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PHARMACOLOGY

TOXICOLOGICAL EVALUATION OF XANTHAN GUM BASED HYDROGEL FORMULATION IN WISTAR RATS USING SINGLE DOSE STUDY

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Abstract: Xanthan gum-based hydrogel formulation FXG3 was prepared by a free radical polymerization technique. To assess the safety of FXG3 hydrogel for potential application as a new drug delivery system, a single oral dose toxicity study was conducted according to OECD guidelines. Female adult rats of Wistar strain were divided into group A and group B. Group A served as the control and was given 1 mL/100 g body weight 0.9% saline. Group B received a dose of 5 g/kg body weight of FXG3 hydrogel. Rats were observed continuously for 14 days for clinical signs, and prior to terminal sacrifice, blood samples were taken to assess for hematology and biochemical parameters. Selected organs (heart, liver, lung, kidneys, spleen, and stomach) were removed and examined macroscopically, washed, sliced and stained with hematoxylin-eosin for histopathological investigation. No mortality or any signs of acute toxicity was observed during the observation period. No macroscopic alteration was found in the selected organs. Histopathological examination did not show any pathological changes. Thus, the maximal tolerated dose of FXG3 was calculated to be higher than 5 g/kg body weight. It can be concluded that FXG3, a xanthan gum-based hydrogel formulation, was non-toxic after acute oral administration at 5 g/kg body weight, and thus may be a promising candidate in controlled drug delivery system.

Keywords: acute toxicity, histopathology, hydrogel, maximal tolerated dose, rats

Hydrogels have drawn significant attention over the past few decades due to their biocompatibility, biodegradability and resemblance to biological tissues. They are regarded as a special class of polymers that imbibe a substantial amount of water while maintaining their shape and physical and chemical cross-linking (1). The research on hydrogels with respect to drug delivery and biomedical devices has been extensive. In general, hydrogels as oral drug delivery carriers are regarded as safe and non-toxic (2). However, the biocompatibility of hydrogels is limited by the release of their unreacted components in the body especially monomers. Small, unpolymerized residual monomer, if persist in polymeric network, has a tendency to leach throughout body fluids and may cause toxicity to host cells. Moreover, enzymatic hydrolysis of hydrogels may also contribute to toxic effect due to diffusion in surrounding tissues. It is, therefore, important to investigate potential toxicity of hydrogels as a new drug delivery system (3).

Xanthan gum-based hydrogels were developed, characterized and evaluated via free radical polymerization technique as reported in our previous study (4). Out of all developed hydrogels, we have chosen FXG3 hydrogel to determine the potential toxicity as a new drug delivery system. The reason for choosing FXG3 hydrogel was its maximum drug entrapment efficiency, good swelling dynamics and excellent drug release profile among all devel-

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oped formulations (4). In the present work, we will discuss safety evaluation of previously developed FXG3 hydrogel that was conducted using a single oral dose toxicity study according to "Organization of Economic Co-operation and Development (OECD)" guidelines (5). As the ultimate goal for any toxicological testing is to establish a safe level for human exposure to the products tested, this safety evaluation of FXG3 hydrogel could be used as a basis for future application in clinical studies.

MATERIALS AND METHODS

Composition of hydrogels

Xanthan gum derived hydrogels were developed, synthesized and evaluated by already reported method (4). Hydrogel formulation FXG3 having maximum drug loading efficiency and ultimately cumulative drug release was selected for toxicological evaluation via acute oral toxicity study. FXG3 hydrogel was developed using xanthan gum (XG), acrylic acid (AA), hydroxyethylmethacrylate (HEMA), methylenebis-acrylamide (MBA) potassium persulfate (KPS) and sodium metabisulfite (SMBS). XG along with AA and HEMA were purchased from Sigma-Aldrich, UK. MBA was obtained from Sigma-Aldrich, USA. KPS and SMBS were obtained from Fluka. First of all, XG (3%) was weighed and dissolved in distilled water with stirring on aluminum hot plate magnetic stirrer. Then analytical grade redox initiator pair of KPS and SMBS (0.3%) was added to the XG solution at 25^{o}C with continuous stirring at 300 rpm until the formation of a homogeneous and viscous solution. Monomers were mixed separately in a beaker using AA (24%) and HEMA (4%). Crosslinker MBA (0.5%) was added into monomers solution with continuous stirring at 40^{o}C until a clear solution was attained. Then monomer solution and polymer solution were mixed in a separate beaker and the final volume was adjusted using distilled water. This mixture was placed on a water bath at 60^{o}C for 12 h. After 12 h, FXG3 hydrogel was removed and cut into uniform size i.e. 8 mm. The developed blank hydrogels were further dried in lyophilizer at -55^{o}C for 24 h. For ACV loading or entrapment in developed hydrogel discs, 0.8% ACV drug solution was prepared by dissolving ACV in phosphate buffer solution (0.2 M). Drug loading was done by immersing blank FXG3 hydrogel in above prepared ACV drug solution for 72 h. ACV loaded swollen hydrogel discs were removed after the specified time and lyophilized at -55^{o}C (4).

Selection of animals

Wistar strain of adult albino female rats (obtained from Animal Facility Centre, Faculty of Pharmacy and Alternative Medicine, The Islamia University of Bahawalpur, Pakistan) were used for the study. They were divided into two groups (n = 5). Animals were housed under a controlled environment (room temperature: 22 ± 2^{o}C, relative humidity: 65 ± 5%, 12 h day/night cycle) with a balanced diet and water *ad libitum*. The experimental protocol used in this study was approved by the Pharmacy Research Ethics Committee of the Islamia University of Bahawalpur, Pakistan (23-2016/PREC). Female rats were chosen randomly, marked for identification, and kept in cages for at least 5 days in an animal transient room. All the animals were nulliparous, non-pregnant, 9-10 weeks old with weight ranged approximately 210 g ± 10. Animals were fasted overnight prior to dosing but the water supply was not withheld. Animal Welfare Act guidelines and the recommendations in the Guide for Care and Use of Laboratory Animals were strictly followed (CCAC, 1993) (6).

Preparation of doses

A single dose toxicity study was carried out by adapting OECD guideline no. 420 (OECD, 2001). Moreover, a dose of 5 g/kg body weight was selected for FXG3 hydrogel as it proved to be safe in a similar study conducted on acyclovir based â-cyclodextrin hydrogel microparticulate system (7). Hence, rats were administered FXG3 hydrogel at a dose of i.e. 5 g/kg body weight. The mortality rate of rats remains zero after being treated with the highest tested dose of FXG3 i.e. 5 g/kg body, thus no lethal dose or median lethal dose (LD50) could be established. Hence, Maximal Tolerated Dose (MTD) method was used in adult female albino rats of Wistar strain to evaluate the safety of FXG3 hydrogel. FXG3 hydrogel, unlike conventional formulations, when coming in contact with liquid medium demonstrates swelling. The swelling dynamics increases with the increase in dose. A higher dose may produce adverse effects due to the limited gastric capacity of rats and the high swelling behavior of FXG3 formulation rather than its toxicity. Although it has been assumed that Maximal Tolerated Dose (MTD) of FXG3 hydrogel could be higher than 5 g/kg body weight in rats, above mentioned limitations deterred us from taking dose higher than 5 g/kg body weight for the experiment. Thus, in order to ensure that toxicity, if happens, has been caused by FXG3 hydrogel instead of excessive volume, we have chosen to work with a maximum dose of 5 g/kg body weight in this study.

Experimental animals were divided into two groups (n = 5). Group A (control) was considered control and was given 1 mL/100 g body weight 0.9% saline per orally. For group B, (5 g/kg) body weight of hydrogel was weighed and crushed to powder. The powdered hydrogel was added to 0.9% saline and administered at the same quantity as the control group i.e. 1 mL of saline / 100 g body weight in the form of suspension by oral gavage.

Clinical observations

Animals were observed individually shortly after administration of dose with special attention given during the first 48 h up to 14 days. Animals were checked physically for any morbidity or any mortality during the whole observation period on a daily basis. Non-specific neurologic effects such as anxiety, salivation, tremor, and coma were noted on a regular basis. Changes in skin, fur, mucus membranes, and eyes were also observed. Gastrointestinal symptoms observed as a part of clinical observations included nausea, vomiting, anorexia, and diarrhea. Animals feces were checked for mucus or pus or blood. Stereotypic activities like excessive grooming, repetitive circling, changes in gaits, posture, and response to handling, was also observed as a result of central nervous system excitation in animals. The time of onset, intensity and duration of such symptoms, if any, were recorded. Furthermore, individual weights of animals were also determined shortly before the start of the experiment and thereafter on the 7th and 14th day of study. Weight changes were calculated and recorded. The amount of feed and water consumed by each cage of animals was also recorded weekly. Feed intake was calculated as g/animal/day for the corresponding bodyweight intervals. The water intake was calculated as mL/animal/day. It was decided to kill animals humanely if they show severe pain or signs of severe distress during the course of study.

Hematology and biochemical blood analysis

Animals in group A (control) and group B (5 g/kg) were anesthetized with ketamine (22 mg/ kg body weight) at the end of the study period on day 15th. Later, cervical decapitation was used to sacrifice animals. For the analysis of hematological parameters, the collection of blood was done in a heparinized tube through cardiac puncture from the posterior vena cava. Hematology analyses were performed on whole blood to assess erythrocyte count, hemoglobin, and hematocrit, mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), platelet count, monocytes, neutrophils, and lymphocytes. For biochemical estimations, the collected blood sample was centrifuged for 15 min at 3500 rpm to obtain the serum. The biochemical parameters were determined enzymatically using specific kits to measure serum creatinine, glucose, cholesterol, triglycerides, urea, alkaline phosphatase (ALP), alanine aminotransferase (ALT), aspartate transaminase (AST) and creatinine kinase (CK) by measuring optical density at the corresponding wavelength with a spectrophotometer.

Macroscopic observations, organ weight and histopathological study

On the 15th day, animals in both groups were sacrificed to obtain tissue samples. The vital organs were collected i.e. heart, liver, kidney, spleen, stomach, and lungs and washed with ice-cold saline. All organs were weighed. Any abnormality or lesions were checked through gross observation. 10% buffered formaldehyde solution was used to store tissues for 48 h. The tissues were then fixed in molten paraffin wax and sliced into 4-5 µm thickness. Staining was performed using hematoxylin and eosin. After histological H–E staining, the slides were observed by a veterinary pathologist and the photos were taken using an optical microscope.

Statistical analysis

All the results are expressed as Mean ± Standard error of mean (SEM). Statistical analysis was performed by one-way analysis of variance (ANOVA) with Tukey's test to evaluate significant differences between groups. A value of $p < 0.05$ was considered significant.

RESULTS AND DISCUSSION

Clinical observations

In the present study, developed hydrogel FXG3 at a dose of 5 g/kg body weight did not produce any visible sign of toxicity nor mortality (8). Table 1 showed the effect of oral intake of normal saline and FXG3 hydrogel on body weight, food and water consumption and behavior of rats in group A (control) and group B (5 g/kg), respectively.

An alteration in total body weight is a simple and sensitive index of toxicity. In our study, all of the rats gained weight due to normal physiological growth as shown in Table 1. Moreover, the difference in the body weights between control group A (control) and FXG3 hydrogel treated group B (5 g/kg) was insignificant. This suggests that the FXG3 hydrogel intake did not interfere with the normal

growth pattern of rats, indicating a lack of any impairment on their health (9-11).

Physical features such as skin, fur, eyes and physical activity in both groups were found to be normal. No signs of illness (lacrimation, salivation, convulsions, and hyperactivity) were observed. Rats were sensitive to sound, touch, light, and other stimulations and they were full of energy, had normal behavior and showed free movements. Corneal reflex, gripping strength, righting reflex were present. Animal feces were in regular form and normal color without showing any mucus or pus, indicating undamaged gastrointestinal mucosa (12).

Table 1. Total body weight, water and food intake and clinical observations of group A and group B rats.

Clinical Observation	Group A (Control)	Group B (5g/kg)
	Mean ± SEM	Mean ± SEM
Signs of illness	-	-
Body Weight (g)		
Pre-treatment	213 ± 2.2	217 ± 4.3
Day 1	213 ± 2.7	216 ± 3.3
Day 7	225 ± 3.4	233 ± 1.8
Day 14	240 ± 3.6	244 ± 2.3
Water Intake (mL/animal/day)		
Pre-treatment	44 ± 1.8	46 ± 1.6
Day 1	43 ± 2.4	37 ± 1.7
Day 7	42 ± 1.9	44 ± 1.2
Day 14	38 ± 2.5	40 ± 1.9
Food Intake (g/animal/day)		
Pre-treatment	15 ± 1.4	16 ± 1.4
Day 1	16 ± 1.9	13 ± 1.5
Day 7	18 ± 1.5	15 ± 1.3
Day 14	19 ± 1.7	18 ± 1.8
Clinical sign		
Dermal irritation	-	-
Other observations		
Ocular Irritation	-	-
Lacrimation	-	-
Salivation	-	-
Convulsions	-	-
Hyperactivity	-	-
Touch response	+	+
Corneal reflex	+	+
Righting reflex	+	+
Gripping strength	+	+
Alertness	+	+
Mortality	-	-

Values are expressed as Mean ± SEM of 5 rats in each group. Group A-Control, Group B-FXG3 hydrogel at a dose of 5g/kg bodyweight. - sign indicates a lack of specified observation. + sign indicates the presence of specified observation.

Table 2. Hematological and biochemical blood analysis of group A and group B rats.

Biochemical blood analysis			
Hematology parameters	Unit	Group A (Control)	Group B (5 g/kg)
		Mean ± SEM	Mean ± SEM
Haemoglobin	g/dL	14.5 ± 0.89	15.3 ± 0.82
Haematocrit	%	44.5 ± 2.02	42.3 ± 3.16
White Blood Cells	103 / µL	4.2 ± 0.63	4.1 ± 0.54
Red Blood Cells	106 / µL	8.82 ± 1.04	8.15 ± 0.974
Platelets	103 / µL	884 ± 16.67	889 ± 15.30
Monocytes	%	2.3 ± 0.34	2.5 ± 0.29
Neutrophils	%	25 ± 1.23	27 ± 1.18
Lymphocytes	%	78 ± 3.06	76 ± 2.99
Mean Corpuscular Volume (MCV)	fL(µm3)	54.5 ± 2.18	54 ± 2.11
Mean Corpuscular Haemoglobin (MCH)	pg	18.2 ± 1.04	17.2 ± 0.97
Mean Corpuscular Haemoglobin Concentration (MCHC)	g/dL	35.3 ± 1.48	34.8 ± 1.41
Serum Biochemistry Parameters			
Triglyceride	mg/dL	78 ± 2.622	73 ± 2.57
Cholesterol	mg/dL	60 ± 1.46	56 ± 1.43
Glucose	mg/dL	108 ± 3.73	111 ± 3.72
Creatinine	mg/dL	0.59 ± 0.034	0.67 ± 0.028
Urea	mg/dL	20 ± 0.58	17 ± 0.59
Alkaline Phosphatase(ALP)	U/L	117 ± 3.48	114 ± 3.45
Aspartate Transaminase (AST)	U/L	109 ± 3.04	103 ± 3.018
Alanine Aminotransferase (ALT)	U/L	28 ± 1.28	25 ± 1.00
Creatinine Kinase(CK)	U/L	620 ± 0.58	617 ± 0.59

Values are expressed as Mean ± SEM of 5 rats in each group. Group A-Control, Group B-FXG3 hydrogel at a dose of 5g/kg bodyweight.

Temporary nausea was observed in the first 4 h after administration of FXG3 hydrogel in group B (5 g/kg) which was absent in group A (control). However, this nausea settled itself without any treatment and might be attributed due to a reflux action and swelling behavior of hydrogels rather than any toxic effects. Moreover, no diarrhea, loss of fluids and shedding of the epithelial cell were observed in feces in any group indicating a lack of toxicity.

Food intake and water consumption were found to be normal. A slight decrease in food and water intake was observed at day 1 in group B (5 g/kg) which might be due to swelling of FXG3 hydrogel producing a feeling of fullness in the stomach. However, this decrease in food intake was insignificant when compared with control group A (control). Food and water intake on day 7^{th} and day 14^{th} in both groups were found to be in the normal range, indicating a lack of any abnormality.

All these observations depicted that oral administration of the FXG3 hydrogels has not produced any harmful effect on the growth, physical characteristics and behavior of the rats in group B (5 g/kg) similar to control group A (control) (13-14). According to globally harmonized system (GHS), if tested chemical has LD50 value higher than the 2000 mg/kg, it is categorized under “category 5”. According to the CLP Regulation (EC No 1272/2008), this compound is not classified as a toxic (15).

Hematological and biochemical blood analysis

Blood is an important index of pathological status in both humans and animals and the parameters usually measured are hemoglobin, hematocrit, white blood cell count, red blood cells, platelets count, etc. The normal ranges of these parameters are usually altered by the ingestion of toxic or poisonous substances. In our study, all hematology and

Table 3. Relative organ weight of group A and group B rats.

Groups	Heart	Liver	Lung	Kidney	Stomach	Spleen
Group A (Control)	0.37 ± 0.020	3.62 ± 0.24	0.78 ± 0.033	0.87 ± 0.044	1.38 ± 0.19	0.46 ± 0.018
Group B (5g/kg)	0.41 ± 0.016	3.11 ± 0.19	0.72 ± 0.029	0.84 ± 0.048	1.79 ± 0.13	0.52 ± 0.015

Values are expressed as Mean ± SEM of 5 rats in each group. Group A-Control, Group B-FXG3 hydrogel at a dose of 5 g/kg bodyweigh.

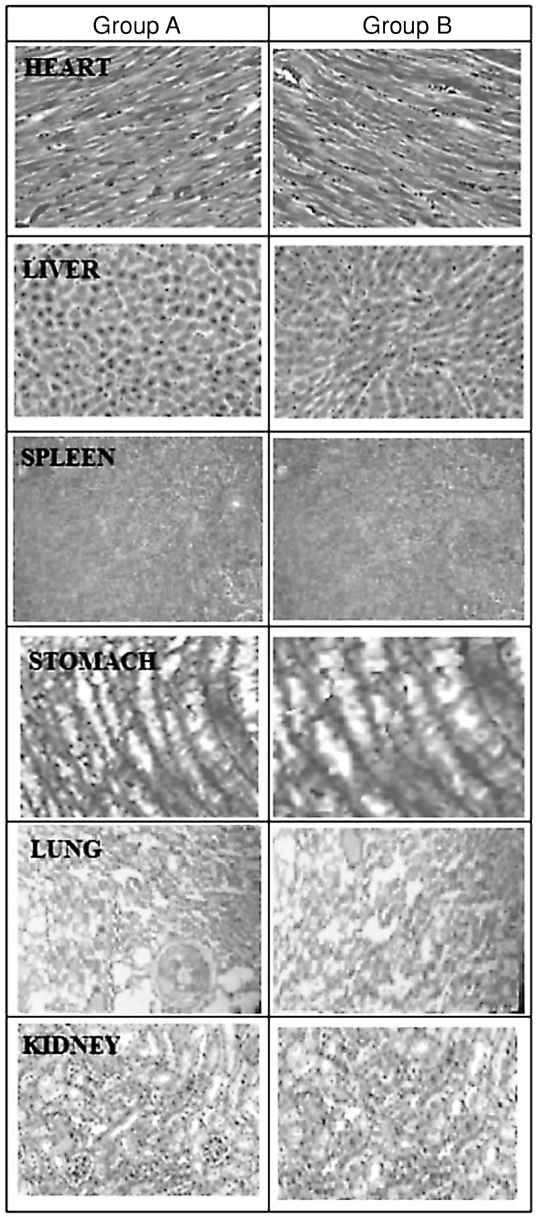

Figure 1. Histopathological observations of tissues from selective organs of group A (control) and group B (5 g/kg) rats.

biochemical blood parameters of group B (5g/kg) did not show any significant alterations from group A (control) as shown in Table 2, indicating that the FXG3 hydrogel is likely to be non-toxic (16).

Liver function was evaluated with serum levels of alkaline phosphatase (ALP), Alanine Aminotransferase (ALT) and Aspartate Transaminase (AST). Nephrotoxicity was determined by urea and creatinine. The enzymes creatinine kinase (CK) was assayed for evaluating cardiac damage (17). Triglycerides, cholesterol and glucose were also evaluated. It has been observed that the administration of FXG3 hydrogel did not alter any of the above biochemical parameters in FXG3 hydrogel treated group B (5 g/kg) from control group A (control). Although creatinine level was slightly elevated, this elevation was insignificant from the normal value. The increase in the serum level of creatinine might be a reflection of an increased catabolic state in the rats but a lack of any toxicity (18). The normal levels of blood urea and insignificant elevation of serum creatinine indicate that the FXG3 hydrogel did not interfere with renal function and that renal integrity was preserved (19).

As a matter of fact, toxic or poisonous substances usually accumulate in the liver. Hence, any significant increase in the level of liver enzymes in the blood serum is an indication of marked necrosis of the liver cells due to toxicity (20-21). In our study, the liver enzymes indicated a lack of any alterations in their level. The lack of this effect on the liver enzymes showed that the FXG3 hydrogel was non-toxic on the hepatocytes (22-23). Similarly, Creatinine Kinase (CK) level was also in the normal range, demonstrating FXG3 hydrogel was safe on the cardiac muscles. Thus, biochemical blood analysis represents insignificant alterations of all calculated parameters, indicating excellent non-toxic nature of xanthan gum-based FXG3 hydrogel after a single oral dose of 5g/kg body weight (24).

Macroscopic observations and organ weights

Gross examination of internal organs of group B (5 g/kg) rats revealed no detectable abnormalities. Moreover, the relative weight of the heart, kidneys, liver, lung, stomach, and spleen of the group B (5 g/kg) rats treated with FXG3 hydrogel was similar to the control group A (control) as shown in Table 3. These results suggest that the administration of FXG3 hydrogel did not produce any detectable abnormality on internal organs. Moreover, relative organ weights of both groups are in normal range of healthy animals (25). Thus, it can be concluded that developed FXG3 hydrogel is non-toxic, safe and biocompatible.

Histopathological study

Microscopic examination of samples obtained from control group A (control) and FXG3 hydrogel treated group B (5 g/kg) revealed the absence of any pathological lesions on vital organs (heart, liver, kidney, stomach, lung, and spleen). Figure 1 exhibited the comparison between the optical microscopic images of tissues of selective organs obtained from control group A (control) and FXG3 hydrogel treated group B (5 g/kg).

Cardiac myocytes were displayed orderly without showing any inflammatory exudate, necrosis, or hemorrhage in both control group A (control) and FXG3 treated group B (5 g/kg), indicating lack of any toxic effect (26).

Optical liver micrography in group B (5 g/kg) animals treated with FXG3 hydrogel indicated no obvious degeneration and necrosis similar to control group A (control). The dividing lines of liver lobules were clear. No hypertrophy was observed on hepatic sinusoid. No neutrophil, lymphocyte, or macrophage infiltration was found, indicating a lack of any toxicity (27).

Spleen sinus was absolutely normal, without showing any pathologic changes in both control group A (control) and FXG3 hydrogel treated group B (5 g/kg). The mucosa of the stomach was normal in both groups A (control) and B (5 g/kg), devoid of any pathological changes. Moreover, gastric glands were in a regular arrangement and mucosa cells were clear. Additionally, the tissue structure of lung of FXG3 hydrogel treated group B and control group A (control) were almost similar and no inflammatory cell infiltration surrounding the bronchus was identified, representing normal physiology as obvious in Figure 1. The optical micrograph of kidneys of group B (5 g/kg) rats treated with FXG3 hydrogel was in normal shape similar to group A (control) rats. No degeneration, bleeding, necrosis was showed within renal glomerulus and various kidney tubes, thus representing a lack of any toxicity (28-29). All findings observed were consistent in both control group A (control) and FXG3 hydrogel treated group B (5 g/kg). These results suggest that the administration of FXG3 hydrogel at dose levels up to 5g/kg body weight to rats did not produce any macroscopic or microscopic alterations on internal organs and are well tolerated for the 14th-day study period. Therefore, developed FXG3 hydrogel formulation is well tolerated and has the potential for safe use as a drug delivery system (30).

CONCLUSION

Acute administration of xanthan gum-based FXG3 hydrogel as a new drug delivery system to adult female rats of Wistar strain produces no significant changes in the behavior pattern of animals and no adverse effects on hematological and biochemical parameters. The level of the marker enzymes in the vital organs was also found to be normal. Histopathological studies also showed the appearance of the normal architecture of the vital organs of the FXG3 hydrogel treated rats similar to the control group. The dose administered 5 g/kg body weight is well tolerated. The results of acute oral toxicity evaluation showed that the FXG3 hydrogel can be used as a potential candidate for application in the biomedical field, especially in the controlled drug delivery system.

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Declaration of interest statement

The author reports no conflict of interest.

Animal studies

All institutional and national guidelines for the care and use of laboratory animals were followed.

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