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L-glutamic acid-g-poly hydroxyethyl methacrylate nanoparticles: acute and sub-acute toxicity and biodistribution potential in mice

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The aim of this safety study in mice was to determine *in vivo* toxicity and biodistribution potential of a single and multiple doses of L-glutamic acid-g-p(HEMA) polymeric nanoparticles as a drug delivery system. The single dose did not cause any lethal effect, and its acute oral LD_{50} was >2.000 mg/kg body weight (bw). Multiple doses (25, 50, or 100 mg/kg bw) given over 28 days resulted in no significant differences in body and relative organ weights compared to control. These results are supported by biochemical and histological findings. Moreover, nanoparticle exposure did not result in statistically significant differences in micronucleus counts in bone marrow cells compared to control. Nanoparticle distribution was time-dependent, and they reached the organs and even bone marrow by hour 6, as established by *ex vivo* imaging with the IVIS® spectrum imaging system. In conclusion, L-glutamic acid-g-p(HEMA) polymeric nanoparticles appear biocompatible and have a potential use as a drug delivery system.

KEY WORDS: biocompatibility; blood biochemistry; genotoxicity; histology; *in vivo* toxicity; micronucleus test; polymers

Poly(2-hydroxyethyl methacrylate) [p(HEMA)] is a biocompatible and a biodegradable polymer used in biomedicine and pharmaceutics (1–4), as it contains hydroxyl and carboxyl groups that can bind essential amino acids such as L-glutamic acid to carry molecules such as drugs. For this reason, it has widely been researched as a potential drug delivery nanosystem (5–9). Our *in vitro* toxicity and characterisation study (6) has shown that L-glutamic acid-g-p(HEMA) nanoparticles have an average size of ~194.6 nm, negative surface charge of -18 mV, and a nearly spherical shape. Another *in vitro* study of ours (7), in turn, has shown no genotoxic or cytotoxic effects of p(HEMA) (grafted on lysine) on 16HBE and THP-1 cell lines and no haemolytic activity in rabbit blood at high doses.

The aim of this study was to take a step further and determine the toxicity and the biodistribution of a single and multiple doses of L-glutamic acid-g-p(HEMA) nanoparticles in mice over 28 days.

MATERIALS AND METHODS

Materials

We used the L-glutamic acid-g-p(HEMA) nanoparticles synthesised and characterised in our previous study (6). All other chemicals were obtained from Sigma Chemical Co. (St. Louis, MO, USA).

Animals

For the study we used 6–8 weeks old BALB/c albino mice (n=44) obtained from the Bornova Veterinary Control and Research Institute, Izmir, Turkey. During the 7 days of acclimation and throughout the experiment, the mice had free access to standard laboratory pellet feed (Arden Araştırma & Deney, Ankara, Turkey) and drinking water under the following controlled ambient conditions: temperature 22±2 °C, humidity 55±5 %, and 12/12 h day-night light cycles. The protocol was approved by the Ege University Ethics Committee for Animal Experiments (approval No. 2015–044 of 25 November 2015).

Single-dose toxicity test

To determine the acute oral lethal dose (LD_{50}) of the L-glutamic acid-g-p(HEMA) nanoparticles, we followed the OECD Guideline 425: Acute Oral Toxicity: Up-and-Down Procedure (10). First, we administered 2.000 mg/kg per kg of body weight (bw) of L-glutamic acid-g-p(HEMA) dissolved in saline to one mouse by gavage, and 24 h later to the remaining three mice in a 24-hour sequence (two male and two female mice weighing 15–20 g each). At the end of

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the experiment (Day 14), blood was collected (100–150 μL) with cardiac puncture from all mice under the ketamine+xylazine (50+10 mg/kg ip) anaesthesia, and their organs were removed and weighed to calculate relative organ weights. Collected blood was analysed immediately after collection using the VetScan VS2 analyser (Abaxis Inc. Union City, CA, USA) for biochemical parameters and VetScan HM5 analyser (Abaxis Inc.) for haematological parameters.

28-day repeated-dose toxicity test

Repeated-dose toxicity testing included 6–8 weeks BALB/c albino mice (n=40) and followed the OECD Guideline 407 (11). The mice were randomised in three exposed groups (10 mice each; 5 male and 5 female) and one unexposed control group. The exposed groups received oral doses of either 25 mg/kg, 50 mg/kg, or 100 mg/kg bw [calculated based on oral LD_{50} (>2.000 mg/kg bw) and body weight] by gavage five days a week for 28 days (four weeks). The control group was receiving saline (0.1 mL), also by gavage.

The mice were weighed each week before and after the current round of 5-day exposure. On Day 29, the mice were sacrificed under ketamine+xylazine (50 +10 mg/kg ip) anaesthesia by taking 0.7–1.0 mL of blood from the heart. The organs were removed and weighed.

Blood biochemistry was determined for 12 parameters at baseline and upon sacrifice with a Vetscan VS2 analyser as follows: albumin (ALB), alkaline phosphatase (ALP), alanine aminotransferase (ALT), total bilirubin (TBIL), blood urea nitrogen (BUN), calcium (Ca), phosphate (PHOS), creatinine (CRE), glucose (GLU), potassium (K), total protein (TP), and globulin (GLOB).

Brain, liver, and kidney tissues for microscopy were fixed in 4 % paraformaldehyde for 24 h and processed for embedding in paraffin. Histological sections were cut into 5 μm thick slices and stained with haematoxylin and eosin (H&E). Tissue images were then taken with an Olympus C-5050 digital camera (Tokyo, Japan) mounted on an Olympus BX5 microscope (20× magnification).

The micronucleus (MN) test followed the OECD Guideline 474 (12) and procedure described by Schmid, with slight modifications (13). For it we took bone marrow cells, removed from the posterior foot tibia and femur with 1 mL of foetal calf serum. The cells were then pipetted into tubes and centrifuged at 450 *g* for 10 min and the supernatant discarded. The pellet was carefully resuspended, and one drop was placed and spread on a clean slide. After overnight drying, the slides were stained in May Grünwald solution for 3 min and then in May Grünwald + demineralised water (1:1 v/v) solution for another 2 min. Followed washing with distilled water, staining in 10 % Giemsa solution for 10 min, and washing again. After air-drying, the slides were fixed in methanol for 10 min and mounted with entellan. At least 1000 polychromatic erythrocytes per animal were scored for the presence of micronuclei.

In vivo biodistribution study

L-glutamic acid-g-p(HEMA) was labelled with IRD800-CW (Perkin Elmer Inc., Waltham, MA, USA) dye following the protocol described by Wu et al. (14) with minor changes. The nanoparticles (1 mg/mL) in PBS (pH 7.5) and 50 μL IRD800-CW (5 mg/mL) were incubated in the dark at room temperature for 3 h, centrifuged at 10,000 *g* for 10 min to remove unbound dye, and washed with 5 mL of PBS three times.

Then we injected 0.2 mL of stained nanoparticles into the tail vein of mice for optical imaging under isoflurane anaesthesia using the IVIS® Spectrum *in vivo* imaging system (Perkin Elmer Inc.). After imaging, the animals were sacrificed with a ketamine/xylazine overdose and the liver, kidney, heart, lung, spleen, stomach, and small and large intestine were imaged *ex vivo* (7). We also imaged bone marrow cells and examined them under an Olympus BX5 fluorescence microscope (Tokyo, Japan) (40× magnification).

Statistical analysis

Statistical analyses were run on the SPSS for Windows 10.0 (IBM Corp., Armonk, New York, USA) and GraphPad Prism 7 (GraphPad Software, LLC, Boston, MA, USA) statistical analysis programs. Total and relative organ weight and biochemical values were calculated, and results compared with the control group using one-way analysis of variance (ANOVA) followed by the LSD test. All values are expressed as means ± standard deviation (SD). Statistical significance was set to $p<0.05$.

RESULTS AND DISCUSSION

Overall, our findings confirm reports of most studies that polymeric nanoparticles are biologically inert and therefore suitable for application for drug and gene delivery or diagnosis (15–19).

Nanoparticle single-dose toxicity

Acute exposure to nanoparticles did not cause any lethal effect or significant changes in total body and (relative) organ weights (data not shown) or biochemical parameters (Table 1). The same is true for the haematological parameters with the exception of a significant decrease in white blood cell and significant increase in platelet counts (Table 2). Rodents show variations in many clinical chemistry values. White blood cell counts in mice vary between 2 and $10 \times 10^3/mm^3$ (20). For this reason, we disregarded the difference between the baseline and final (day 14) WBC counts in the single-dose toxicity test, as they were within the reference range.

28-day repeated-dose toxicity

The 28-day dosing with 25, 50, or 100 mg/kg bw of polymeric nanoparticles for five days a week did not cause any morphological or behavioural adverse effects, and the study was completed with

Table 1 Biochemical parameters in BALB/C mice (n=5) after acute single-dose exposure to 2000 mg/kg of L-glutamic acid-g-p(HEMA) nanoparticles

Biochemical parameters	Baseline (Mean±SD)	Day 14 post-exposure (Mean±SD)
ALB (g/dL)	3.6±0.4	3.3±0.1
ALP (U/L)	64±0.4	60±5.5
ALT (U/L)	36±0.4	35±2.5
TBIL (mg/dL)	5.0±0.4	5.0±0.5
BUN (mg/dL)	9.0±0.4	8.5±1.6
Ca (mg/dL)	2.37±0.4	2.75±0.3
PHOS (mg/dL)	2.88±0.4	3.35±0.7
CRE (mg/dL)	2.90±0.4	2.20±0.1
GLU (mg/dL)	136±0.4	136±8
K (mmol/L)	5.3±0.4	8.0±0.1
TP (g/dL)	4.5±0.4	5.5±0.4
GLOB (g/dL)	1.2±0.4	2.2±0.3

ALB – albumin; ALP – alkaline phosphatase; ALT – alanine aminotransferase; BUN – blood urea nitrogen; Ca (mg / dl) calcium; CRE – creatinine; GLOB – globulin; GLU – glucose; K – potassium; PHOS – phosphate; TBIL – total bilirubin; TP – total protein

Table 2 Haematological test results in BALB/C mice (n=5) after acute single-dose exposure to 2000 mg/kg of L-glutamic acid-g-p(HEMA) nanoparticles

Haematological parameters	Baseline (Mean±SD)	Day 14 post-exposure (Mean±SD)
WBC (10^9 cells/L)	8.68±2.1	**3.21±1.1***
LYM (10^9 cells/L)	5.43±0.8	2.41±0.9
MON (10^9 cells/L)	0.23±0.1	0.10±0.1
NEU (10^9 cells/L)	1.36±1.02	0.70±0.9
RBC (10^{12} cells/L)	8.02±1.3	10.41±1.2
HGB (g/L)	14.52±1.3	14.5±1.1
HCT (%)	38.63±3.9	45.65±4.1
MCV (fl)	48.0±2.9	44.0±2.8
MCH (pg)	12.26±0.7	13.90±0.7
MCHC (pg)	28.04±1.1	31.70±0.8
PLT (10^9 cells/L)	294.60±25.1	**975±35.8***

* significantly different from baseline ($p<0.05$). HCT – haematocrit; HGB – haemoglobin; LYM – lymphocytes; MCH – mean corpuscular haemoglobin; MCHC – mean corpuscular haemoglobin concentration; MCV – mean corpuscular volume; MON – monocytes; NEU – neutrophils; PLT – platelets; RBC – red blood cells (erythrocytes), WBC – white blood cells (leukocytes)

Table 3 Organ and relative organ weights in female (n=5) and male mice (n=5)

	Absolute organ weights (Mean±SD)				Relative organ weights (%)			
	Lung	Liver	Kidney*	Brain	Lung	Liver	Kidney*	Brain
Female mice								
Control	0.183±0.02	1.095±0.04	0.318±0.04	0.362±0.02	0.0083	0.0073	0.0074	0.0095
25 mg/kg	0.173±0.03	1.322±0.16	0.312±0.01	0.37±0.09	0.0497	0.0563	0.0518	0.0551
50 mg/kg	0.186±0.03	1.286±0.13	0.295±0.03	0.378±0.05	0.0144	0.0133	0.0118	0.0123
100 mg/kg	0.248±0.08	1.436±0.05	0.322±0.03	0.408±0.04	0.0164	0.0157	0.0152	0.0156
Male mice								
Control	0.222±0.03	1.536±0.11	0.47±0.05	0.37±0.06	0.0084	0.0077	0.0077	0.0069
25 mg/kg	0.228±0.05	1.818±0.21	0.468±0.05	0.374±0.03	0.0581	0.0615	0.0550	0.0548
50 mg/kg	0.225±0.04	1.596±0.17	0.462±0.04	0.358±0.06	0.0178	0.0158	0.0159	0.0157
100 mg/kg	0.205±0.03	1.615±0.27	0.465±0.06	0.397±0.03	0.0140	0.0126	0.0123	0.0134

* Kidney weights are given as the total weight of both organs

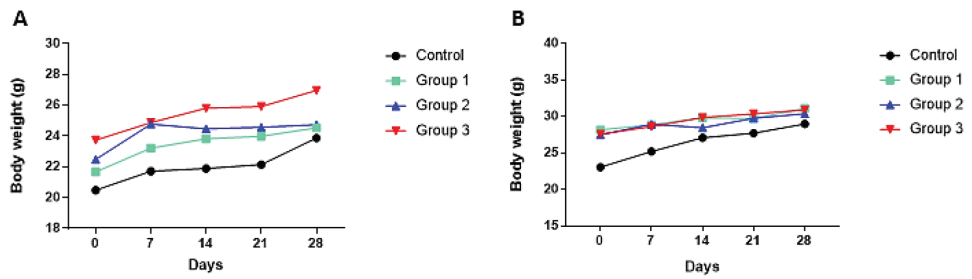

Figure 1 Body weight changes in control mice and those exposed to 25, 50, or 100 mg/kg bw of L-glutamic-g-p(HEMA) for five days a week for four weeks. A – female mice; B – male mice

the initial number of mice. Their body weights increased in a similar way in all groups, including control (Figure 1). No significant difference in (relative) organ weights (Table 3) or biochemical parameters (Table 4) was found between control and any of the exposed groups.

Furthermore, we found no significant histological changes in the exposed groups. Brain tissue (Figure 2) showed no abnormalities; the pia mater, the cortex, and medulla underneath appeared normal, as did the neurons and neuroglia. Liver tissue (Figure 3) had normal stromal and parenchymal areas and cells. The same is true for the kidneys (Figure 4), whose glomeruli, proximal and distal tubules, and other parenchymal, stromal, and perivascular areas and cells appeared normal.

These results are well in line with Malonne et al. (21), who reported no differences between control mice and those receiving 2.000 mg/kg bw of PNIPAAm, PNIPAAm-co-NVA, or PNIPAAm-co-AAc nanoparticles a day over 28 days.

In contrast to polymeric nanoparticles, metallic and metal-oxide nanoparticles have been reported to deposit in the brain and disturb the normal metabolism of neurotransmitters (22) or induce liver and spleen injury, lung inflammation, and cardiac toxicity (23).

The bone marrow micronucleus test found no significant differences in MN counts between controls and exposed animals (Table 5). These results are supported by Karlsson et al. (24).

Table 4 Changes in biochemical parameters (means ± SD) in female (n=5) and male (n=5) mice after exposure to L-glutamic acid-g-p(HEMA) polymeric nanoparticles over 28 days

Biochemical parameters	Groups							
	Control		Group 1 (25 mg/kg)		Group 2 (50 mg/kg)		Group 3 (100 mg/kg)	
	Baseline	Post-exposure	Baseline	Post-exposure	Baseline	Post-exposure	Baseline	Post-exposure
Female mice								
ALB (g/dL)	3.9±0.6	3.4±0.30	3.7±0.4	3.3±0.3	4.1±0.1	3.5±0.30	4.1±0.2	3.6±0.23
ALP (U/L)	81.2±4.7	65±15.8	82.2±5.2	54±6.02	50±9.1	53±8.50	50.6±4.1	51±19.4
ALT (U/L)	61.0±7.5	37±5	57.0±12.9	31±15.8	44±16.1	45.5±16.9	40.6±14.6	35±7.54
TBIL (mg/dL)	0.3±0.1	5±0.57	0.3±0.1	5±0.57	3.0±0.1	5±0.57	3.0±0.1	5±0.57
BUN (mg/dl)	12.0±1.8	9.0±1.19	12.2±1.6	8.7±0.1	6.0±3.2	7±1.17	7.1±2.7	6.4±0.40
Ca (mg/dL)	10.3±0.8	2.37±0.6	4.0±0.6	2.36±0.4	2.51±0.8	2.48±0.11	3.9±0.4	2.41±0.8
PHOS (mg/dL)	8.3±1.4	2.88±0.2	8.1±1.6	2.51±0.2	8.2±1.3	2.36±0.21	3.8±1.4	2.04±0.4
CRE (mg/dL)	0.2±0.1	2.9±0.95	0.2±0.1	1.8±1.2	0.3±0.0	2.15±0.20	2.5±0.0	3.6±0.41
GLU (mg/dL)	214±53	136±31.0	216±19	112±60	214±16	135±26.7	215±54	118±6.6
K (mmol/L)	6.1±1.6	5.3±0.23	6.1±1.8	4.8±0.5	6.0±1.4	5.4±0.95	4.0±2.0	3.9±1.12
TP (g/dL)	5.4±0.4	4.5±0.37	5.3±0.5	5.0±0.1	5.7±0.4	4.9±0.20	5.5±0.5	4.9±0.32
GLOB (g/dL)	2.2±0.7	1.2±0.4	2.3±0.6	1.7±0.2	2.4±0.5	1.4±0.25	2.8±0.4	1.2±0.32
Male mice								
ALB (g/dL)	3.7±0.6	3.0±0.30	3.3±0.1	2.9±0.37	3.0±0.1	3.0±0.30	3.0±0.4	36±0.23
ALP (U/L)	62.2±3.7	56±15.8	87.2±2.7	53±6.02	86.4±6.6	48±8.50	84.8±5.7	57±19.4
ALT (U/L)	31.0±6.5	29.5±5	57.4±2.0	58.5±15	56.6±2.5	39±16.9	55.6±4.3	42.5±7.5
TBIL (mg/dL)	3.4±0.8	4.5±0.57	0.2±0.1	4.5±0.57	0.2±0.1	6±0.57	0.2±0.05	5±0.57
BUN (mg/dl)	7.50±1.8	6.95±1.1	10.2±1.6	8.7±0.15	10.8±1.3	7.7±1.17	10.6±0.8	6±0.40
Ca (mg/dL)	3.58±1.7	2.47±0.6	10.6±0.3	2.43±0.4	10.2±0.5	2.42±0.1	9.8±0.4	2.47±0.8
PHOS (mg/dL)	4.3±1.4	2.5±0.26	8.1±0.7	2.86±0.2	8.2±1.6	2.22±0.2	8.1±0.8	2.33±0.4
CRE (mg/dL)	21.8±0.98	27.5±0.9	0.1±0.1	32±1.20	0.2±0.1	18±0.20	0.2±0.1	35±0.41
GLU (mg/dL)	214±53	157±31	223±8	101±6.0	227±17	117±26.7	226±9	118±6.6
K (mmol/L)	6.1±1.6	4.9±0.23	5.2±0.1	4.7±0.50	5.2±0.1	4.7±0.95	5.2±0.3	5±1.12
TP (g/dL)	5.4±0.4	4.7±0.37	6.2±0.2	4.8±0.17	6.2±0.4	4.8±0.20	6.3±0.2	5.2±0.32
GLOB (g/dL)	2.2±0.7	1.8±0.4	2.8±0.5	1.9±0.20	2.8±0.3	1.8±0.25	2.8±0.4	1.5±0.32

ALB – albumin; ALP – alkaline phosphatase; ALT – alanine aminotransferase; BUN – blood urea nitrogen; Ca – calcium; CRE – creatinine; GLOB – globulin; GLU – glucose; K – potassium; PHOS – phosphate; TBIL – total bilirubin; TP – total protein

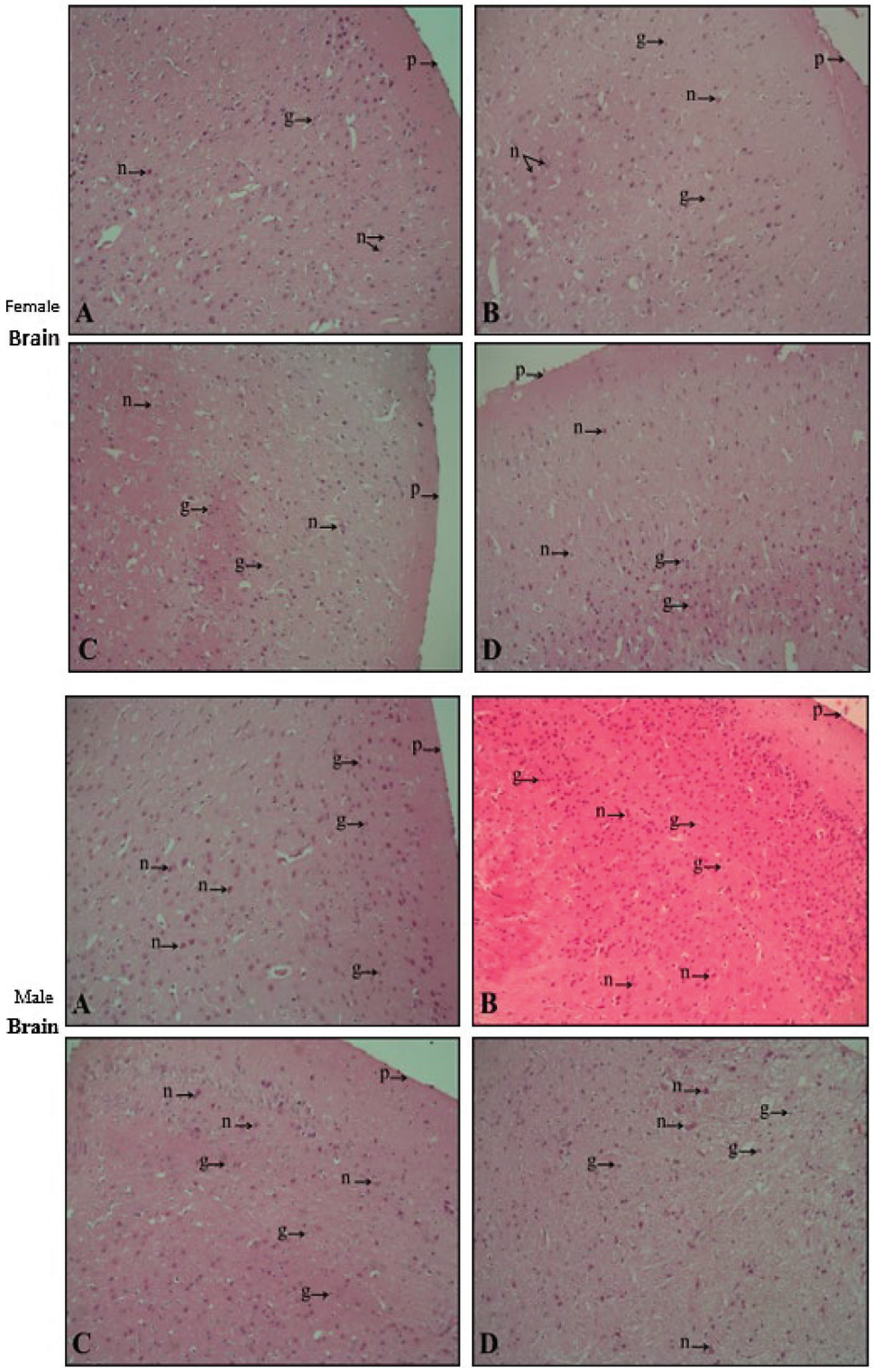

Figure 2 Brain histology with haematoxylin & eosin staining in female and male mice receiving L-glutamic-g-p(HEMA) polymeric nanoparticles as follows: 25 mg/kg (A); 50 mg/kg (B); 100 mg/kg (C); negative control (D). The arrows represent glia (g), neuron (n), and pia mater (p) (20× magnification)

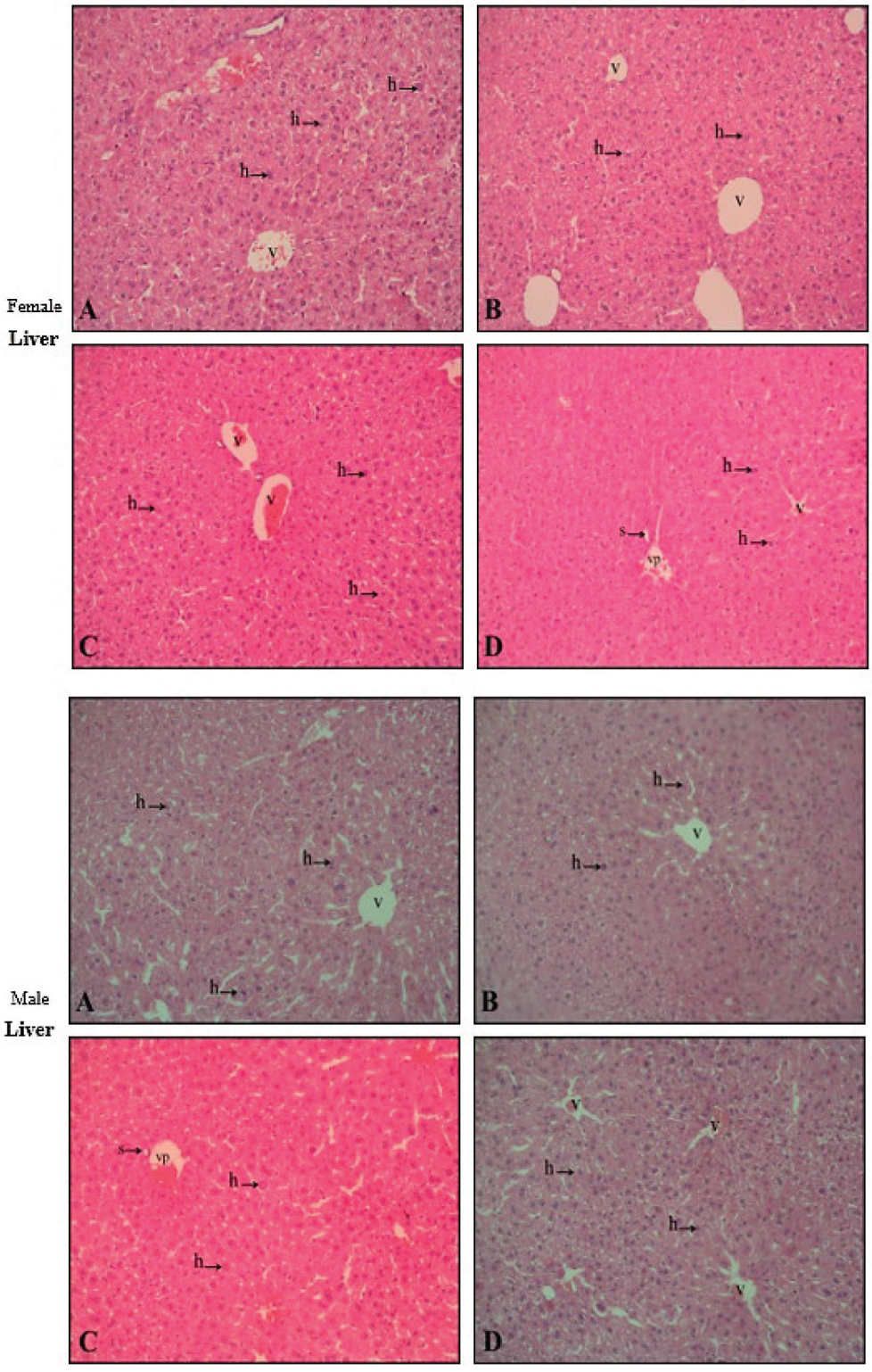

Figure 3 Liver histology with haematoxylin & eosin staining in female and male mice receiving L-glutamic-g-p(HEMA) polymeric nanoparticles as follows: 25 mg/kg (A); 50 mg/kg (B); 100 mg/kg (C); negative control (D). The arrows represent hepatocytes (h), vena centralis (v), ductus biliferi interlobularis (s), and vena porta interlobularis (vp) (20× magnification)

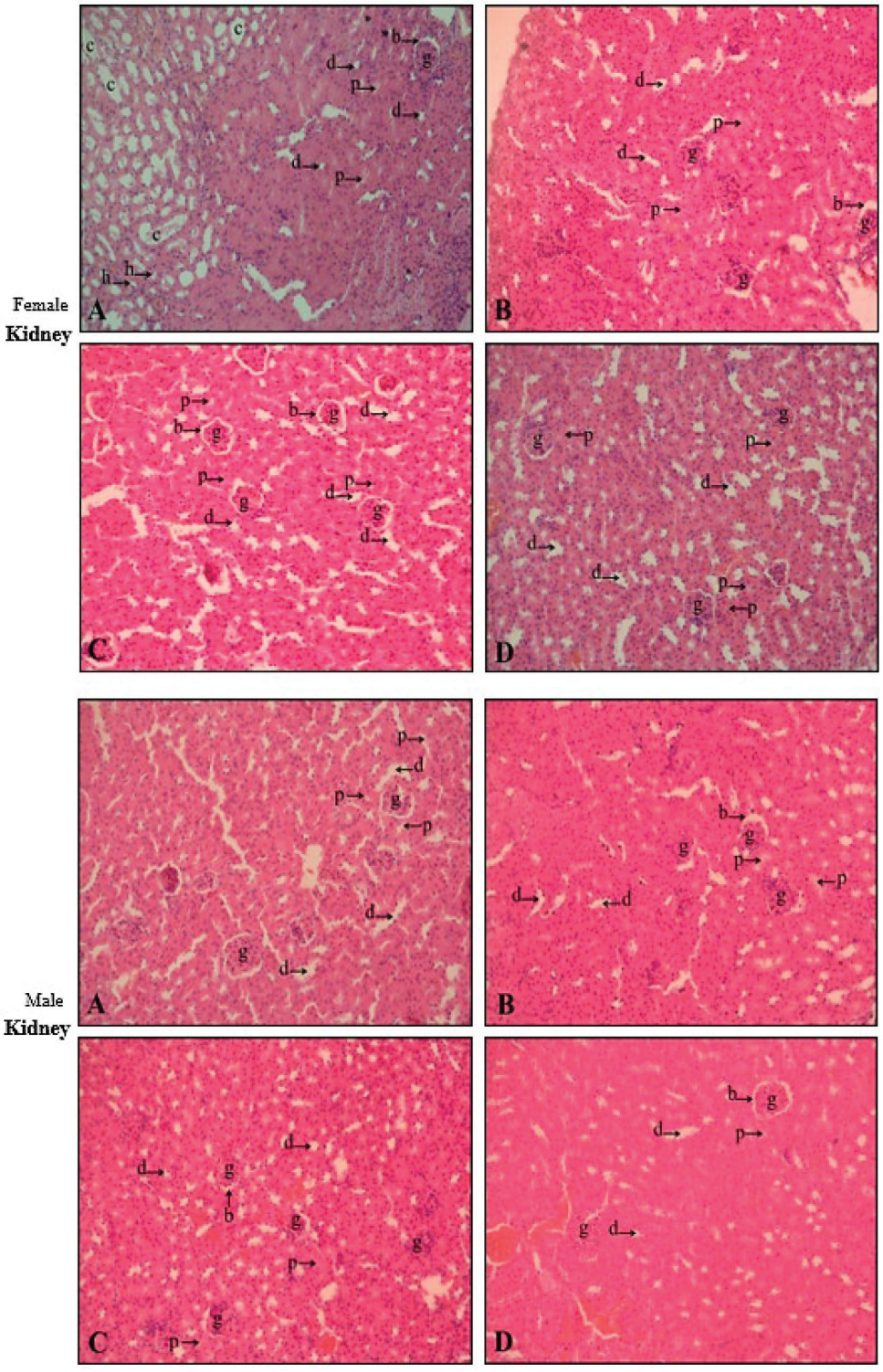

Figure 4 Kidney histology with haematoxylin & eosin staining in female and male mice receiving L-glutamic-g-p(HEMA) polymeric nanoparticles as follows: 25 mg/kg (A); 50 mg/kg (B); 100 mg/kg (C); negative control (D). The arrows represent glomeruli (g), Bowman's capsule (b), distal tubule (d), proximal tubule (p), collective tubule (c), and Henle's handle (h) (20× magnification)

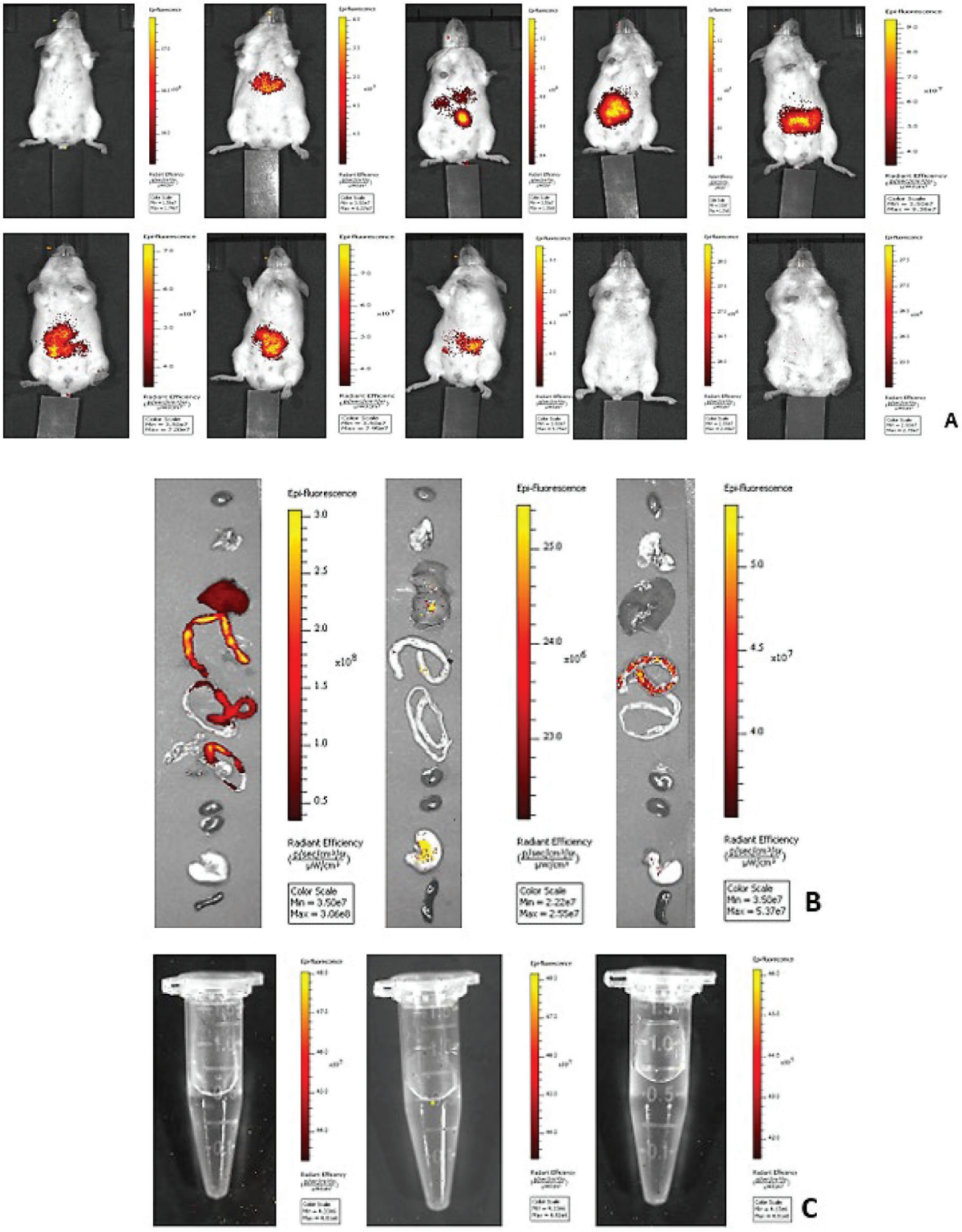

Figure 5 A. Time-course fluorescence imaging in living mice after intravenous injection of L-glutamic-g-p(HEMA) polymeric nanoparticle (n=3); B. *Ex vivo* imaging of major organs of mice at 6, 12, and 24 h; C. Optical imaging of bone marrow cells by IVIS® spectrum at 6, 12, and 24 h (excitation at 745 nm, emission at 850 nm)

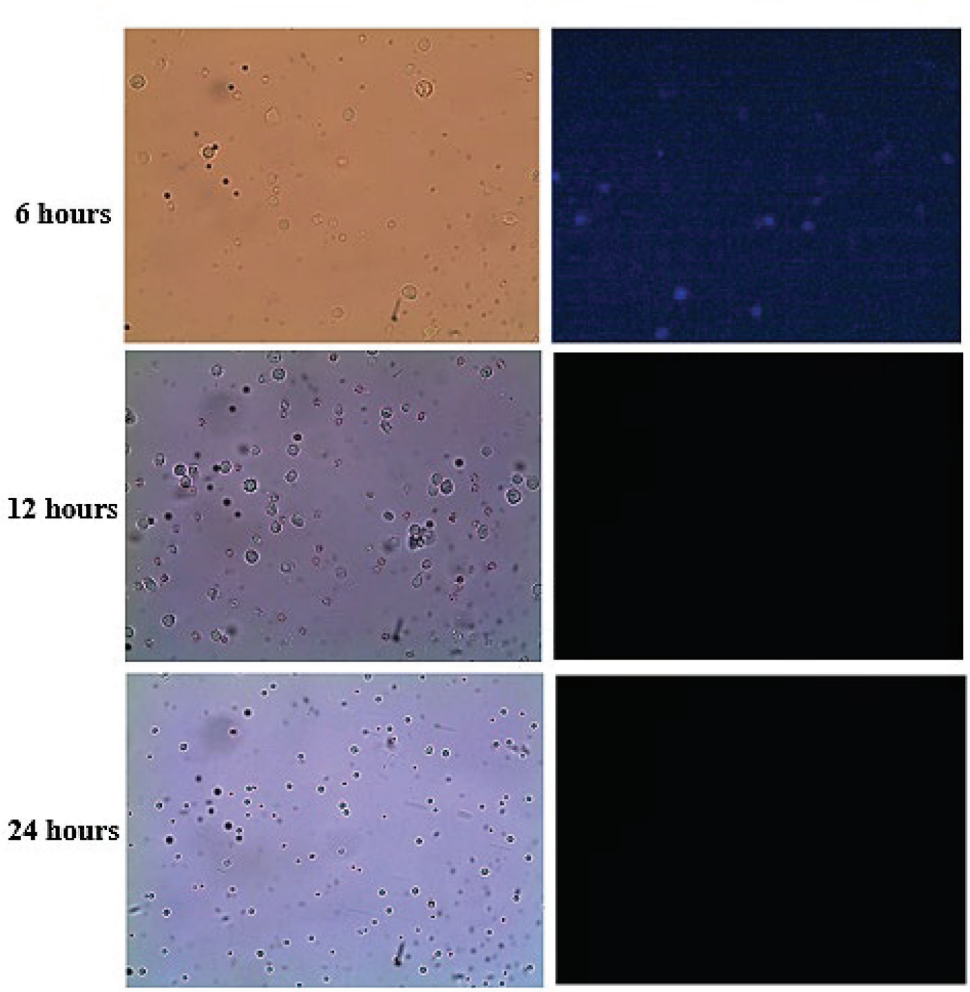

Figure 6 Images of bone marrow cells at 6, 12, and 24 h under the light (left side) and fluorescence (right side) microscope (40× magnification). Fluorescent microscopy revealed a fluorescent signal at hour 6 only.

In vivo nanoparticle biodistribution

Intravenously injected nanoparticles were absorbed by the organs in a time-dependent manner and most of them eliminated between 12 and 24 h after dosing (Figure 5A). *Ex vivo* images show relatively high nanoparticle amounts in the liver and intestine at 6 h after dosing. The signal became weaker at 12 h, and completely disappeared 24 h after dosing (Figure 5B). Although IVIS® spectral imaging showed no fluorescent signal in the bone marrow (Figure 5C), fluorescent microscopy revealed a fluorescent signal at hour 6 (Figure 6).

Intravenous L-glutamic acid-g-p(HEMA) polymeric nanoparticles seem to enter the organs by hour 6 of exposure and are eliminated from between hours 12 and 24. This may point to their metabolism, as suggested by Durner et al. (25) and elimination by macrophages, as suggested by He et al. (17) and Arnida et al. (26).

CONCLUSION

To the best of our knowledge, this is the first *in vivo* study of oral biocompatibility and biodistribution properties of L-glutamic acid-g-p(HEMA) polymeric nanoparticles given orally, and these properties look quite promising in terms of their pharmaceutical application. We intend to expand our research to include the effectiveness of the nanosystem in drug delivery and treatment.

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Conflict of interests

None to declare.

Table 5 Micronucleus test results in mice (5 per group) after exposure to L-glutamic acid-g-p(HEMA) polymeric nanoparticles over 28 days

Groups	Micronuclei (% per 1,000 erythrocytes per animal)
Negative control (saline)	3
Group 1 (25 mg/kg)	2
Group 2 (50 mg/kg)	3
Group 3 (100 mg/kg)	5

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Nanočestice L-glutaminske kiseline-g-polihidroksietilnoga metakrilata – akutna i subakutna toksičnost i bioraspodjela u miševa

Cilj je ovoga istraživanja bio utvrditi *in vivo* toksičnost i raspodjelu jednokratne i višekratnih doza polimernih nanočestica L-glutaminske kiseline-g-p(HEMA) u funkciji isporuke lijekova. Jednokratna oralna doza nije prouzročila smrtnost, a akutni LD_{50} iznosio je >2,000 mg/kg tjelesne težine. Višekratne oralne dnevne doze od 25 mg, 50 mg i 100 mg/kg tjelesne težine, davane 28 dana, nisu dovele do značajnih razlika u ukupnoj tjelesnoj i relativnoj težini organa u usporedbi s kontrolnom skupinom. Ove rezultate potvrđuju biokemijski i histološki nalazi. Izloženost nanočesticama nije uzrokovala statistički značajne razlike u broju mikronukleusa u stanicama koštane srži miševa u odnosu na kontrolu. Raspodjela nanočestica u tijelu mijenjala se s vremenom, a nanočestice bi doprle u organe, pa i u koštanu srž, unutar prvih šest sati od primitka doze, što je utvrđeno *ex vivo* snimkama dobivenim pomoću sustava IVIS® Spectrum. Naši rezultati upućuju na to da su polimerne nanočestice L-glutaminske kiseline-g-p(HEMA) biokompatibilne i imaju potencijala za primjenu kao sustav isporuke lijekova.

KLJUČNE RIJEČI: biokompatibilnost; biokemija krvi; genotoksičnost; histologija; *in vivo* toksičnost; mikronukleus test; polimeri