Interdiscip Toxicol. 2021; **Vol. 14**(1): 9–19.
doi: 10.2478/intox-2021-0002

sciendo

interdisciplinary toxicology

ORIGINAL ARTICLE

Physicochemical properties and toxicity of soluble polypyrrole: A study of its potential for biomedical applications

Ana Paula PEREIRA ALVES [1], Rodolfo DE MORAES PEIXOTO [2], Renata DE FARIA SILVA SOUZA [1], Helinando Pequeno DE OLIVEIRA [3], Fernando Antônio Gomes DA SILVA JÚNIOR [3], Kátia Simoni BEZERRA LIMA [1], Cynthia Layse Ferreira DE ALMEIDA [1], Ricardo Santana DE LIMA, Larissa Rolim ARAÚJO [1], Alane Pains Oliveira DO MONTE [1], Isabela Araujo E AMARIZ [1], Valéria PEREIRA ALVES [1], João Paulo DA SILVA RODRIGUES [1], Mateus Matiuzzi DA COSTA [1]

[1] Universidade Federal do Vale do São Francisco, Universidade Federal do Vale do São Francisco, Petrolina, Brazil
[2] Instituto Federal de Educação, Ciência e Tecnologia do Sertão Pernambucano, Instituto Federal de Educação, Ciência eTecnologia do Sertão Pernambucano, Petrolina, Pernambuco, Brazil
[3] Universidade Federal do Vale do São Francisco, Universidade Federal do Vale do São Francisco, Juazeiro, Bahia, Brazi

ITX140121A02 • Received: 12 March 2021 • Accepted: 29 May 2021

ABSTRACT
Polypyrrole is one of the most promising polymers for biomedical applications, and its low solubility in most common solvents led to the development of soluble forms of this conducting polymer. In this study, the physicochemical properties and the *in vivo* toxicity of soluble polypyrrole were evaluated, aiming to validate its potential for biomedical applications. The pH, density, and miscibility in different solvents were determined in two soluble polypyrrole samples synthesized on different dates. Two nonclinical studies on the toxicity of soluble polypyrrole were conducted with Swiss mice distributed into groups according to the dose administered. The single-dose toxicity study (acute toxicity) consisted of a single intraperitoneal application of a soluble polypyrrole solution at the doses of 500 and 1,000 mg/kg, followed by observation during 14 days. The repeated-dose toxicity study lasted for 28 days with daily intraperitoneal applications of the soluble polypyrrole solution, at the doses of 250, 500, and 1,000 mg/kg. Parameters such as mortality, behavioral screening, water and ration intake, body mass evolution, organ indices, and the macroscopic evaluation of the organs were observed in both studies. Additionally, hematological and histopathological analyses were performed in the repeated-dose toxicity study. In the acute toxicity study, no mortality was verified during the 14 days of observation at any of the doses tested, indicating that soluble polypyrrole has an LD_{50}>1,000 mg/kg. The main alterations observed in the repeated-dose toxicity study involved the liver changes, such as in the increase of its relative weight, the presence of necrosis, and granulomatous inflammation. Considering the high doses administered and the time of application, the results indicate low toxicity of soluble polypyrrole when administered by the intraperitoneal route.

KEY WORDS: Polymer; nanoparticles; toxicology; mice

Introduction

Polypyrrole (PPy) is an organic polymer based on pyrrole. It is one of the most promising conducting polymers due to its high conductivity, chemical and environmental stability, relatively simple synthesis in aqueous or organic media by chemical and electrochemical methods, and optical and electrical properties that highlight its potential for several applications (Arantes *et al*., 2008; Zhang *et al*., 2018).

Due to these characteristics PPy is the most studied conducting polymer, with its relevance reflected in the number of publications addressing its properties and biomedical applications, including tissue engineering and regenerative medicine (Asadi *et al*., 2020; Qin *et al*., 2020; SU *et al*., 2019; Talikowska *et al*., 2019), biosensors (Lee *et al*., 2021), and drug administration (Hsiao *et al*., 2020; Ryan & Breslin, 2019).

In general, PPy is known for being insoluble and infusible due to the strong inter and intramolecular interactions and crosslinkings, which is why its poor processability has limited its applications (Lee *et al*., 1995).

Correspondence address:
Dr. Mateus Matiuzzi da Costa
Universidade Federal do Vale do São Francisco,
Petrolina, Brazil
E-MAIL: mateus.costa@univasf.edu.br

The low solubility in most common solvents and the low thermoplasticity stimulated studies on the development of soluble conducting polymers (Kumar & Sharma,1998). Currently, there are several synthetic routes for the production of soluble polypyrrole.

Silva Junior *et al.* (2016) reported the synthesis of highly-soluble polypyrrole through chemical polymerization and demonstrated the antimicrobial potential of this polymer against several bacteria, such as *Staphylococcus aureus, Escherichia coli,* and *Klebsiella pneumoniae*. In view of the worldwide bacterial resistance scenario (WHO, 2014), soluble polypyrrole appears as a potential antimicrobial alternative, widening its possibilities in biomedical applications.

Some studies verified the good biocompatibility of polypyrrole in *in vivo* systems through different toxicity studies (Ramanaviciene *et al*., 2007; Wang *et al*., 2004). However, considering that, in these studies, polypyrrole was synthesized in different ways, the conducting of toxicity studies to evaluate soluble polypyrrole occurs as a safety measure.

However, in order to validate the potential of soluble polypyrrole in biomedical applications further studies on its physicochemical properties and toxicity are still necessary. Knowledge about the physicochemical properties of xenobiotics is essential for pharmacokinetics and the analytical project (Avdeef, 2003; Takács-Novák, 2012). For drug formulation, for example, aspects such as bioavailability, expiration date, and even industrial processing are affected by the physicochemical properties (Maximiano *et al*., 2010).

The absence of studies that relate toxicity and bioavailability hinders the introduction of new molecules to the market (Pereira *et al*., 2005). These complementary physicochemical and biological approaches are essential to develop safer projects. Therefore, aiming at future biomedical applications, the objective of this study was to investigate some of the physicochemical properties of soluble polypyrrole and its toxicological profile when administered to mice in a single dose and in repeated doses.

Materials and methods

Characterization of the physicochemical properties of soluble polypyrrole

The physicochemical characterization of soluble polypyrrole was performed at the Center for Drug, Medication, and Food Analysis (CAFMA) of the Federal University of São Francisco Valley (UNIVASF), Brazil. To perform these analyses, two soluble polypyrrole samples were provided by the Laboratory of Impedance Spectroscopy and Organic Materials (LEIMO) of UNIVASF. The same synthesis process was used in the two samples tested, although with a manufacturing interval of one year, to identify whether the physicochemical properties of soluble polypyrrole change throughout time. The soluble polypyrrole used in this study has a particle size around 72.39 nm, verified with a Zetasizer Nano-ZS90 Malvern device (Malvern, United Kingdom) (Silva Junior *et al*., 2016). All analyses for the characterization of the physicochemical properties of soluble polypyrrole were performed according to the guidelines of the Brazilian Pharmacopeia 6th Edition (ANVISA, 2019).

Direct and indirect pH measurement

The pH of soluble polypyrrole was determined using an MS Tecnopon mPA 210 pH meter (MS Tecnopon, Piracicaba, Brazil). The pH meter was calibrated with buffer solutions, the electrodes were washed with distilled water beforehand, which were then dipped in the buffer solution (pH 7.00). The electrode-washing procedure was repeated with distilled water and this time the electrode was dipped in a pH 4.00 buffer solution. Finally, the electrodes were inserted in the soluble polypyrrole samples (A and B) for pH reading, and the data were recorded (ANVISA, 2019).

Density measurement

The density determination of soluble polypyrrole was made with the pycnometer method (Fort Labor, São Paulo, Brazil). For this, a clean, dry, and previously calibrated 25-ml pycnometer was used. Subsequently, the soluble polypyrrole samples were transferred to the pycnometer and the samples were weighed. Sample weight was determined as the difference in weight between the full and empty pycnometer. Finally, the soluble polypyrrole density was calculated by determining its mass to volume ratio (ANVISA, 2019).

Miscibility determination

The miscibility test of soluble polypyrrole was performed with 1 ml of soluble polypyrrole in 10 ml of the following solvents: water, ethyl alcohol (Synth, Diadema, Brazil), and n-hexane (Dinâmica, Indaiatuba, Brazil). The degrees of miscibility were visualized with the naked eye and recorded.

Nonclinical toxicology studies of soluble polypyrole

For the nonclinical toxicology studies of soluble polypyrrole, a single-dose toxicity study (acute toxicity) and a repeated-dose toxicity study were performed. Both tests were based on the Guide for Conducting Nonclinical Toxicology Studies and Pharmacological Safety Necessary for Drug Development (ANVISA, 2013). Some complementary guidelines from international regulatory agencies such as the Organisation for Economic Co-operation and Development (OECD) and the European Medicines Agency (EMA) were adopted. The acute toxicity and repeated-dose studies of soluble polypyrrole were evaluated and approved by the Ethics Commission on Animal Use (CEUA) of UNIVASF, under registration number 0003/261118.

The toxicology studies with soluble polypyrrole were conducted with *Mus musculus* (Swiss mouse), a rodent mammal species provided by the Bioterium of the Center of Agricultural Sciences (CCA) of UNIVASF. These animals were healthy, young adults (6–8 weeks old), and the females were nulliparous and non-pregnant (OECD 423, 2001).

Regarding environmental conditions, all toxicity studies were performed in the Bioterium of UNIVASF. The room temperature for the animals under experimentation remained around 22 °C (+3 °C), with artificial lighting consisting of 12 hours of light and 12 hours of darkness. The animals were kept in laboratory cages (BioXP, São Paulo, Brazil) and were grouped per dose so that it did not interfere with the observations of each animal. The animals were fed a conventional diet based on a commercial ration (Presence, Paulínia, Brazil), and drinking water provided *ad libitum* (OECD 423, 2001; OECD 407, 2008).

The animals were randomly selected, marked to allow individual identification, and kept in their cages for at least five days before the beginning of dose application for acclimation to the laboratory conditions (OECD 423, 2001; OECD 407, 2008).

Single-Dose Toxicity Study (Acute Toxicity)

The single-dose toxicity study (acute toxicity) consisted of evaluating the toxicity produced by soluble polypyrrole when administered in a single dose for a period not longer than 24 hours, followed by observation of the animals for 14 days after administration (ANVISA, 2013).

The experimental substance (soluble polypyrrole) and the control substance (0.9% sterile sodium chloride solution (Fresenius Kabi, Aquiraz, Brazil)) were administered in a single dose by the intraperitoneal route. The test substance was administered in a constant volume, using sodium chloride solution as an excipient. The limit dose tested was 1,000 mg/kg of soluble polypyrrole, resulting in a margin of 10 times the intended clinical exposure and not exceeding the recommended limit dose of 1,000 mg/kg/day (ANVISA, 2013). The doses were prepared just before administration (OECD 423, 2001).

Considering the guidelines of the European Medicines Agency (2010), the acute toxicity study comprised a total of 30 animals (mice) divided into three experimental groups, each composed of five males and five females (n=10/group). The control group received the vehicle solution (0.9% sterile sodium chloride solution (Fresenius Kabi)), while the experimental groups received soluble polypyrrole at the doses of 1,000 mg/kg and 500 mg/kg, all by the intraperitoneal route.

After administering the single dose of soluble polypyrrole and the control substance, the animals were observed daily for 14 days to evaluate the presence of death and signs of toxicity according to the behavioral screening adapted from Almeida *et al*. (1999). Furthermore, other parameters such as body weight and ration and water intake were evaluated throughout the study using forms. On the day of administration, the behavioral parameters were evaluated at 30, 60, 120, 180, and 240 minutes after the treatments (experimental and control). Afterward, these parameters were evaluated once a day. All observations were systematically recorded in forms kept for each subject (ANVISA, 2013; OECD 23, 2001).

At the end of 14 days of observation, the animals were euthanized by cervical dislocation after being anesthetized with 2% xylazine % (Syntec, Santana de Parnaíba, Brazil) at the dose of 10 to 15 mg/kg, and 10% ketamine (Syntec, Santana de Parnaíba, Brazil) at the dose of 100 to 150 mg/kg, applied by the intraperitoneal route (Greene, 2004). These procedures were based on the Brazilian Guide for Good Practices on Animal Euthanasia (CFMV, 2012).

After euthanizing the animals, the organs (heart, lungs, kidneys, liver, and spleen) were analyzed macroscopically, removed, and weighed. Subsequently, the relative weight of the organs was calculated by dividing the weight of each organ (g) by the body weight of the animal (g) and multiplying by 100.

Repeated-Dose Toxicity Study

The doses used in this study were formulated based on information produced in the acute toxicity study. Three doses were used, the highest of which was intended to produce observable toxic effects but cause neither death nor intense suffering, and observing a maximum limit of 1,000 mg/kg/day in rodents. The other doses were established in a decreasing sequence by suggesting intervals from 2 to 4 times (ANVISA, 2013).

Based on that, the repeated-dose study design was as follows: the mice were divided into four groups, each group composed of five males and five females (n/group=10). The control group received a 0.9% sodium chloride solution (Fresenius Kabi) by the intraperitoneal route, while the remaining experimental groups received doses of 1,000, 500, and 250 mg/kg of soluble polypyrrole. All groups received the experimental and control doses daily for 28 days.

Throughout this period, the parameters evaluated were mortality, clinical signs (including behavioral parameters), duration and reversibility of toxicity, and variations in body weight and ration and water intake. At the end of 28 days, the animals were euthanized as described in the acute toxicity study, followed by the analysis of hematological parameters, macroscopic and relative weight analysis of the organs, and histopathological analysis (ANVISA, 2013; Klaassen & Watikins III, 2012).

Analysis of hematological parameters

For the evaluation of hematological parameters, blood was collected from the brachial plexus and stored in microtubes containing the EDTA anticoagulant (Vacuplast, Cotia, Brazil). These tubes were sent to the laboratory for full blood count using a HematoClin 2.8 Vet automated hematology analyzer (Mindray, Shenzen, China). The red blood cell count (10^6/µl), hemoglobin (g/dl), hematocrit (%), mean corpuscular volume (MCV, fl), mean corpuscular hemoglobin (MCH, pg), mean corpuscular hemoglobin concentration (MCHC, %), red cell distribution width (RDW, %), total leucocyte count (10^9/l), lymphocytes (10^9/l), (%) monocytes (10^9/l), and granulocytes (10^9/l) were evaluated.

Macroscopic and relative organ weight analysis

After euthanizing the animals, the organs (heart, lungs, kidneys, liver, and spleen) were macroscopically analyzed, removed, and weighed. Subsequently, the relative organ weight was calculated by dividing the weight of each organ (g) by the body weight of the animal (g), multiplied by 100.

Table 1. Values of direct pH, indirect pH, and density of soluble polypyrrole (Samples A and B)

Soluble polypyrrole	Direct pH	Readings	Indirect pH	Volume(ml)	Mass(g)	Density
Sample A	2.58	1st	2.97	25	26.799	1.07196
		2nd	3.06			
		3rd	3.04			
		Mean	3.02±0.03			
Sample B	2.34	1st	2.80	25	26.747	1.06982
		2nd	2.99			
		3rd	2.97			
		Mean	2.92±0.08			
Mean	2.46±0.12	Mean	2.97±0.05	Mean		1.07089±0.001

Table 2. Effect of the intraperitoneal polypyrrole administration (500 and 1,000 mg/kg) on the ration and water intake, body mass evolution, and organ indices of mice for 14 days.

Parameters	Treatments		
Males	**Control**	**Polypyrrole (500 mg/kg)**	**Polypyrrole (1,000 mg/kg)**
Ration intake (g)	31.50±0.83	30.29±0.77	32.57±1.06
Water intake (ml)	53.69±1.32	53.21±1.67	48.69±1.82
Mass evolution (g)			
Initial	34.20±1.43	36.20±1.99	36.60±1.81
Final	38.40±1.60	39.80±2.60	39.60±1.44
Organ indices (g/100 g)			
Liver	5.59±0.23	6.65±0.22*	5.79±0.12
Kidneys	1.74±0.07	1.86±0.08	1.66±0.07
Spleen	0.41±0.04	0.38±0.02	0.36±0.02
Lungs	0.51±0.07	0.60±0.05	0.56±0.03
Heart	0.49±0.02	0.54±0.02	0.52±0.02
Females			
Ration intake (g)	29.86±0.69	28.00±0.35	24.86±0.68***
Water intake (ml)	52.00±1.37	52.57±1.51	46.25±1.26*
Mass evolution (g)			
Initial	31.40±0.93	30.60±1.78	28.80±0.58
Final	33.80±0.96	33.40±1.44	31.40±0.81
Organ indices (g/100 g)			
Liver	5.73±0.31	5.66±0.25	5.55±0.07
Kidneys	1.25±0.04	1.24±0.04	1.20±0.06
Spleen	0.49±0.02	0.45±0.04	0.47±0.04
Lungs	0.60±0.05	0.54±0.04	0.62±0.04
Heart	0.48±0.02	0.50±0.01	0.50±0.02

Results are expressed as mean±s.e.m. One-way analysis of variance (ANOVA), followed by Tukey's test, compared with the control group (n=5), *p<0.05, ***p<0.001.

Histopathological analysis

After excision, the organs (heart, lungs, kidneys, liver, and spleen) were washed with sodium chloride solution (Fresenius Kabi) and kept in 10% formalin (Dinâmica, Indaiatuba, Brazil) – 0.1 M phosphate buffer, pH 7.2, for 12 hours. After this time, the fragments were transferred to 70% alcohol (Dinâmica), where they were kept for a week. Subsequently, the organs of all animals were dehydrated with increasing alcohol concentrations (Dinâmica) (80%, 90%, 95%, and 100%), followed by diaphanization in xylol (Synth) (xylol:alcohol (1:1 v/v), xylol I, II, and III), and impregnation and inclusion in paraffin (Synth) to obtain histological blocks (Tolosa *et al.*, 2001).

The histological cuts were obtained by microtomy (Easy Path, São Paulo, Brazil), at 4–5 µm, and stained by the Hematoxylin and Eosin technique (Vetec, Duque de Caxias, Brazil). Detailed microscopic examination of organ fragments was performed in the control and treatment groups (one slide per organ of the 40 animals (5 organs × 39 animals = 195 slides)) using a CM 2000 biological microscope (Brax). Images were captured using a digital camera (Toupcam) and the software Toupview.

Statistical analysis

The results were expressed as mean ± standard error of the mean. The differences between groups were determined by one-way analysis of variance (ANOVA), followed by Tukey's *post hoc* test. The Kruskal-Wallis test was used for the hematological analyses, followed by Dunn's *post hoc* test. The statistical analysis was performed using the software GraphPad Prism, version 5.0 (San Diego, California, USA). The minimum level of significance was $p \leq 0.05$.

Results

Characterization of the physicochemical properties of soluble polypyrrole

The results of direct pH, indirect pH, and density of the two soluble polypyrrole samples are shown in Table 1. Based on the miscibility study, it was observed that soluble polypyrrole is immiscible in n-hexane, poorly miscible in ethyl alcohol, and highly miscible in water.

Single-Dose Toxicity Study (Acute Toxicity)

The single-dose administration of soluble polypyrrole, at the tested doses, did not cause death. Therefore, soluble polypyrrole resulted in LD_{50}>1,000 mg/kg when administered by the intraperitoneal route. In the pharmacological behavioral screening, it was observed that the animals treated with soluble polypyrrole, by the intraperitoneal route at the doses of 500 and 1,000 mg/kg, showed up to 60 minutes of

constant licking of the limbs and abdomen, these effects ceased in the remaining observation time.

Regarding the remaining parameters evaluated, Table 2 shows the ration and water intake, the initial and final body weight, and the relative organ weight of the mice from the first to the fourteenth day after the application of soluble polypyrrole. No significant statistical differences were observed between groups for most of the parameters evaluated, with some exceptions.

It was observed that the experimental group of females that received 1,000 mg/kg of soluble polypyrrole showed a significant reduction in the mean water (46.25±1.26) and ration intake (24.86±0.68) compared with the control group.

After euthanizing the animals, the macroscopic analysis indicated that all the organs from the control group and the group treated with soluble polypyrrole (by the intraperitoneal route) had a preserved architecture, normal color and consistency. A significant increase was observed only in the relative liver weight of males treated with 500 mg/kg of soluble polypyrrole by the intraperitoneal route (6.65± 0.22) compared with the group that received the sodium chloride solution (5.59±0.23).

Repeated-dose toxicity study

The treatment with different doses of soluble polypyrrole throughout the 28 days did not cause mortality, except for one death among the male mice belonging to the group treated with 1,000 mg/kg. In this case, death occurred due to an accidental injection that caused hemorrhage in the abdominal cavity. The data from this animal were excluded.

In the daily behavioral screening, no signs of toxicity were observed in the experimental and control groups except for the manifestation of limb and abdomen licking after the intraperitoneal polypyrrole administration, for one hour until the third day of application in all experimental groups.

Regarding the remaining parameters evaluated, Table 3 shows the effects on the ration and water intake, body mass evolution, and relative organ weight after 28 days of daily intraperitoneal soluble polypyrrole applications. No significant statistical differences were observed between groups for most of the parameters evaluated, with some exceptions.

It was observed that the male experimental group that received 1,000 mg/kg of soluble polypyrrole showed

Table 3. Effect of the intraperitoneal polypyrrole administration (250, 500, and 1,000 mg/kg) on the ration and water intake, body mass evolution, and organ indices of mice subjected to the toxicity of repeated doses for 28 days.

Parameters	**Treatments**			
Males	**Control**	**Polypyrrole (250 mg/kg)**	**Polypyrrole (500 mg/kg)**	**Polypyrrole (1000 mg/kg)**
Ration intake (g)	32.04±0.37	31.93±0.78	30.50±0.44	27.00±0.98***
Water intake (mL)	40.52±1.58	37.92±0.65	36.82±0.87	36.58±1.56
Mass evolution (g)				
Initial	42.60±0.40	39.40±1.20	38.60±1.03	38.40±1.63
Final	46.75±0.25	42.40±1.69	44.40±0.97	42.60±1.36
Organ indices (g/100g)				
Liver	4.07±0.09	4.02±0.05	4.12±0.14	4.76±0.06***
Kidneys	1.28±0.05	1.29±0.06	1.25±0.04	1.33±0.07
Spleen	0.34±0.022	0.36±0.019	0.37±0.016	0.39±0.014
Lungs	0.49±0.017	0.52±0.016	0.55±0.017	0.52±0.014
Heart	0.40±0.014	0.42±0.017	0.40±0.021	0.40±0.015
Females				
Ration intake (g)	28.43±0.37	27.00±0.33	2.86±0.36	27.79±0.61
Water intake (mL)	43.65±1.36	45.81±1.60	48.77±1.16*	46.56±1.33
Mass evolution (g)				
Initial	31.40±1,17	32.00±1.10	31.80±1.83	31.80±1.02
Final	34,40 ±1.50	34.60±1.17	36.00±2.47	35.60±1.50
Organ indices (g/100g)				
Liver	4.89±0.34	4.19±0.15	4.47±0.17	4.89±0.16
Kidneys	1.13±0.06	0.99±0.01	1.10±0.05	1.21±0.06
Spleen	0.50±0.04	0.43±0.04	0.51±0.03	0.54±0.03
Lungs	0.65±0.08	0.58±0.02	0.70±0.06	0.64±0.02
Heart	0.43±0.03	0.44±0.03	0.45±0.02	0.46±0.01

Results are expressed as mean±s.e.m. One-way analysis of variance (ANOVA), followed by Tukey's test, compared with the control group (n=5), *p<0.05, ***p<0.001.

a significant reduction in the mean ration intake (27.00±0.98) compared to the control group. In turn, the female group that received 500 mg/kg of soluble polypyrrole showed a significant increase in the water intake (48.77±1.16) compared to the control group.

During necropsy, polypyrrole deposition was observed in the abdominal cavity due to its accumulation throughout the 28 days. Nevertheless, the macroscopic analysis indicated that all organs from the control groups and those treated with soluble polypyrrole had a preserved architecture, normal color and consistency.

Regarding the relative organ weight, a significant increase was observed only in the liver of male animals treated with 1,000 mg/kg of soluble polypyrrole by the intraperitoneal route (4.76±0.06) compared to the group that received the sodium chloride solution (4.07±0.09).

Analysis of hematological parameters

Regarding the peripheral blood erythrogram of the mice (Table 4), no significant alterations were observed in any of the experimental and control groups of females and male animals treated with 250 and 500 mg/kg of soluble polypyrrole. However, significant alterations were verified in the red blood cell count, hematocrit, MCH, and MCHC values of the male mice treated with 1,000 mg/kg of soluble polypyrrole compared to the group treated with the sodium chloride solution.

The leukogram analysis of the females treated with soluble polypyrrole at all experimental doses indicated no significant statistical alterations in all cell types evaluated compared to the control group. In turn, the groups of male animals showed significantly increased values of total leukocytes and lymphocytes at all experimental doses compared to their control group (Table 4).

Histopathological analysis

No relevant histological changes were identified in the heart and lungs of animals treated with the sodium chloride solution and with soluble polypyrrole, in both sexes, at the doses of 250, 500, and 1,000 mg/kg.

Regarding the liver tissue evaluation, the control group treated with the sodium chloride solution showed no alterations in the parenchyma and liver structures; the hepatocytes showed no atypia and were arranged as hepatic cords directed toward the centrilobular veins, and there was no hyperemia in the hepatic vessels. In the group treated with 250 mg/kg of soluble polypyrrole, evidence of necrosis associated with soluble polypyrrole

Table 4. Effect of the intraperitoneal polypyrrole administration (250, 500, and 1,000 mg/kg) on the hematological parameters of mice subjected to the toxicity of repeated doses for 28 days.

Parameters	**Treatments**			
Males	**Control**	**Polypyrrole (250 mg/kg)**	**Polypyrrole (500 mg/kg)**	**Polypyrrole (1,000 mg/kg)**
Red blood cells (x10^6/µl)	9.16±0.24	10.64±0.20	10.10±0.34	13.01±1.53**
Hemoglobin (g/dl)	14.16±0.61	15.90±0.41	14.58±0.41	14.00±1.48
Hematocrit (%)	49.34±1.43	55.90±1.42	53.82±2.45	70.35±6.83**
MCV (fl)	53.94±0.76	52.56±0.84	53.26±0.69	53.95±0.48
MCH (pg)	15.40±0.35	14.90±0.31	14.44±0.53	11.08±1.09**
MCHC (%)	28.62±0.59	28.40±0.53	27.26±1.26	20.58±2.05**
RDW (%)	14.38±0.75	13.38±0.37	13.20±0.32	14.18±0.35
Leukocytes (x10^9/l)	5.08±0.92	9.15±0.72*	10.35±1.32**	14.67±0.59***
Lymphocytes (x10^9/l)	3.75±0.55	7.13±0.68*	7.78±0.85**	11.53±0.65***
Monocytes (x10^9/l)	0.20±0.04	0.20±0.03	0.26±0.04	0.28±0.05
Granulocytes (x10^9/l)	2.15±0.61	1.80±0.14	1.98±0.57	2.33±0.41
Females				
Red blood cells (x10^6/µl)	10.49±0.44	12.41±1.51	9.70±0.34	12.17±1.05
Hemoglobin (g/dl)	15.06±0.34	15.58±0.09	10.30±1.98	13.43±1.31
Hematocrit (%)	55.78±2.25	67.72±8.65	52.38±1.98	66.03±6.17
MCV (fl)	53.24±0.24	54.50±0.68	54.76±0.84	54.34±0.51
MCH (pg)	14.44±0.84	13.18±1.48	11.57±2.68	13.48±1.04
MCHC (%)	27.16±1.52	24.37±2.83	18.23±6.08	25.03±2.56
RDW (%)	13.04±0.12	13.16±0.34	14.34±0.67	13.72±0.54
Leukocytes (x10^9/l)	6.36±1.12	8.42±0.59	4.46±0.80	6.58±0.45
Lymphocytes (x10^9/l)	5.06±1.01	6,94±0.47	3.64±0.64	5.28±0.43
Monocytes (x10^9/l)	0.16±0.02	0.18±0.04	0.13±0.02	0.14±0.02
Granulocytes (x10^9/l)	1.14±0.02	1.30±0.11	0.72±0.15	1.00±0.18

Results are expressed as mean±s.e.m. Kruskal-Wallis test, followed by Dunn's *post hoc* test, compared with the control group (n=5), *p<0.05, **p<0.01,***p<0.001.

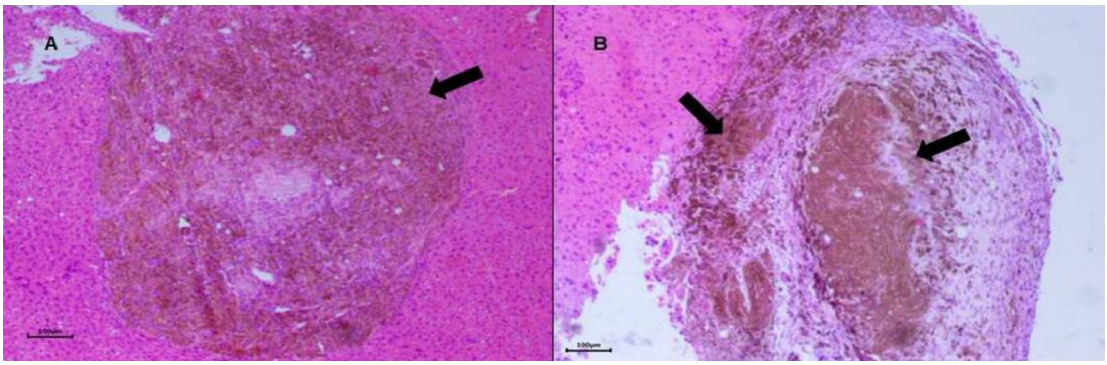

Figure 1. Histopathological analysis of the livers of Swiss mice injected with a polypyrrole solution by the intraperitoneal route. In **(A)**, there is an area of soluble polypyrrole deposition in the parenchyma of the liver tissue (arrow), with central necrosis in the impregnated region, at the dose of 250 mg/kg of soluble polypyrrole. In **(B)**, there is soluble polypyrrole impregnation at the edge of the liver tissue (arrows), with a granulomatous inflammatory reaction around the impregnated material, at the dose of 1,000 mg/kg of soluble polypyrrole. In both, 40X magnification.

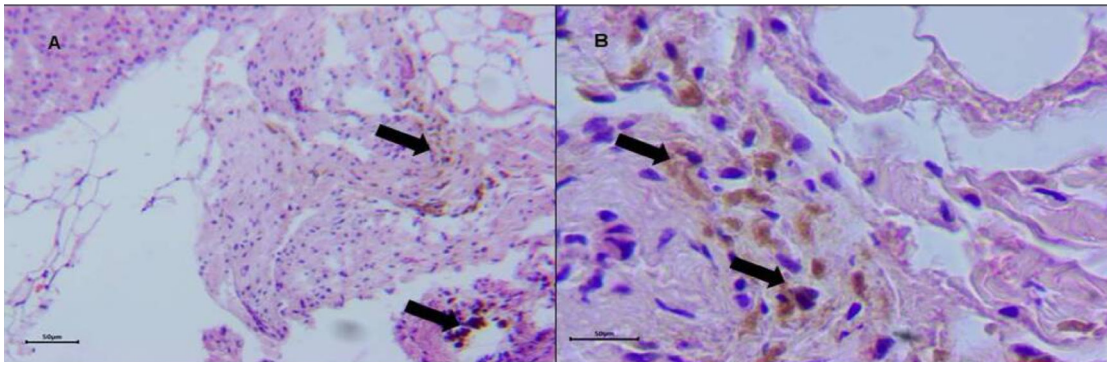

Figure 2. Histopathological analysis of the kidneys of Swiss mice injected with a polypyrrole solution by the intraperitoneal route. In **(A),** areas of soluble polypyrrole deposition in the tissue adjacent to the kidney, with a clear brown color (arrows), 100X magnification. In **(B**), intracellular aspect of soluble polypyrrole impregnation in the kidney, brown cells (arrows), 400X magnification. In both, 1,000 mg/kg of soluble polypyrrole.

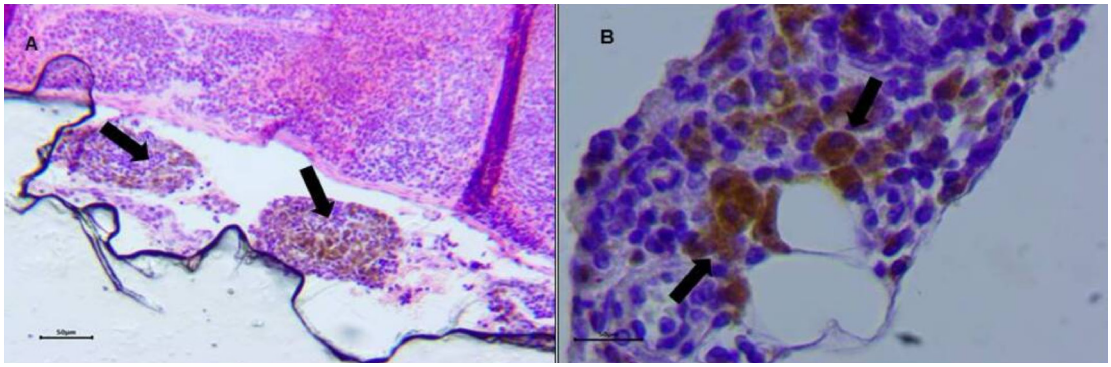

Figure 3. Histopathological analysis of the spleens of Swiss mice injected with a polypyrrole solution by the intraperitoneal route. In **(A**), soluble polypyrrole impregnation in cells adjacent to the spleen capsule (arrows), dose of 500 mg/kg of soluble polypyrrole, 100X magnification. In **(B)**, peritoneal macrophages with intracellular polypyrrole deposition, brown cells (arrows), 250mg/kg of soluble polypyrrole, 400X magnification.

deposition was found in the central area of the liver tissue of 10% of the animals (Figure 1A). The same finding was verified in 10% of the animals that received 500 mg/kg of soluble polypyrrole.

When observing the group of animals that received the dose of 1,000 mg/kg, 2.2% of the animals showed soluble polypyrrole deposition in the liver capsule, 11.11% of the animals showed small polypyrrole deposition in a small area of the parenchyma, although without inflammatory reaction of the tissue, and 11.11% showed polypyrrole impregnation in the liver parenchyma, evidencing a granulomatous reaction in the tissue (Figure 1B).

Aiming to evaluate the effect of soluble polypyrrole treatment on renal histology, the microscopic analysis of this organ was performed. Under microscopic examination, the renal tissue of the control groups and those treated with 250 and 500 mg/kg showed a normal and preserved architecture and regularly-distributed corpuscles and renal convoluted tubules. In the group of animals that received the dose of 1,000 mg/kg, 33% of the animals showed soluble polypyrrole deposition adjacent to the renal tissue (Figures 2A and 2B).

Aiming to evaluate the effect of soluble polypyrrole treatment on spleen histology, the microscopic

analysis of this organ was performed. Under microscopic examination, the spleen tissue of the control groups and the group treated with 1,000 mg/kg showed a normal and preserved architecture. In the group that received the dose of 500 mg/kg, 60% of the animals showed moderate soluble polypyrrole accumulation in the spleen tissue (interstitial or parenchyma), while 40% showed soluble polypyrrole accumulation in the spleen capsule (Figure 3A). In the group treated with 250 mg/kg of soluble polypyrrole, 50% showed moderate soluble polypyrrole deposition in the spleen tissue, 40% showed evidence of peritoneal macrophages full of soluble polypyrrole at the periphery of the spleen (Figure 3B), and 10% showed no alterations.

Discussion

The standard deviation values of pH and density in samples A and B indicate that soluble polypyrrole has good stability, maintaining its properties over a year. The low pH may be related to the ammonium persulfate added during its synthesis (Silva Junior *et al*., 2016). It is important to verify the pH in order to justify the possible acidification or alkalinization of pharmaceutical bases with its addition (Alencar Filho *et al*., 2020).

The mean density of soluble polypyrrole was 1.070±0.001 g/ml. Density is determined to facilitate possible future industrial operations, such as converting mass into volume and facilitating the work with liquid samples (Alencar Filho *et al*., 2020).

Soluble polypyrrole is readily miscible with water. Aqueous miscibility is essential for drugs and excipients and can influence the formulation, production, and pharmacokinetics, directly affecting the dissolution and absorption rates and, consequently, the *in vivo* efficacy (Kalra & Jain, 2011).

No mortality was observed during the 14 days at any dose of the soluble polypyrrole in the acute toxicity study. Ferraz *et al*. (2012) also observed no mortality when administering 50 ml/kg of nanocellulose and polypyrrole compounds. Another study did not observe any deaths after a single intraperitoneal injection of 50 ml/kg of an extraction solution of polypyrrole (Wang *et al*., 2004). Given the possibility of thrombotic events in mice after a single intravenous injection of nanocomposites of PPy/Cu-MOF, other researchers observed only one death on the seventh day after the injection among all the mice in a study (Neisi *et al*., 2019).

The licking of the abdomen and limbs after the intraperitoneal injection of polypyrrole can be related to its low pH. It is assumed that polypyrrole injection caused irritation and/or pain in the abdomen during the application, triggering licking movements of the spot to alleviate the discomfort.

Still regarding acute toxicity, the reduction in water and food intake is considered an irrelevant finding since there was no statistical difference in the evolution of the body mass parameter. Furthermore, the OECD guideline 407 (2008) states that reduced food intake may not be an indication of toxicity. Researchers have observed no body weight changes in mice over 15 days after intratumoral injection of 4m/kg of polypyrrole nanoparticles (PPyNP) (Lu *et al*., 2021).

Regarding the relative organ weight, a significant enlargement was verified in the livers of male mice treated with 500 mg of soluble polypyrrole. This finding does not necessarily indicate a toxic response since no alterations were found in the macroscopic evaluation of these organs in the mentioned group.

When evaluating hepatotoxicity seven days after intravenous injection of 5 mg/kg of PPy/*x* %Cu-MOF composites, no significant effects were observed in the serum levels of ALT (alanine aminotransferase) and AST (aspartate aminotransferase) of mice (Neisi *et al*., 2019).

The repeated-dose toxicity study did not cause mortality over 28 days, suggesting that soluble polypyrrole has low toxicity. Significant changes in food and water intake constitute irrelevant results when highlighting the absence of changes in the mass evolution parameter. Zhang *et al.* (2020) found no significant changes in the body weight of mice 25 days after intratumoral injection of Selenium/Polypyrrole nanocomposites (Se@PPy).

Polypyrrole deposition in the abdominal cavity represented the main macroscopic finding during necropsy. A similar finding was obtained in another study when observing AgNP residues in the abdominal cavity six hours after single intraperitoneal injections (Cho *et al*., 2018). The macroscopic evaluation of the present study indicated that all organs of the experimental groups showed typical architecture, color, and consistency.

A significant enlargement was only observed in the liver of the animals treated with 1,000 mg/kg of soluble polypyrrole. Considering the exposure period and the high doses of the studied compound, this isolated result does not constitute an indication of toxicity since it does not support the findings of the macroscopic evaluation; furthermore, liver enlargement can be related to physiological overload due to metabolization of the drug.

The high red blood cell count and hematocrit values correspond to clinical pictures of erythrocytosis, which can be attributed to dehydration or diversion of body fluids (Thrall *et al*., 2015). It is suggested that the accumulation of high doses of soluble polypyrrole in the abdominal cavity affected the osmotic balance of fluids and solutes in the organism, changing the proportion of plasma and erythrocytes. Erythrocytosis is often observed along with dehydration in preclinical studies, especially in rodents under prolonged fasting (Weiss *et al*., 2010).

In a blood compatibility evaluation of polypyrrole nanocomposite and copper metal-organic frameworks (PPy/Cu-MOF), the addition of PPy to Cu-MOF increased red blood cell viability (RBCs) from 92% to more than 98% (Neisi *et al*., 2019). Another study verified that the hemolytic activity of *Pseudomonas aeruginosa* was highly influenced by the addition of chitosan and polypyrrole nanocomposites (CS-PPy NCs) and individually by PPy (Khan *et al*., 2019).

Regarding the significant decrease in the MCHC and MCH values in male mice treated with 1,000 mg/kg of soluble polypyrrole, the fraction between the hemoglobin and hematocrit values is considered to obtain the MCHC. Since there was no difference in the hemoglobin concentration of this group in relation to the control group, the low MCHC is attributed to the high hematocrit value. The same applies to the MCH index, which value is obtained based on the hemoglobin concentration and the erythrocyte count, being considered a redundant parameter (Thrall *et al*., 2015).

In the leukogram, the male mice groups showed significantly higher values of total leukocytes and lymphocytes. Lymphocytes predominate in the peripheral blood of mice, representing from 70 to 80% of the leukocyte population (Thrall *et al*., 2015). Therefore, the significantly higher number of total leukocytes can be attributed to the increased number of lymphocytes.

Physiological leukocytosis is often observed in excitation states that induce a rapid increase in the number of lymphocytes in mice (Weiss *et al*., 2010). Researchers have observed increased lymphocytes in mice after an accumulated dose of 125 mg/kg of polyethylene glycol-coated hollow gold nanospheres. The authors do not consider these alterations as toxicologically important (You *et al*., 2014).

Regarding histopathology, the alterations observed in the livers consisted of soluble polypyrrole deposition in the capsule and in the parenchyma (without inflammation, with granulomatous reaction and necrosis). Granulomas are common in the liver and can reflect a systemic or specific alteration in the organ. These injuries can be induced by drugs or toxins (Kumar *et al*., 2013). Soluble polypyrrole may have induced the formation of liver granulomas, although the incidence of this alteration was low in the experimental groups.

The deposition of a brown pigment in the Kupffer cells and focal necrosis in the liver were observed after the intraperitoneal injection of silver nanoparticles in mice (Cho *et al*., 2018). Pigments were visualized in liver Kupffer cells of mice treated with polyethylene glycol-coated hollow gold nanospheres. In the same study, inflammatory liver injuries and necrosis were observed, although with minimal incidence and severity in both sexes; therefore, they were not considered an adverse effect by the authors (You *et al*., 2014).

Soluble polypyrrole visualization adjacent to the renal tissue was the only alteration observed regarding renal histopathology. This finding may result from the daily intraperitoneal administration of high doses of soluble polypyrrole, leading to its accumulation under some abdominal organs. Organs such as the liver and spleen also showed polypyrrole deposition in their capsules. Ppy particles have been verified to adhere to the peritoneum organs and be phagocyted by macrophages (Ramanaviciene *et al*., 2007). In the absence of relevant findings for the relative weight analysis and the macroscopic evaluation of the kidneys of treated animals, it is supposed that polypyrrole deposition under the organs does not result in adverse effects on renal physiology.

As main microscopic finding, the spleens revealed a moderate soluble polypyrrole accumulation in the tissue or capsule, besides peritoneal macrophages full of polypyrrole at the periphery of the spleen. The microscopic analysis showed a significant increase of peritoneal cells after three days of treatment with polypyrrole particles (5 mg/kg). After six weeks, the number of macrophages in the peritoneum decreased considerably (Ramanaviciene *et al*., 2007). An *in vitro* study verified that polypyrrole-polyethyleneimine nanocomplexes (Ppy-PEI NCs) could be absorbed by macrophages via endocytosis (Burnouf *et al*., 2019). The ability of macrophages to capture foreign micro/nanomaterials is well known and occurs due to the positive charge of the particles (Gustafson *et al*., 2015).

Lu *et al*. (2021) showed that polypyrrole-based nanoformulations, administered via intratumoral injection, were beneficial for not causing toxicity to surrounding healthy organs such as the liver, heart, kidneys, lungs, and spleen. A similar result was found in another study when verifying the absence of tissue damage in these organs after photothermal therapy using selenium/polypyrrole (Zhang *et al*., 2020).

Conclusions

The physicochemical characterization of soluble polypyrrole will allow its pharmacotechnical development, subsidizing the establishment of a profile for its quality control and other bioapplications that use it as a base.

In the acute toxicity study, it was verified that soluble polypyrrole has an LD_{50}>1,000 mg/kg since no mortality was observed in the mice until the 14th day after the single administration by the intraperitoneal route. In the repeated-dose toxicity study, when evaluating mortality, behavioral screening, body weight, water and ration intake, and hematology, it was verified that none of the treatments implied systemic adverse effects or results in the mice treated with the different doses of soluble polypyrrole. When evaluating the histopathological parameters, these showed significant alterations, even if most of the findings in these organs were within an expected standard.

Even when considering the high doses administered (up to 1,000 mg/kg) and the time of application (28 days) of soluble polypyrrole, the incidence and intensity of the alterations induced by it can be considered minimal, allowing to classify polypyrrole as a compound of low or no toxicity. No significant findings were observed to imply adverse effects due to PPy application in mice. It should be noted that, in this study, the intended clinical or therapeutic doses were extrapolated, resulting in a wide range of doses that can be used as a reference in further studies on the bioapplication of soluble polypyrrole.

Acknowledgments

This study was funded in part by the Coordination for the Improvement of Higher Education Personnel - Brazil (CAPES) - Finance Code 001. The authors also thank the financial support of the Foundation for the Support of Science and Technology of the State of Pernambuco (FACEPE) and the National Council for Scientific and Technological Development (CNPq - Process 305755/2016-1).

REFERENCES

Alencar Filho JMT, Sampaio PA, de Carvalho IS, Guimarães AL, e Amariz IA, Pereira ECV, Rolim-Neto PJ, Rolim LA, Araújo ECC. (2020). Flavonoid enriched extract of Alternanthera brasiliana with photoprotective effect: Formulation development and evaluation of quality. *Ind Crops Prod* **149**: 112371.

Almeida RN, Falcão ACGM, Diniz RST, Quintans-Júnior LJ, Polari RM, Barbosa-Filho JM, Agra MF, Duarte JC, Ferreira CD, Antoniolli AR, Araújo CC. (1999). Metodologia para avaliacao de plantas com atividade no Sistema Nervoso Central e alguns dados experimentais. *Rev Bras Farm* **80**: 72–76.

ANVISA. (2013). Guia para a condução de estudos não clínicos de toxicologia e segurança farmacológica necessários ao desenvolvimento de medicamentos. Anvisa, Brasília. Disponível em: www.anvisa.gov.br

ANVISA. (2019). Farmacopeia Brasileira. Anvisa, Brasília. Available from: : https: //www.gov.br/anvisa/pt-br/assuntos/farmacopeia/farmacopeia-brasileira

Arantes C, Rocco MLM, da Cruz AGB, Rocco AM. (2008). Dessorção iônica e degradação de filmes de polipirrol dopado com dodecilsulfato induzidas por elétrons de alta energia. *Quim Nova* **31**: 61–65.

Asadi N, Bakhshayesh ARD, Davaran S, Akbarzadeh A. (2020). Common biocompatible polymeric materials for tissue engineering and regenerative medicine. *Mater Chem Phys* **242**: 122528.

Avdeef A. (2003). *Absorption and Drug Development: Solubility, Permeability, and Charge State*. Wiley, New York.

Burnouf T, Chen C, Tan S, Tseng C, Lu K, Chang L, Nyambat B, Huang S, Jheng P, Aditya RN, Mi F, Chuang E. (2019). A bioinspired hyperthermic macrophage-based polypyrrole-polyethylenimine (Ppy-PEI) nanocomplex carrier to prevent and disrupt thrombotic fibrin clots. *Acta Biomater* **96**: 468–479.

CFMV. (2012). Guia brasileiro de boas práticas para a eutanásia em animais - Conceitos e procedimentos recomendados. Conselho Federal de Medicina Veterinária, Brasília. Available from: : https: //www.cfmv.gov.br/guia-brasileiro-de-boas-praticas-para-a-eutanasia-em-animais/comunicacao/publicacoes/2020/08/03/#1

Cho Y, Muzuta Y, Akagi J, Toyoda T, Sone M, Ogawa K. (2018). Size-dependent acute toxicity of silver nanoparticles in mice. *J Toxicol Pathol* **31**: 73–80.

EMA. (2010). Guideline on Repeated Dose Toxicity. European Medicines Agency - Science Medicines Health, Reino Unido. Available from: : https: //www.ema.europa.eu/en/repeated-dose-toxicity

Ferraz N, Stromme M, Fellstrom B, Pradhan S, Nyholm L. (2012). In vitro and in vivo toxicity of rinsed and aged nanocellulose-polypyrrole composites. *J Biomed Mater Res A* **100A**: 2128–2138.

Greene SA. (2004). *Segredos em anestesia veterinária e manejo de dor*. Artmed, Porto Alegre.

Gustafson HH, Holt-Casper D, Grainger DW, Ghandehari H. (2015). Nanoparticle uptake: The phagocyte problem. *Nano Today* **10**: 487–510.

Hsiao PF, Anbazhagan R, Tsai H, krishnamoorthi R, Lin S, Lin S, Lee K, Kao C, Chen R, Lai J. (2020). Fabrication of electroactive polypyrrole-tungsten disulfide nanocomposite for enhanced in vivo drug release in mice skin. *Mater Sci Eng C Mater Biol Appl* **107**: 110330.

Kalra K, Jain DA. (2011). *Solubilty Enhancement of Poorly Water Soluble Drugs-Techniques to overcome poor water solubility*. LAP Lambert Academic Publishing.

Khan F, Manivasagan P, Pham DTN, Oh J, Kim S, Kim Y. (2019). Antibiofilm and antivirulence properties of chitosan-polypyrrole nanocomposites to Pseudomonas aeruginosa. *Microb Pathog* **128**: 363–373.

Klaassen CD, Watikins III JB. (2012). *Fundamentos em toxicologia de Casarett e Doull*. AMGH, Porto Alegre.

Kumar D, Sharma RC. (1998). Review Article: Advances in Conductive Polymers. *Eur Polym J* **34**: 1053–1060.

Kumar V, Abbas AK, Aster JC. (2013). *Robbins Patologia Básica*. Elsevier, Rio de Janeiro.

Lee JY, Kim DY, Kim CY. (1995). Synthesis of soluble polypyrrole of the doped state in organic solvents. *Synth Met* **74**: 103–106.

Lee M, Kim S, Jang M, Park HS, Lee JY. (2021). One-Pot electrochemical fabrication of high performance amperometric enzymatic biosensors using polypyrrole and polydopamine. *J Ind Eng Chem* **97**: 316–325.

Lu K, Jheng P, Lu L, Rethi L, Mi F, Chuang E. (2021). Enhanced anticancer effect of ROS-boosted photothermal therapy by using fucoidan-coated polypyrrole nanoparticles**.** *Int J Biol Macromol* **166**: 98–107.

Maximiano FP, Costa GHY, de Souza J. (2010). Caracterização físico-química do fármaco antichagásico benznidazol. *Quim Nova* **33**: 1714–1719.

Neisi Z, Ansari-Asla Z, Jafarinejad-Farsangib S, Tarzi ME, Sedaghat T, Nobakht V. (2019). Synthesis, characterization and biocompatibility of polypyrrole/Cu(II) metal-organic framework nanocomposites. *Colloids Surf B Biointerfaces* **178**: 365–376.

OECD 407. (2008). Repeated dose 28-day oral toxicity study in rodents. OECD Guidelines for the testing of chemicals. Available from: https: //www.oecd-ilibrary.org/environment/test-no-407-repeated-dose-28-day-oral-toxicity-study-in-rodents_9789264070684-en

OECD 423. (2001). Acute Oral Toxicity - Acute Toxic Class Method. OECD Guideline for Testing of Chemicals. Available from: : https: //www.oecd-ilibrary.org/environment/test-no-423-acute-oral-toxicity-acute-toxic-class-method_9789264071001-en

Pereira AS, Bicalho B, Lilla S, De Nucci G. (2005). Desafios da química analítica frente às necessidades da indústria farmacêutica. *Quim Nova* **28**: S107–S11.

Qin C, Yue Z, Chao Y, Forster RJ, Maolmhuaidh FO, Huang XF, Beirne S, Wallace GG, Chen J. (2020). Bipolar electroactive conducting polymers for wireless cell stimulation. *Appl Mater Today* **21**: 100804.

Ramanaviciene A, Kausait A, Tautkus S, Ramanavicius A. (2007). Biocompatibility of polypyrrole particles: an in-vivo study in mice. *J Pharm Pharmacol* **59**: 311–315.

Ryan EM, Breslin CB. (2019). The incorporation of drug molecules with poor water solubility into polypyrrole as dopants: Indomethacin and sulindac. *Electrochim Acta* **296**: 848–855.

Silva Junior FAG, Queiroz JC, Macedo ER, Fernandes AWC, Freire NB, da Costa MM, de Oliveira HP. (2016). Antibacterial behavior of polypyrrole : The influence of morphology and additives incorporation. *Mater Sci Eng C* **62:** 317-322.

Su D, Zhou J, Ahmed KS, Ma Q, Lv G, Chen J. (2019). Fabrication and characterization of collagen-heparin-polypyrrole composite conductive film for neural scaffold. *Int J Biol Macromol* **129**: 895–903.

Takács-Novák K. (2012). Physicochemical profiling in drug research and development, In: *Physico-chemical Methods in Drug Discovery and Development* (Mandic Z ed) pp. 3–52, IAPC Publishing, Zagreb.

Talikowska M, Fu X; Lisak G. (2019). Application of conducting polymers to wound care and skin tissue engineering: A review. *Biosens Bioelectron* **135**: 50–63.

Thrall MA, Weiser G, Allison RW, Campbell TW. (2015). *Hematologia e Bioquímica Clínica Veterinária*. Guanabara Koogan, Rio de Janeiro.

Tolosa EMC, Rodrigues CJ, Behmer OA.; Freitas Neto AG. (2001). *Manual de técnicas para histologia normal e patológica*. Manole, Barueri.

Wang X, Xiaosong G, Yuan C, Chen S, Zhang P, Zhang T, Yao J, Chen F, Chen G. (2004). Evaluation of biocompatibility of polypyrrole in vitro and in vivo. *J Biomed Mater Res A* **68**: 411–422.

Weiss DJ, Wardrop KJ, Schalm OW. (2010). *Schalm's veterinary hematology*. Willey-blackwell, Yowa.

WHO. (2014). Antimicrobial Resistance: Global Report on Surveillance. World Health Organization, França.

You J, Zhou J, Zhou M, Liu Y, Robertson JD, Liang D, Pelt CV, Li C. (2014). Pharmacokinetics, clearance, and biosafety of polyethylene glycol-coated hollow gold nanospheres. *Part Fibre Toxicol* **11**: 1–14.

Zhang J, Qiu T, Lan Y, Tu C, Shang T, Lin Y, Mao G, Yang B. (2020). Autocatalytic polymerization of selenium/polypyrrole nanocomposites as functional theranostic agents for multi-spectral photoacoustic imaging and photothermal therapy of tumor. *Mater Today Chem* 17.

Zhang Y, Chen Z, Zhang D, Zhao Y, Wu P, Wang F. (2018). Diversified applications of polypyrrole/graphene aerogel in supercapacitors and three-dimensional electrode system. *Mater Lett* **227**: 158–160.