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Acute and Subacute Toxicity of Sorbitan Monostearate (Span 60) Non-ionic Surfactant Vesicles (Niosomes) in Sprague Dawley Rats

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Authors' contributions

All authors contributed significantly to the study. Author JS developed the planning protocols and executed the studies under the mentorship of authors PPL, ASA and PG. Author JJ was the veterinarian who monitored the animals and performed the histological examination of tissues and managed animal post mortems. Author ASA handled the technical and editorial aspects of the study. All authors read and approved the manuscript.

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ABSTRACT

This study was designed to determine the acute and subacute toxicity of non-ionic surfactant, sorbitan monostearate (Span 60) vesicles in a Sprague Dawley rat model. The primary aim was to investigate the acute toxicity of Span 60 niosomes after single intraperitoneal (IP) as well as once daily bolus dose for 5 days. Niosomes were prepared by the thin-film hydration method and

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subjected to ultracentrifugation to produce a final concentration of 30 mg of span 60 in 1 ml of niosome suspension. Acute toxicity study was performed following OECD test guideline 423 with modifications. Animals were divided into four groups, two control groups and two treatment groups. Group 1 animals were administered single 600 mg/kg IP bolus dose, whilst group 2 animals received 120 mg/kg/day IP for 5 days. The controls for each treatment group was administered an equivalent volume of phosphate buffered saline (PBS). Their body weight, food intake, water intake, fecal mass and urine output were measured daily. All clinical signs, time of onset, duration, and reversibility of toxicity or mortality were documented. Gross necropsies were performed on all animals terminated at 14 days post injection. There was no treatment related deaths and no toxic signs were observed in the two treatment groups. There was an initial decrease in food intake and, hence, body weight with IP niosome injection. However, weight loss was less than 10 percent in both groups of animals. All other parameters measured showed no statistical significance between niosome treated group and placebo. Necropsy performed at day 14 showed no signs of local reaction and there was no discernible effect on major organs. Results indicated that the LD_{50} of IP injected Span 60 was greater than 600 mg/kg. Therefore, Span 60-based niosomes appear to be non-toxic at the tested doses and experimental conditions and may not contribute to the potential toxicity of drug-loaded niosomes of this surfactant.

Keywords: Acute; sub-acute toxicity; span 60 niosomes; Sprague Dawley rat.

1. INTRODUCTION

Non-ionic surfactants vesicles (niosomes) are microscopic lamellar structures formed on admixture of non-ionic surfactant and cholesterol with subsequent hydration in an aqueous media [1]. These drug carriers have the ability to encapsulate both hydrophobic and hydrophilic drugs. Although numerous studies have been published regarding administration of niosome-encapsulated drugs [2-4], there is limited data on the safety of surfactants administered intraperitonealy. Although Span 60 is known to be safe for use in many non-parenteral preparations, its application in formulations intended for parenteral administration requires the establishment of its safety and toxicity via the route in an animal model.

Acute toxicity is the toxicity produced by a substance when it is administered in one or more doses during a period not exceeding 24 hours while subacute toxicity is the toxic effect occurring as a result of exposure to repeated daily dosing of a drug substance or exposure to a chemical [5]. Usually, the test compound is administered to animals to identify the maximum tolerable dose causing no adverse effect, and the minimum dose causing major (life-threatening) toxicity using a control group to confirm the absence of treatment-dependent outcomes.

Acute toxicity studies in animals falls under the pre-clinical phase of new drug development process and provides useful information for the determination of doses that can be used for multiple-dose studies, for preliminary identification of target organs for toxicity and for identifying delayed toxicity. Acute toxicity studies may also aid in the selection of start doses for Phase 1 human studies, and provide information relevant to acute overdosing in humans. This study was designed to determine the acute and subacute toxicity of non-ionic surfactant, Sorbian monostearate (Span 60) vesicles, in a Sprague Dawley rat model. The specific aims are to investigate acute toxicity of single IP bolus dose and subacute toxicity of IP once daily dose for 5 days.

2. MATERIALS

All materials for niosome preparation - span 60, cholesterol and dicetylphosphate - were purchased from Sigma Chemicals, Canada. All other reagents including methanol and chloroform were of analytical grade from Acros Organics, USA. Female Sprague Dawley, aged 8-12 weeks and weighing 200-250 g were obtained from the Animal House, School of Veterinary Medicine (SVM), University of the West Indies (UWI), Trinidad. They were weighed and marked to permit individual identification, then transported to the animal room at the SVM.

3. METHODS

Animals were allowed to acclimatize to the local conditions of the room for seven days before any experimental work was conducted. During the acclimatization period, animals were handled daily by the same animal care facility personnel

who trained them to enter into a manual restrainer.

3.1 Housing and Feeding Conditions

The ambient temperature was maintained in the experimental animal room. Lighting was set to the sequence of 12 hours light, 12 hours dark. Animals were provided with conventional laboratory diets and drinking water *ad libitum*. Animals were individually housed in metabolic cages so their food and water consumption as well as urine and fecal output could be measured.

3.2 Preparation of Niosomes

Niosomes were prepared using surfactant (Span 60), cholesterol and dicetylphosphate in a molar ratio of 7.5:7.5:1 and by the thin film hydration method [6]. All procedures for niosomes preparation followed strict aseptic technique principles. Before administration, 100 µl of niosome was streaked onto blood agar plates and incubated for 18-24 hours at 37℃. Plates were observed for bacterial growth (to check contamination). Once no growth was observed, the sample was used for intraperitoneal injection.

3.3 Preparation of Animals

Animals were weighed daily during the acclimatization period, and after this initial seven-day period, animals were categorized into two groups with each comprising three animals (n=3). Animals were placed in a manual restrainer and injected intraperitoneally (IP) with a single dose of niosomes of 600 mg/kg per dose (5 ml), the other group (control) was injected with 5 ml of phosphate buffered saline (PBS, pH 7.4) (stage 1 trial). At the end of the stage 1 trial, a new group of animals was used for stage 2 trials. This involved IP injections of niosomes at 120 mg/kg per dose (2 ml) once daily for 5 days. Like stage one trials, a control group was also utilized; these were injected with 2 ml PBS. Stage 2 tests were designed to simulate a typical treatment scenario where the drug encapsulated in niosomes would be administered once daily for 5 days.

After niosomes injection, each animal was carefully returned to its metabolic cage and observed for 14 days. Animals' body weight, food intake, water intake, fecal mass and urine output were measured daily. All mortalities, clinical signs, time of onset, duration, and reversibility of toxicity were recorded. Gross necropsies were conducted on all animals at the end of the 14-day observation.

3.4 Study Approval

The study protocol was approved by the Department of Graduate Studies, University of the West Indies, St Augustine. The application for animal research was approved by the Animal Ethics Committee, Faculty of Medical Sciences, UWI.

3.5 Statistical Analysis

Minitab 16 statistical package was utilized for data treatment and analysis. Significance of any difference between test and control animals was determined using the student t-test for independent samples at 95% confidence level ($p < 0.05$).

4. RESULTS AND DISCUSSION

The dose of niosome used was based on the surfactant content of Span 60. The Organization for Economic Cooperation and Development (OECD) guidelines [7] recommends doses of 5, 50, 300 and 600 mg/kg/dose for acute toxicity studies of small molecule drug substances [8,9]. However, significant increase in viscosity of the niosome preparations required to deliver the high doses made manipulation of the injection syringe with a 23-gauge needle and hence, application of such doses, impractical. Preliminary screening indicated that a maximum concentration of 30 mg/ml of span 60 in niosomes has acceptable viscosity. In stage 1 studies, 5 mL injection volume was given as a single bolus dose while in stage 2 studies, 2 mL injection volume was administered once daily for 5 days. These dosing volumes appeared to be the maximum tolerable volumes for the age and weight of the animals used and were therefore applied to the rest of the studies. Volumes greater than 2 mL could dilute the animals' body fluid significantly to cause acid and electrolyte imbalance which would be lethal to the animals.

In Stage 1 of this study, the control group (PBS treated) showed no abnormal gross findings. In the second group, which was given the single bolus dose of niosomes, two of the three animals showed similar results as the control, in which no gross changes were observed. The third animal showed a very small white patchy scaring (2 mm X 2 mm, Fig. 1) on the right lobe of the liver

parenchyma. There were neither attachments nor fibrin tags noticed. The white patch observed could be a deposition of niosomes onto the liver, which was not completely absorbed. It also could be breakdown of niosomes into its components of surfactant and cholesterol followed by deposition of the surfactant onto the liver. However, histology of liver sections, examined at 100X magnification, showed no abnormality (Fig. 1). There was also no noticeable difference in animal behavior.

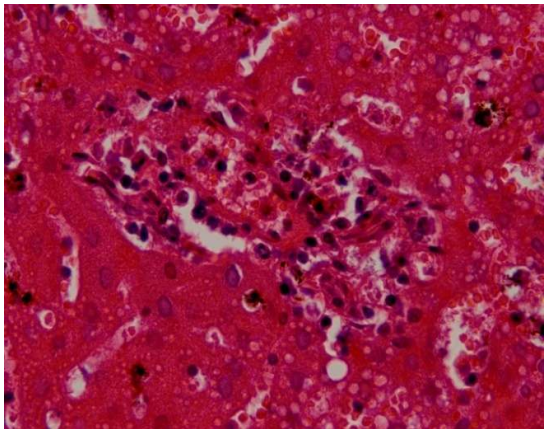

Fig. 1. Histology of liver portion with white deposition after IP administration of niosomes showing normal histology for rats aged 8-12 weeks (X100)

Stage 2 studies showed no signs of toxicity. The liver was neither swollen nor enlarged, and it did not show signs of rounded edges. Both the control and treated groups showed no gross changes. Unlike the group 2 animals, where one animal showed deposition, all animals in the niosome group showed no white patchy deposition and the livers appeared normal as in the control group. There was no enlargement of the spleen and no discoloration of the kidneys. There was also no fibrin tags or attachment of tissue suggesting no local reaction to the niosome injection. Presence of fibrin tags usually indicate introduction of a toxic compound into the peritoneum with the resulting injury to the organs present. Deposition of toxic substance into the peritoneum usually induces release of fibrin and development of fibrin tags between organs. Absence of fibrin tags is an indication that the test substance was indeed non-toxic. Moreover, injections of the niosomes did not produce any sign of local irritation at the injection sites.

The volumes chosen for IP administration were carefully selected based on animal welfare. The study protocol dictated that injection volumes must be within the guidelines which are safe for IP administration in the test subjects. Specific reference was made to rat species at 8-12 weeks old and weighing 200-250 g. For stage 1 studies, a 5 mL maximum volume of injection was administered as single bolus dose and it has been shown that this volume represented the maximum tolerable dose for the test animals [10]. Stage 2 studies involved IP injections over a five-day period. A total of 10 ml was injected into the animals over a 5-day period. This volume represented the maximum weekly volume that could be tolerated by the test animals [11]

During the initial treatment period, there was a decline in weight of both the niosome and control group. This may be due to an added stressor on the animal to which they responded by consuming less food, and as a result, experienced decrease in weight. The decrease appeared to be slightly sharper in the niosome treated group than in the control. This may be due to the content of the niosomes as they are absorbed after IP administration, primarily via the portal circulation before reaching systemic circulation and other organs [12]. Drug administered IP diffuses across the peritoneal membrane into the systemic circulation where it can be eliminated, or distributed to the extravascular space where it may be eliminated by non-renal mechanisms [13]. The blood vessels supplying and draining the abdominal viscera, musculature and mesentery, constitute a blood-filled compartment into which compounds can diffuse from the peritoneum [14]. Therefore, intraperitoneally administered niosomes are cleared via metabolism in plasma or non-renal mechanism. As previously reported by Jankie et al. [6] the sizes of the niosome particles were between 8 – 15 µm in diameter, were spherical in shape and the niosomes were also highly polydispersed. The particle size and shape qualifies them as potential candidates for removal from circulation via endocytosis by macrophages of the reticuloendothelial system [15,16].

When niosomes were injected IP, the absorption and elimination of the test compound was probably prolonged when compared to the PBS treated animals. As a result, there could have been reduced food consumption, which ultimately led to a slightly greater reduction in weight. It should be noted however that the patterns of weight variation in both groups were almost identical and percentage weight loss in

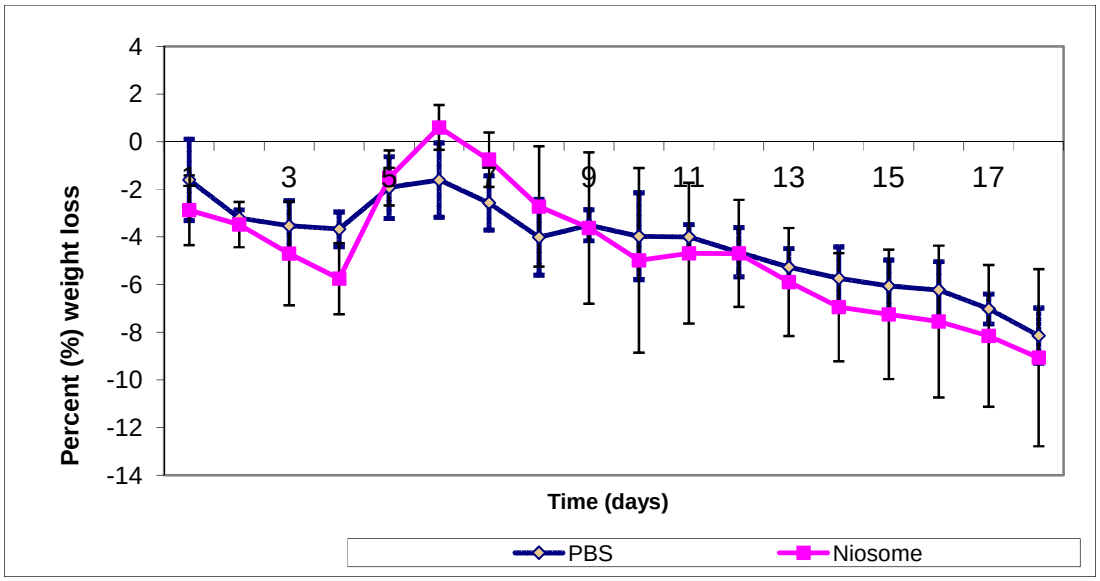

Fig. 2. Effect of daily injections of niosome (600 mg/kg) vs phosphate buffered saline on day 4 on weight loss in rats (p = 0.516)

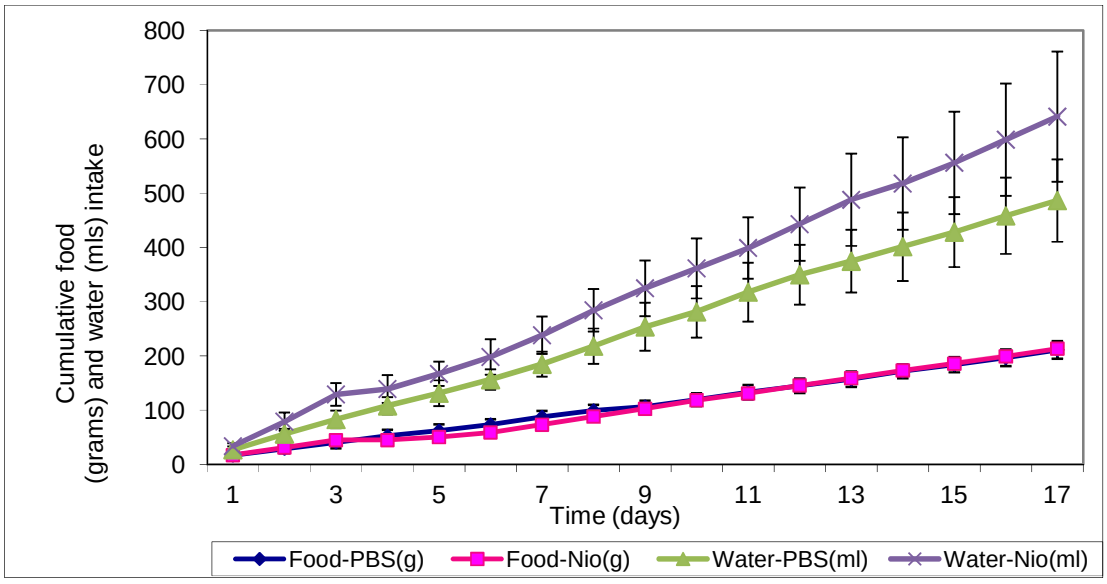

Fig. 3. Comparative effects of daily intraperitoneal injection of phosphate buffered saline or niosome (600 mg/kg/day bolus dose) on day 4 on food (p=0.893) and water intake(p=0.207)

both groups were not statistically significant. Fig. 2 (p = 0.516) & Fig. 5 (p = 0.278). The OECD guideline states that weight loss of greater than 10% is a marker for toxicity in an animal model [9]. Weight loss in an animal of such magnitude indicates the negative effects of the test compound and suggests euthanasia of the animal in light of good animal welfare protocol. In this study, the percentage weight loss was significantly less than the 10% limit set by the guidelines. Food intake and weight gain were actually similar in both treatment and control groups indicating non-toxicity of the niosomes.

The effect on food and water intake was also similar in treatment and control groups in stages 1 and 2 (Figs. 3 and 6). After the single bolus dose, there was a reduction in food intake for all niosome treated rats. Injection with PBS did not affect food consumption significantly.

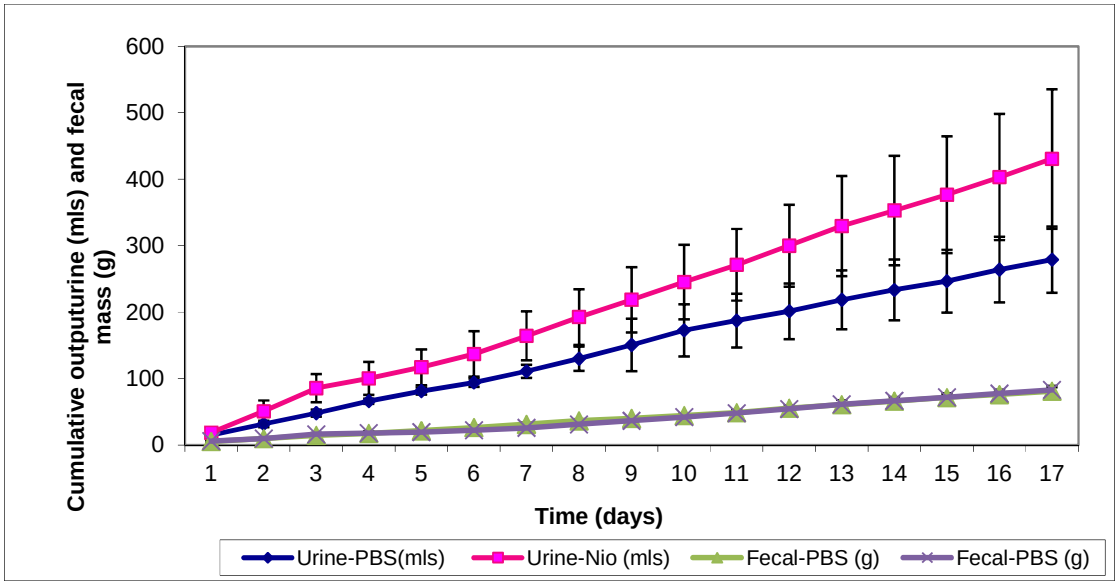

Fig. 4. Comparative effects of daily intraperitoneal injection of phosphate buffered saline or niosome (600mg/kg/day bolus dose) on day 4 on fecal mass (p=0.902) and urine output (0.058) by rats

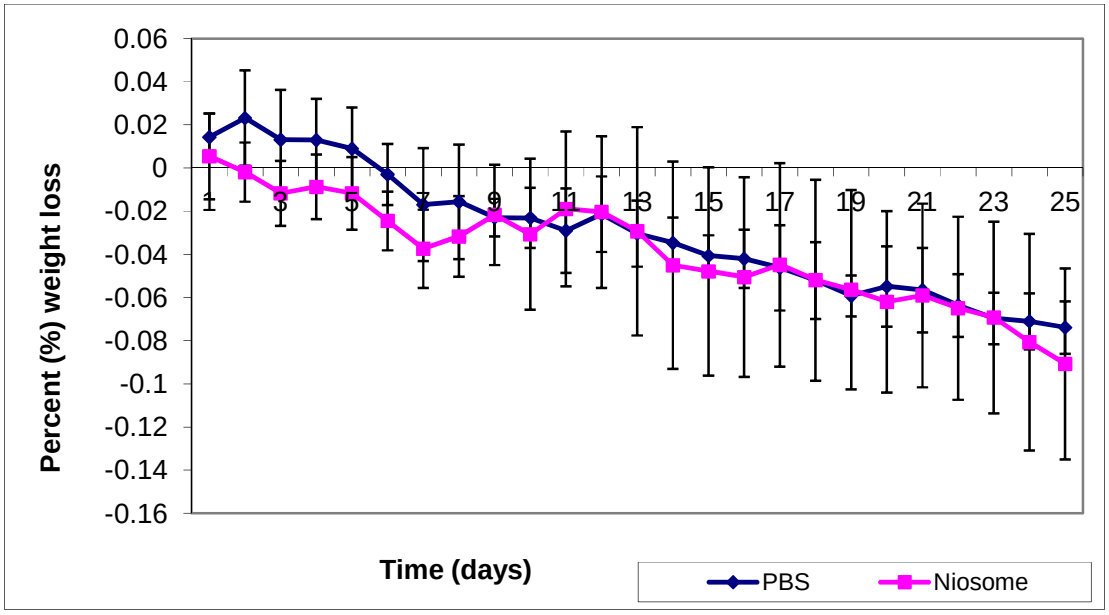

Fig. 5. Effect of daily injections of niosome (120 mg/kg/day on days 7-11) vs. phosphate buffered saline on weight loss in rats (p=0.278)

However, the niosome treated group resumed their normal eating pattern within 3 days of bolus dose (stage 1) and after a slight decrease on the day after injection, the animals appeared to resume their normal consumption of food (stage 2). However, the effect of IP injection on water consumption was slightly different in both stages. In stage 1, with the single bolus dose of niosome, there was an initial decrease in water intake for day 1 but the pattern returned to normal on day 2 post injections. In stage 2, there was no decrease, and the 5-day treatment appeared to have no effect on water consumption compared with control.

There was also a similar effect on urine and fecal output in both phases (Figs. 4 and 7) In stage 1, there was an initial decrease in fecal output which paralleled the decrease in food consumption, but this returned to that of placebo four day post injection. In stage 2, a similar pattern was observed: there was a slight decrease after injection on day 1, but the pattern returned to that of placebo four days after the last injection on day 5.

The stage 1 study, there was no significant difference in urine output of both noisome-treated and placebo treated groups of rats (p = 0.058). Similarly, there was no observable difference between the treatment group and placebo in stage 2.

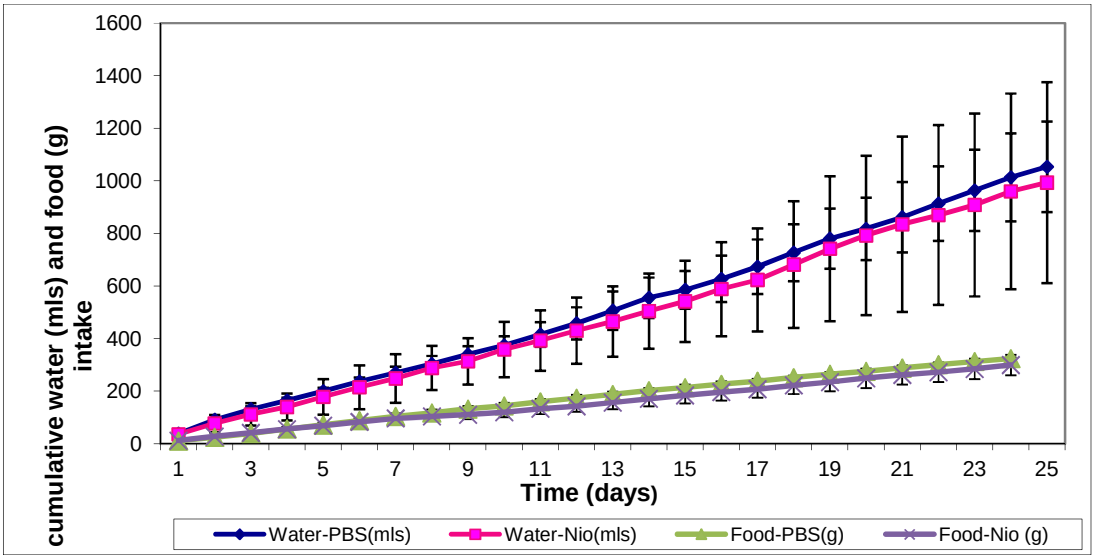

Fig. 6. Comparative effects of daily intraperitoneal injection of phosphate buffered saline or niosome (120 mg/kg/day on days 7-11) on food (p=0.456) and water intake (p=0.708)

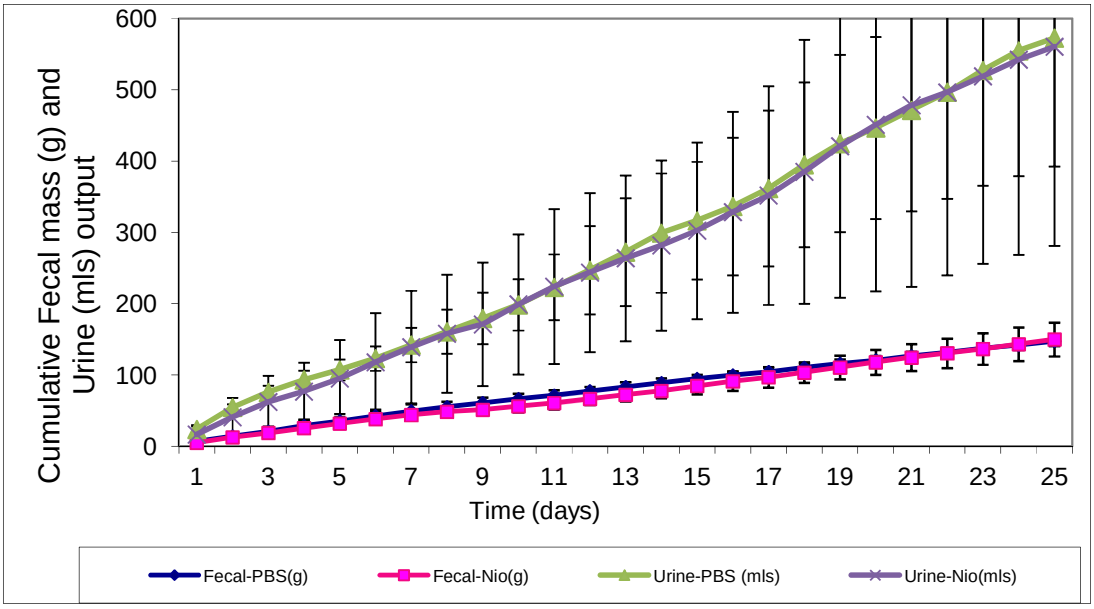

Fig. 7. Comparative effects of daily intraperitoneal injection of phosphate buffered saline or niosome (120 mg/kg/day on days 7-11) on urine (p=0.882) and fecal mass (p=0.663) output

5. CONCLUSION

The acute and sub-acute toxicity studies were done to determine the safety of the drug-free, plain niosomes administered via the intraperitoneal route in Sprague Dawley rats. Results of the acute toxicity study showed that a bolus dose of sorbitan monosterate injected intraperitoneally was well tolerated by the animals. With the oral safety of sorbitan monosterate already established, the intraperitoneal administration of the compound produces similar effects indicating that its effect on body systems were negligible. The effects of the test compound on the treatment group versus the control group were all non-significant indicating lack of toxicity of span 60 niosomes. Similarly, results of subacute toxicity study was about the same for both the control and the treatment groups indicating safety of Span 60-based niosomes for use as drug delivery system via the intraperitoneal route.

CONSENT

It is not applicable.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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