4

Radioactivity count in blood at different time points after oral administration of ⁸⁹SrCl₂ in rats (*p < 0.05, and **p < 0.01).

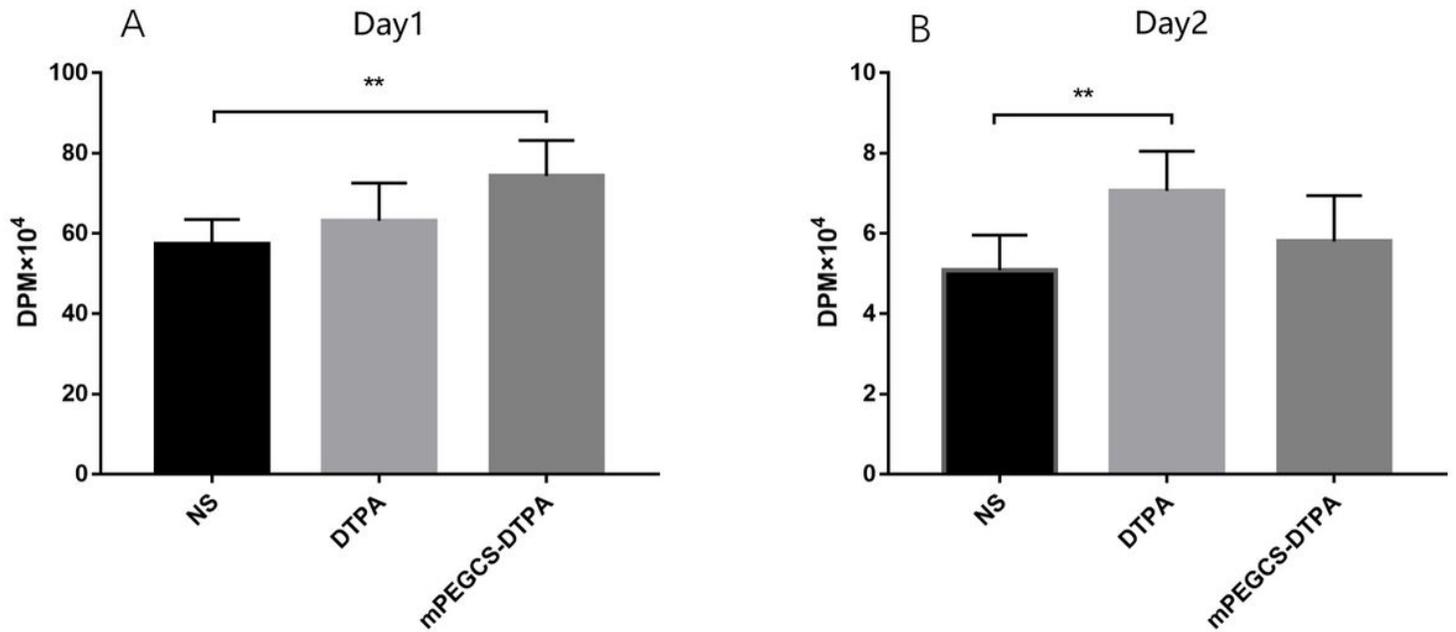


Figure 5

Total fecal radioactivity count of rats 24h after oral gavage of ⁸⁹SrCl₂ on Day 1 (Fig. 5A) and Day 2 (Fig. 5B) (*p < 0.05, and **p < 0.01).

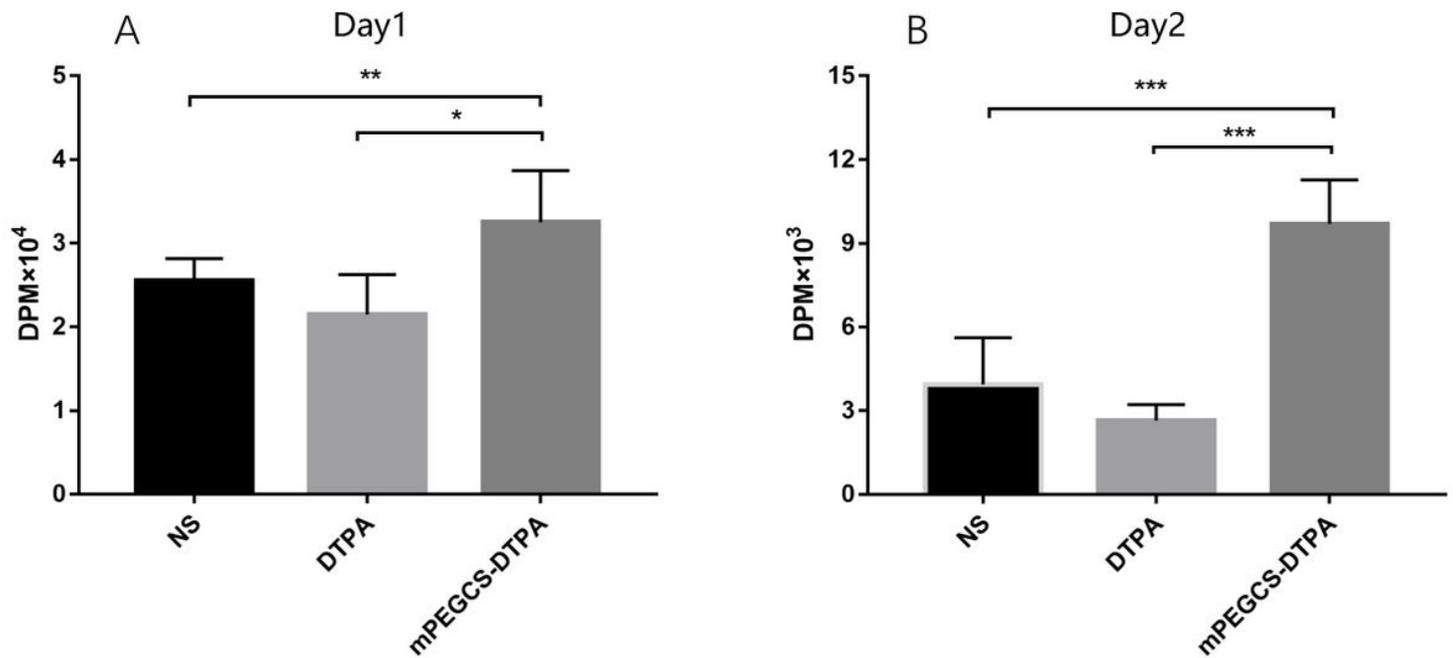


Figure 6

24 h total urine radioactivity count of rats after oral gavage of ⁸⁹SrCl₂ on Day 1 (Fig. 6A) and Day 2 (Fig. 6B). (*p < 0.05, **p < 0.01, and ***p < 0.001).

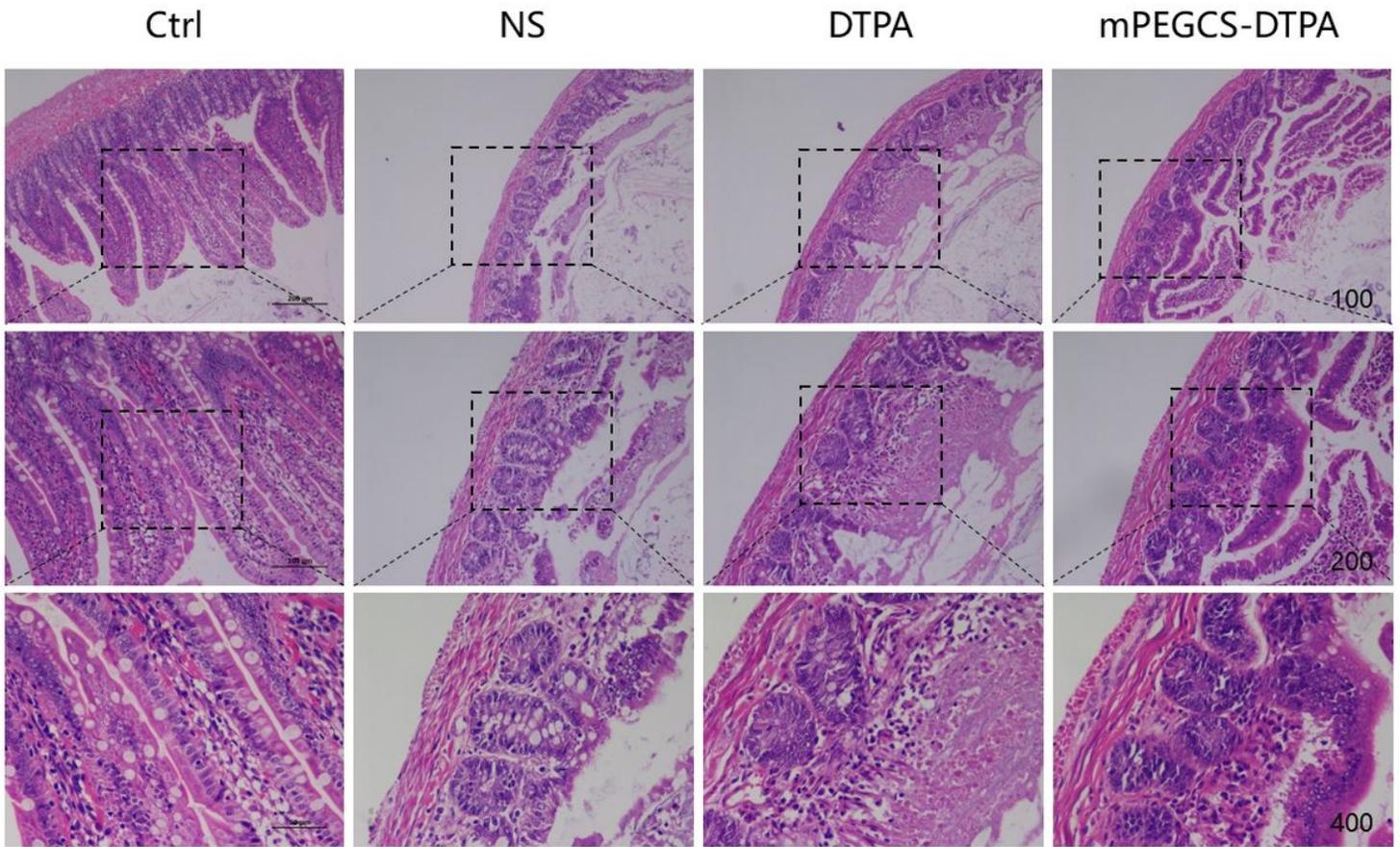


Figure 7

Pathological results of small intestine of rats in each group at 48 h. H&E staining of small intestine, Ctrl (normal without any treatment), NS ($^{89}\text{SrCl}_2$ + NS), DTPA ($^{89}\text{SrCl}_2$ + DTPA), and mPEGCS-DTPA ($^{89}\text{SrCl}_2$ + mPEGCS-DTPA)