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Research paper

Preparation of poly(*N*-isopropylacrylamide) copolymers and preliminary assessment of their acute and subacute toxicity in mice

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Abstract

A subacute toxicity study was conducted to evaluate the oral toxicity profile of poly(*N*-isopropylacrylamide) (PNIPAAm) derivatives. These thermoresponsive polymers may have several potential pharmaceutical applications such as ingredient for oral solid dosage form. A preliminary acute oral toxicity study was performed with one of the polymer (PNIPAAm-co-NVA) at a unique dose of 4000 mg/kg body weight administered to six male and six female mice, to determine the dosage for further evaluation. No treatment-related effect was observed on behavior and health condition of the experimental animals during the 14 days observational period. The autopsy of the treated animals did not revealed any macroscopic changes in major organ aspects. Based on these preliminary results we selected a 2000 mg/kg body weight/day dose for the 28 days long subacute study. Three polymers were tested, namely PNIPAAm, PNIPAAm-co-NVA and PNIPAAm-co-AAc and compared to a saline control. No significant changes in clinical signs, body weight and food consumption, hematology, clinical chemistry or absolute organ weight were observed. Histological examination of excised major organs showed no marked differences between treated and control mice. In conclusion, PNIPAAm-co-NVA is well tolerated up to 4000 mg/kg body weight when administered orally. In addition, the subacute study indicated the absence of cumulative toxicity and a no-observed-adverse-effect level (NOAEL) of 2000 mg/kg was identified for PNIPAAm and its two copolymers. Further studies are mandatory.

Keywords: *N*-isopropylacrylamide; Acute toxicity; Subacute toxicity; Thermoresponsive polymer; PNIPAAm; PNIPAAm-co-NVA; PNIPAAm-co-AAc

1. Introduction

Poly(*N*-isopropylacrylamide) (PNIPAAm) is a thermoresponsive polymer that has met an increasing interest and stirred up the attention of many authors, chiefly in the field of controlled drug delivery [1–8].

Aqueous solutions of thermoresponsive polymers are characterized by an inverse dissolution behavior, their isobaric phase diagrams presenting a lower critical solution temperature [5,9–11] (LCST). The solutions are homogenous at low temperature and a phase separation appears when the temperature exceeds a definite value. The LCST is the minimum of the phase diagram of the system [5,9], and in the practical cases to be treated in the following, the phase separation temperatures at which the phase transition occurs, also called demixtion, will be denoted 'T_d' or CP.

PNIPAAm owes its popularity mainly because of the sharpness of its phase transition, of the closeness of its LCST—about 32 °C—to the physiological temperature, and of the easiness to vary its phase separation temperature by copolymerization [5,12,13], addition of salts [3,14,15] or addition of surfactants [3,16,17] to the polymer solution.

In our previous works, we designed two new drug delivery concepts that, for the very first time, make thermosensitive (co)polymers suitable for an effective in vivo application [2,4,6]. That might be a new approach to specific drug targeting into the GI tract, mainly for

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colonic drug delivery, which seems quite difficult to be achieved by classical means. Considering that it is practically unfeasible to change the temperature of the human body, the proposed concepts offer the advantage of controlling drug delivery without having to vary the medium temperature as is currently reported in some other works over in-vitro studies [1,7,8].

Although those thermoresponsive materials are designed for in vivo applications, none at this time has made in vivo toxicity tests yet; only Hsiue et al. [18] performed in vitro tests on cell cultures. Considering the high toxicity of acrylic monomers [19] such as those used in this study, it is of outstanding importance to evaluate the potential toxicity of the polymeric material before venturing into the public health area.

In this work, in vivo toxicity evaluations were performed on mice with PNIPAAm and two copolymers, poly(*N*-isopropylacrylamide)-co-*N*-vinylacétamide (PNIPAAm-co-NVA) and poly(*N*-isopropylacrylamide)-co-acrylic acid (PNIPAAm-co-AAc), designed to be suitable at physiological temperature [5]. As the ultimate goal for any toxicological testing is to establish a safe level for human exposure to the products tested, we focused our study on establishing a no-observed-adverse-effect level (NOAEL) according to a similar approach to the guidelines published by the Organization for Economic Cooperation and Development [19]. This work was intended to give us only a first assessment of the toxicological pattern of the polymers (preliminary results).

2. Material and methods

2.1. Test material

2.1.1. Preparation of the polymers

N-isopropylacrylamide (NIPAAm) monomer and diethyl ether were purchased from Acros (Belgium). *N*,*N*′azobisisobutyronitrile (AIBN) was purchased from Fluka (Belgium). *N*-vinylacetamide (NVA), acrylic acid (AAc) comonomers and *N*,*N* dimethylformamide (DMF) were purchased from Aldrich (Belgium). Unstabilized 1,4-dioxane and *N*-hexane were purchased from Lab-Scan (Belgium). D_2O was purchased from Cortec (France). The other materials were of analytical reagent grade.

N-isopropylacrylamide (NIPAAm) was purified by recrystallization from *N*-hexane, the other materials were used as received.

About 20 g of PNIPAAm and PNIPAAm copolymers were prepared by free radical polymerization in 1,4-dioxane. AIBN was used as an initiator (1 mol%) and polymerizations were carried out under nitrogen atmosphere and magnetic stirring at 70 °C for 5 h. Prior to polymerization, the monomer solutions (1 mol/l) were bubbled with nitrogen for 20 min in order to remove the remaining oxygen.

The comonomers percentages incorporated in the reaction media were, respectively, 12.9 and 3.9 mol% for NVA and AAc.

After polymerization, the obtained polymers were precipitated in diethyl ether by adding the polymeric solutions to an excess volume of diethyl ether (5:1), under agitation at room temperature. The suspensions were filtered and washed with diethyl ether and the recovered polymers were dried in a vacuum oven at 60 °C. They were then further purified by soxhlet extraction in diethyl ether (30 cycles—5 h), using cellulose cartridge filled with 10 g of sample—diethyl ether is a non-solvent for the polymers but a good solvent for the monomers—so removing unreacted toxic monomers and residual solvents.

2.1.2. Characterization of the polymers

The synthesized polymers were characterized by ^{1}H NMR and gel permeation chromatography, to establish, respectively, their effective comonomer content and molecular weight [6].

The phase transition of the polymer solutions was examined by differential scanning calorimetry (DSC).

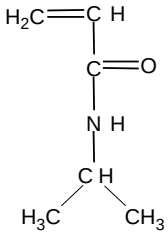
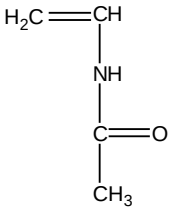
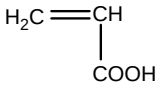
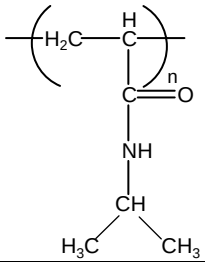
The T_d value was arbitrarily taken as the abscissa of the maximum of the endothermic transition peak (average of two measurements). DSC analyzes were performed using a Perkin Elmer DSC-7 differential scanning calorimeter/TAC-7 thermal analysis controller with an intracooler-2 cooling system (Perkin Elmer Instruments, USA). Aluminum sealed pans containing 10 μl of the various polymer aqueous solutions (56 g/l) were heated at a scanning rate of 2 °C min^{-1}, using nitrogen as blanket gas. Calibration was performed using cyclohexane and indium as standards.

The phase transitions were also evaluated by performing transmittance measurements of the polymer solutions (in duplicate) at 500 nm, using a Shimadzu 160 spectrophotometer (Shimadzu Corp., Japan). The temperature of the polymeric solutions (14 g/l), was raised by 0.1 °C steps, using a Cell positioner with a Peltier temperature controller (Shimadzu CPS-240A). The cloud point (CP) value (average of two measurements) was determined as the abscissa of the inflexion point of the transmittance versus temperature curves [3].

Residual NIPAAm, NVA and AAc monomers and solvent content, still present in the samples, were determined by gas chromatography (GC). A Carlo Erba Instruments 'Auto/HRG/MS' MFC 500 with a CP-Sil 5CB capillary column was used for this purpose. The analyzes were performed using 50 g/l DMF solutions for PNIPAAm and PNIPAAm-co-NVA and 50 g/l acetone solutions for PNIPAAm-co-AAc. Menthone was used as internal standard and samples were heated from 40 to 140 °C with a 15 °C min^{-1} temperature scan.

Standard solutions of NIPAAm, NVA, AAC, dioxane, diethylether, acetone and *N*-hexane were used to calibrate the system.

Table 1
Main physico-chemical characteristics of the thermosensitive polymers used

N-Isopropylacrylamide	*N*-Vinylacétamide	Acrylic acid	Poly(*N*-isopropylacrylamide)
			

Polymer type	T_d (°C)	CP (°C)	% Calculated[a]	$\bar{M}_n$ (g/mol)	$\bar{M}_w$ (g/mol)	$\bar{M}_z$ (g/mol)	$\bar{M}_p$ (g/mol)	*I*
PNIPAAm	31.8	32.5	/	4500	55,200	125,600	80,100	12,3
PNIPAAm-co-NVA	39.0	38.5	13.3	5800	53,500	120,500	81,200	9,2
PNIPAAm-co-AAc	/	38.8[b]	4.5	6500	59,500	135,100	83,200	9,1

[a] By RMN ^{1}H.
[b] At pH 7,0.

We considered the lower detection limit as threefold the background noise and results ($n = 2$) are expressed in ppm. That lower detection limit was found to be 30 ppm for diethyl ether and NIPAAm, 40 ppm for 1,4-dioxane, 50 ppm for hexane, 100 ppm for NVA and 270 ppm for AAc.

The chemical structures of NIPAAm and comonomers, and the main physico-chemical characteristics of the polymers used are presented in Table 1.

2.2. Animals and diet

Swiss NMRI albino mice, 6–8 weeks of age, weighing 18–20 g (Charles River Laboratories, Belgium) were used for the studies. They were individually housed under suitable conditions of temperature (21 °C), relative humidity (60%) and light (12 h) throughout the experiments. Standard mice chow (Carfil, Belgium) and tap water were provided ad libitum. Following a 14-day acclimatization period, animals were randomly assigned to each group and housed individually. All animal procedures were approved by the ethical committee of the Faculty of Medicine of our university (ULB).

2.3. Acute study

PNIPAAm-co-NVA, at a dose of 4000 mg/kg body weight, a single dose of 0.5 mL polymer suspension was administered by gavages to six male and six female mice. The polymers were almost totally dissolved in water before administration (160 mg/ml).

Another group of six males and six females received the same volume of vehicle (0.5 mL of NaCl 9‰ in pure water) that was administered to the test group and served as a control.

The animals were carefully observed for any behavioral change and mortality, immediately after dosing, at 2, 4 and at 12 h intervals, during the subsequent 14 days. No urine or feces were collected; hence no sign of accumulation was observed. Feces macroscopic aspects were similar between groups (treated and control). At the end of the observational period, the animals were sacrificed under CO_2. A thorough autopsy was carried out on all the animals.

2.4. Subacute study

The mice were randomly assigned to four groups. The three experimental groups, consisting of 12 animals each, six males and six females, received either PNIPAAm, PNIPAAm-co-NVA or PNIPAAm-co-AAc at the mean daily dose of 2000 mg/kg body weight orally (in drinking water), for a period of 28 days. This administration was performed individually according to bodyweight with a daily prepared high concentration polymer solution which was diluted with tap water to the minimal daily water ration. A control of the water consumption was performed to insure complete dosage and a complement water feeder was offered to animals if needed (after complete emptying of the administration feeder). A fourth group composed of 11 animals (five males, six females) served as control. The animals were observed twice daily for any signs of morbidity and mortality. Detailed physical examinations for the signs of morbidity were conducted every week during the study period. At the end of the 28-day treatment period, the animals were sacrificed under CO_2, following an overnight fast. Bodyweight and food intake were monitored two times a week.

Table 2
Mean monomer and residual solvents contents (expressed in ppm) ($n=3$)

Polymère type	Ether	*N*-hexane	1,4-dioxane	NIPAAm	NVA	AAc
PNIPAAm not purified	3480±240	<	14940±3900	2760±220	/	/
PNIPAAm purified	<	<	<	<	/	/
PNIPAAm-co-NVA not purified	2850	<	25090	328	435	
PNIPAAm-co-NVA purified	<	<	<	<	<	/
PNIPAAm-co-AAc not purified	2250	<	?	1560	/	?
PNIPAAm-co-AAc purified	<	<	<	<	/	<

<, not detected; /, absent; ?, not determined.

2.5. Hematology/biochemistry

Heparinized whole blood was collected for the assessment of haemoglobin concentration, erythrocyte, platelet and leukocyte counts with a VITROS 250 AT (Ortho Clinical Diagnostics, Raritan, NJ, USA). Assays for total protein, total cholesterol, urea, alanine aminotransferase and bilirubin were performed using a CELL-DYN 3500 (ABBOTT, Chicago, IL, USA). Only these parameters were assessed because they were the only ones validated in our laboratory. Due to troubles during transport, only blood parameters from male mice were assessed. This point represents a limitation to our conclusions and further studies are therefore needed.

2.6. Autopsy and histology

During autopsy, all organs observed macroscopically and selected vital organs (thymus, spleen, lungs, liver and kidneys) were excised, blotted and weighed. The rationale of this selection is that all the organs could not be analyzed separately because of time limitation (sacrifice, dissection and weighting). Tissues were fixed in 10% buffered neutral formalin. Paraffin sections of major organs were prepared and stained with haematoxylin, acid fuschin, orange G and light green SF for histological examination.

2.7. Statistical analysis

Values obtained were expressed as mean−standard deviation. The statistical analysis was done using Fischer's Student's *t*-Test and Kruskall–Wallis for non-parametric values followed by a Dunn's Multiple Comparison Test (performed with GraphPad Prism 4).

3. Results and discussion

The experimental conditions used for the preparation, purification and characterization of thermosensitive polymers were thoroughly discussed in previous works [3]. Besides the PNIPAAm homopolymer, two copolymers with the most interesting phase transition properties within the context of the new drug delivery concept developed [3], were used for the in vivo toxicity evaluation.

As can be seen in Table 1, the three synthesized thermosensitive polymers present nearly the same molecular weight, with a relatively large polydispersity. The presence of hydrophilic comonomers in the structure of the two copolymers, bring about a rise of the phase transition temperature in comparison with the PNIPAAm homopolymer. As PNIPAAm-co-AAc is a thermosensitive copolymer, its T_d varies consequently with pH and with AAc

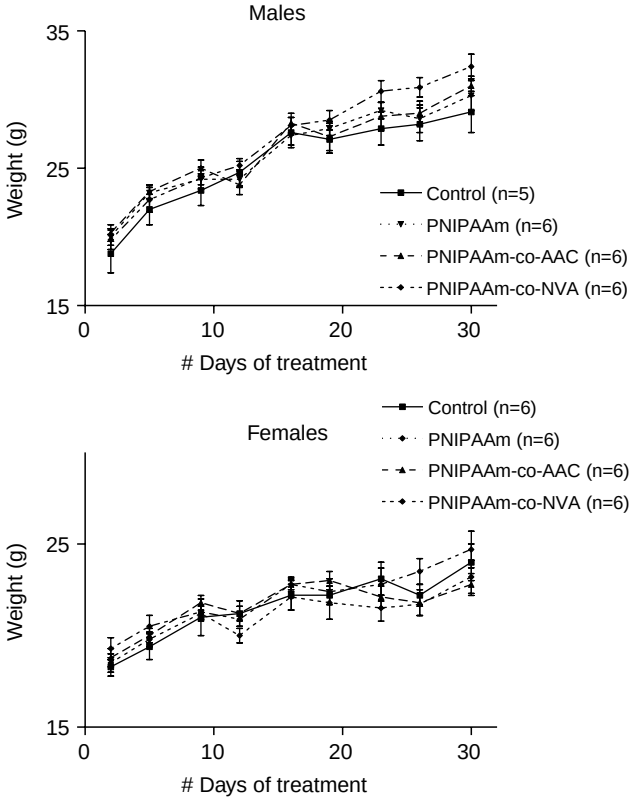


Fig. 1. Mean absolute body weight evolution over time of control or polymer-fed mice. Each value is the mean±SD; *n*, number of mice used.

Table 3
Some major organs weights of treated and control mice

Groups	Thymus (mg)	Liver (g)	Lungs (mg)	Kidneys (mg)	Spleen (mg)
Females					
Control ($n=6$)	52.4±4.8	0.980±0.041	154.1±7.1	344.4±11.9	64.1±6.2
PNIPAAm ($n=6$)	58.4±4.8	1.000±0.042	162.7±8.1	371.3±22.7	85.1±14.1
PNIPAAm-co-AAC ($n=6$)	64.5±7.0	1.065±0.026	173.5±11.1	366.2±10.9	97.7±11.6
PNIPAAm-co-NVA ($n=6$)	53.6±5.7	1.048±0.041	150.0±6.4	373.7±19.6	77.8±6.2
Males					
Control ($n=5$)	69.2±7.1	1.445±0.076	248.2±17.1	559.5±17.8	79.2±6.6
PNIPAAm ($n=6$)	74.4±5.0	1.409±0.056	242.9±11.6	576.2±17.7	87.0±7.7
PNIPAAm-co-AAC ($n=6$)	84.6±5.3	1.386±0.043	247.0±16.2	611.3±35.2	80.4±7.0
PNIPAAm-co-NVA ($n=6$)	80.3±11.6	1.490±0.069	245.0±12.2	637.6±35.7	90.4±4.2

Each value is the mean±SD; *n*, number of mice used. Statistical analysis performed using Kruskall–Wallis followed by a Dunn's Multiple (performed with GraphPad Prism 4) vs control. No significant difference was found between control and treated mice groups.

content. The AAc content thus was adjusted in order to obtain a copolymer with a T_d slightly higher than 37 °C at pH 7.0 (i.e. 38.8 °C in the present case).

Examination of Table 2 shows that residual monomers and solvents totally disappeared thanks to the purification process, i.e. their content has become beneath the detection limit. This suggests that, if any kind of toxicity was detected, it should obviously be attributed to the polymer itself. No observable effects on behavior have been pointed in all the groups. Nevertheless, no functional observation battery or locomotor activity assessment were performed in this study to evaluate neurotoxicity but represent a major step for the future development of these polymers.

During the PNIPAAm-co-NVA acute toxicity study, no treatment-related effect was observed on behavior and health condition of the experimental animals. None of them exhibited any sign of morbidity and all of them survived during the observational period. The thorough autopsy did not revealed any treatment-related macroscopic changes in

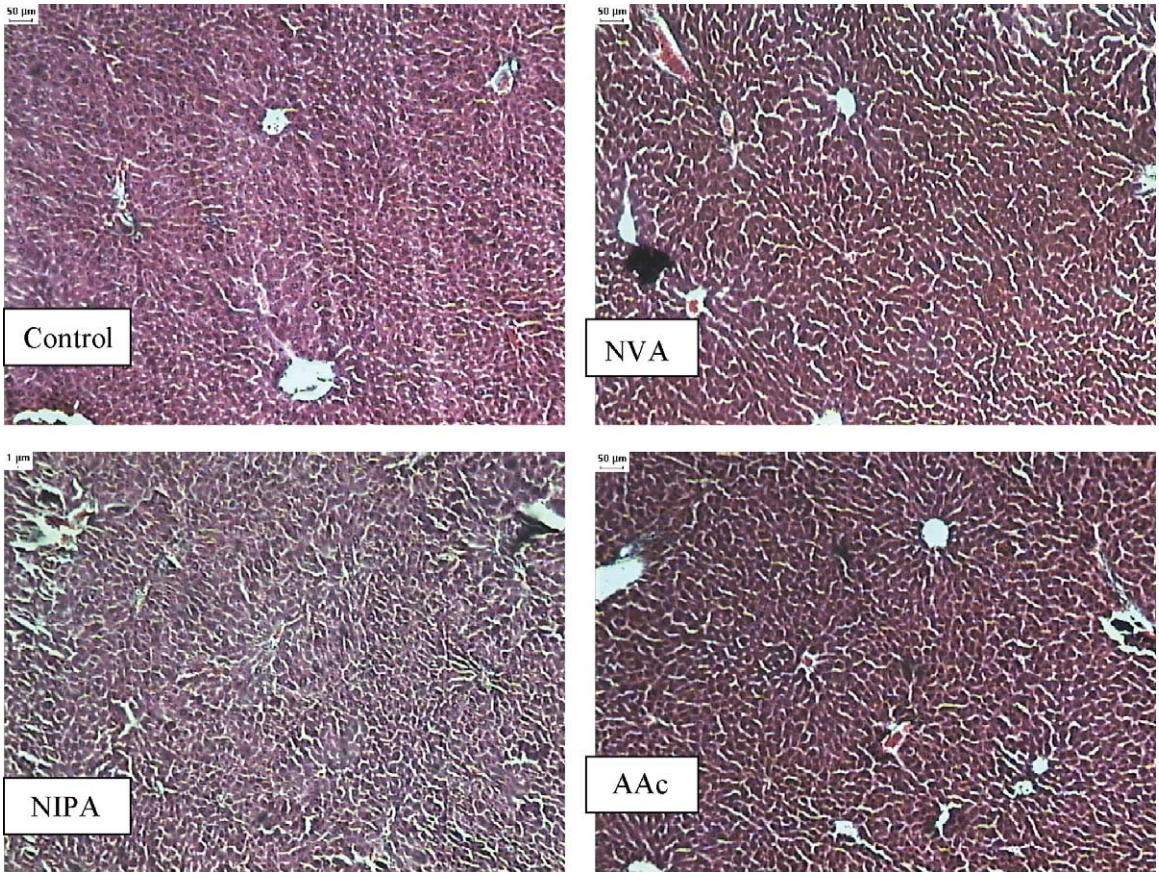


Fig. 2. Histological sections of liver. NVA, PNIPAAm-co-NVA; NIPA, PNIPAAm; AAc, PNIPAAm-co-AAc).

Table 4
Biochemical constituents and serum enzymes of control and treated mice

	Total cholesterol	Total proteins	Urea	Bilirubin	GPT (ALT)
Unit:	mg/dL	g/dL	mg/dL	mg/dL	U/L
Control ($n=5$)	88±7	4,0±0.2	47±6	0,8±0.1	56±4
PNIPAAm ($n=4$)	80±7	4,1±0.1	57±6	0,7±0.2	56±3
PNIPAAm-co-AAC ($n=6$)	105±11	4,6±0.2	44±3	0,7±0.1	43±4
PNIPAAm-co-NVA ($n=5$)	97±5	4,3±0.1	45±2	0,7±0.1	48±4

Each value is the mean±SD; n, number of mice used. Statistical analysis performed using Kruskall–Wallis followed by a Dunn's Multiple (performed with GraphPad Prism 4) vs control. No significant difference was found between control and treated mice groups. Performed only in male mice.

Table 5
Hematological profile of control and treated mice

	Hemoglobin	Red blood cells	White blood cells	Platelets
Unit:	gr/dL	Millions/mm^3	Thousands/mm^3	Thousands/mm^3
Control ($n=5$)	14,8±0.3	9,24±0.26	3,24±0.61	1177±193
PNIPAAm ($n=4$)	14,5±0.1	8,87±0.22	1,23±0.21*	1417±95
PNIPAAm-co-AAC ($n=6$)	14,6±0.4	8,67±0.40	3,29±0.36	1483±119
PNIPAAm-co-NVA ($n=5$)	14,9±0.1	8,97±0.16	2,45±0.70	1513±97

Each value is the mean ±SD; n, number of mice used. Statistical analysis performed using Kruskall–Wallis followed by a Dunn's Multiple (performed with GraphPad Prism 4) vs control. *$P<0.05$ vs control groups. Performed only in male mice.

major organ aspects. Some complementary experiments are needed: female blood samples still need to be assessed.

The subacute administration of the PNIPAAm, PNIPAAm-co-NVA and PNIPAAm-co-AAc polymers at the daily dose of 2000 mg/kg did not cause any appreciable change in the body weight (Fig. 1) or food intake (data not shown) in the treated group when compared to control. Macroscopic appearance and weight (Table 3) of all observed organs was found to be normal.

Although PNIPAAm possesses a T_d value of about 32 °C (meaning it becomes insoluble when the aqueous solution reaches temperatures higher than 32 °C), it did not provoke intestinal occlusion and no trace of it was found during the autopsy. This was neither observed for PNIPAAm-co-AAc which, however, precipitates at 35 °C when pH is lower than 4.0 and may, therefore, have precipitated in mice's stomach. Gastrointestinal tract was observed more precisely after harvesting, and no macroscopic difference was stated between all the groups. Histological examination showed no marked differences between treated and control mice (see Fig. 2).

Similarly, no alteration has been observed in all groups in the biochemical assays (Table 4).

On the hematological parameters, only one statistically significant difference was observed in the leukocyte numeration. Indeed, the number of white blood cells was decreased after PNIPAAm treatment (Table 5, Fig. 3): mean±SEM was 1.23±0.21 ($n=4$) versus 3.24±0.61 ($n=5$) thousands/mm^3 ($P<0.05$). Nevertheless, this result must be taken carefully because the number of mice studied was low linked to some difficulties encountered in blood sampling. Therefore, utmost care must be taken for the interpretation of this result and a greater number of animals must be used in order to validate or reject this observation.

4. Conclusion

This preliminary toxicological study shows that PNIPAAm-co-NVA seems non-toxic up to 4000 mg/kg body weight when administered orally. In addition, the subacute study (28 days) indicated the absence of cumulative toxicity and a no-observed-adverse-effect level (NOAEL) of 2000 mg/kg was identified for PNIPAAm and its two copolymers. Taken all together, our data show that all the animals well tolerated the PNIPAAm polymers.

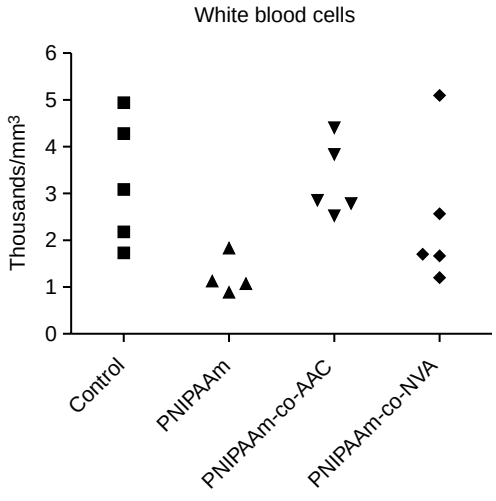


Fig. 3. Distribution of the number of white blood cells in the different groups.

The absence of any adverse effects either with an acute oral dose of 4000 mg/kg or a repeated oral dose of 2000 mg/kg body weight per day during 28 days demonstrates the favorable tolerance profile of our polymers. Due to some troubleshooting, the assessment of the blood parameters needs to be performed in female mice. Moreover, functional observation battery test must be performed. In line with this fact, some further toxicological evaluations are needed in order to validate our results.

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