ORIGINAL ARTICLE

Acute Toxicity Study of Intravenous Administration of Thymoquinone-Loaded Nanostructured Lipid Carrier (TQ-NLC) in *Sprague Dawley* Rats

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ABSTRACT

Introduction: Thymoquinone (TQ), a bioactive compound from *Nigella sativa* is known for its various medicinal properties. Due to the low solubility of TQ, nanostructured lipid carrier (NLC) has been used as a delivery system to improve its efficacy. Nevertheless, the effect of TQ-NLC when administered intravenously is unclear. This study investigated the acute toxicity profile of intravenous administration of TQ-NLC in an in vivo model. **Methods:** Twelve female *Sprague dawley* rats were assigned randomly into two groups (n=6); a control and a treatment group that received normal saline and 25 mg/kg TQ-NLC, respectively, via intravenous injection. The rats were observed for 14 days for any alterations to their usual physical conditions such as behaviour and mortality, body weight, food intake, organ-to-body weight ratio, and haematological, biochemical and histopathological profile. **Results:** There were no significant changes ($p>0.05$) in the body weight, food intake, organ-to-body weight ratio, and haematological, biochemical and histopathological profile between TQ-NLC treatment and the control group. However, inflammation was observed at the site of injection on the rat's tail. **Conclusion:** Intravenous administration of TQ-NLC (25 mg/kg) did not exert acute toxic effect in female *Sprague dawley* rats. The data can be used as a basis to further develop TQ-NLC as a potential therapeutic drug.

Keywords: Thymoquinone, Intravenous administration, Nanostructured lipid carrier, Acute toxicity

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INTRODUCTION

Nigella sativa (*N. sativa*) (Linn.) of the Ranunculaceae family is a medicinal plant commonly known as black cumin. The seeds of this plant and its oil are widely used as home remedy to treat various ailments in regions like the Middle East, India, Bangladesh, Pakistan, Mediterranean and also Europe for centuries. Asthma, inflammation, cough, bronchitis, diabetes, fever, dizziness, eczema, influenza and hypertension are among the diseases treated with black cumin seeds (1). A key bioactive component of *N. sativa* seed volatile oil is 2-isopropyl-5-methyl-benzoquinone, thymoquinone (TQ) (2).

TQ has been widely reported for its vast pharmacological effects in various disease models both in vitro and in vivo. TQ has potent anticancer properties in glioblastoma cells (3), lung cancer cells (4), neuroblastoma cells (5) and myeloblastic leukemia (6). Its anti-inflammatory (7), hepatoprotective (8), antioxidant and antihistaminic (9) activities has also been documented.

TQ has limited bioavailability and solubility due to its hydrophobic properties that eventually decreases its effectiveness in clinical use (10). Application of nanotechnology is an alternative to overcome this problem. Nanocarriers have been used to help the drug to be more soluble in aqueous solution, increasing their bioavailability, and enhancing the drug's half-life in serum (11). Nanoparticle delivery system is usually carried out when an intended drug is dissolved, entrapped, absorbed, attached and/or encapsulated onto or into nano-matrix (12). Lipid nanoparticles have been identified to provide several advantages due to its bioacceptable and biodegradable nature, leading it to have lower toxicity compared to other nanocarriers (13).

TQ has been encapsulated with nanostructured lipid carrier (NLC), herein referred as TQ-NLC, which increased its efficacy and solubility, making injection

of this compound possible (14). Lipid carrier is a drug vehicle that helps in improving the solubility of active compounds and controlling drug release (12). TQ-NLC exhibited anti-proliferative activity towards cervical cancer cell lines (HeLa and SiHa) and breast cancer cell lines (MCF-7 and MDA-MB-231) (14). In order to further establish TQ-NLC as a potential drug, toxicological evaluation is essential to determine the highest exposure of a particular drug that shows no toxicity resulting in the expected efficacy (15).

We have recently reported on the acute and subacute toxicity profiles of oral administration of TQ-NLC (16). Nevertheless, the effect of TQ-NLC when administered intravenously remains unclear. Toxicological profile upon intravenous injection is important as the drug will directly go into the blood system for systemic action (12) bypassing the first-pass metabolism and absorption phase. The effect of intravenous administration is different to oral administration as the drug is vulnerable to the rigorous activity of digestive juices and has to bypass the physiological barriers in the gastric and intestinal environment that includes the mucosal barrier, enterocytes of the intestinal epithelium, tight junctions between epithelial cells and subepithelial tissues (17). Intravenous route of administration is a more reliable route as it is quicker and efficient in exhibiting immediate therapeutic effects because the drugs could also directly reach target organs. Drugs that have short half-life or duration of action is more suitable to be given via this route as it could be delivered in a more uniform rate, thus reaching a bioavailability of 100%. Furthermore, intravenous drug administration is a better option for patients who are uncooperative as they are unconscious or likely to vomit due to the unpleasant taste of the oral drugs. This study investigated the acute toxicity profile of intravenous administration of TQ-NLC in *Sprague dawley* rats.

MATERIALS AND METHODS

Synthesis of TQ-NLC

TQ-NLC was synthesised according to the protocol outlined by Ng et al. (14) by a high-pressure homogenisation process. First, two matrices consisting the lipid and aqueous matrix were prepared. The lipid matrix was prepared by mixing hydrogen palm oil, lecithin and olive oil and melted at 70°C. Aqueous matrix was prepared by mixing sorbitol, polysorbate 80 and thimerosal in deionised water and heated at 70°C. Next, TQ was added to the lipid matrix before dispersion with aqueous matrix. Subsequently, the pre-emulsion was obtained by high-speed stirring by Ultra-Turrax (IKA-werke, GmBH, Germany) for 10 minutes at 13 000 rpm at 70°C. The pre-emulsion was homogenised at 1000 bars for 20 cycles by using a high-speed homogeniser EmulsiFlex-C50 (Avestin, Mannheim, Germany). Immediately, the emulsion obtained was let to crystallise at room temperature to form TQ-NLC (18).

Animal

Female *Sprague dawley* rats with initial body weight ranging from 180 to 220 g and eight weeks of age were used in this experiment. Prior to any procedures, the rats were acclimatised for a minimum of one week. Animals were housed two in a cage under room temperature of 29-32°C and humidity of 70-80% with 12 hours light-dark cycles. The rats had access to standard chow (Specialty Feeds, Glen Forrest, WA, Australia) and tap water ad libitum. The experiments conformed to ethical guidelines and were carried out with the approval of Institutional Animal Care and Use Committee (IACUC) of Universiti Putra Malaysia (Reference number: UPM/IACUC/AUP-R074/2015).

Experimental Design

TQ-NLC at a dose of 25 mg/kg of was administered via intravenous (i.v) injection into the rat's tail, after 8 hours of fasting. Then, at an interval of 48 hours, the same dose was employed to four other rats. In parallel, five rats were administered with normal saline as control group. The animals were observed closely during the first 24 hours and once daily for 14 days (19).

Hippocratic screening (20) was carried out on the rats where the analysed parameters include:
i) conscious state (general activity)
ii) activity and coordination of motor system and muscle toning (response to tail touch and grip, straightening and strength to grab)
iii) reflexes (corneal and headset)
iv) activities on the central nervous system (tremors, convulsions, straub, sedation, anaesthesia and ataxia)
v) activities on the autonomic nervous system (lacrimation, cyanosis, ptosis, salivation and piloerection)

All rats' feed and water consumption, and body weights were recorded on a daily basis (19).

On day 14, all the rats were fasted for one day before they were anaesthetised with ketamine (25 mg/kg; ip) and xylazine (10 mg/kg; ip). Approximately 1 mL of fresh blood was allocated for haematological analysis. The remaining blood were placed into plain tubes and centrifuged at 12 000 rpm (Hettich EBA21, Germany) for 10 minutes to obtain serum for biochemical analysis.

The liver, spleen, lung, heart, and kidneys were dissected out and weighed. The organs were placed onto square grid lines for observation of changes in the gross morphology. Each rat's organ weight-to body weight ratio was calculated. The organs were placed in 10% formalin fixative.

Hematological and Biochemical Analysis

Haematological parameters that include red blood cell (RBC), haemoglobin concentration, haematocrit (packed cell volume, PCV), mean corpuscular volume (MCV), white blood cells (WBC) and platelet counts

(thrombocytes) were analysed using an automated hematology analyser (Sysmex-XT-1800, Norderstedt, Germany).

A chemical analyser (Selectra-XL, Huizen, Netherlands) was used to perform the biochemical analysis. The hepatic function was determined by the level of serum alkaline phosphatase (ALP), aspartate aminotransferase (AST), alanine aminotransferase (ALT), total protein, albumin and total bilirubin. The renal function was evaluated based on the level of blood urea nitrogen and serum creatinine.

Histopathological Analysis

The organs that were fixed in 10% formalin for least 24 hours were processed (Leica TP 1020; Leica Biosystems Nussloch GmbH, Buffalo Grove, IL, USA) and embedded in paraffin wax. Tissues were sectioned at 5 µm thickness, mounted onto glass slides and stained with haematoxylin and eosin. Finally, a total of 10 fields from each slide were observed at 40X magnification under a light microscope (Leica, Wetzlar, Germany) for any histological changes.

Statistical Analysis

All data are expressed as mean ± standard error of mean (S.E.M.) values. GraphPad Prism version 5 was used for statistical evaluation of the data. Statistical significance was determined by two way Analysis of Variance (ANOVA) followed by Bonferroni post hoc analysis for behaviour and histopathological study. Hematological and biochemical test were analysed using Mann-Whitney test. p value less than ≤0.05 was considered statistically significant.

RESULTS

Table 1 shows the body weight of rats throughout the experimental period. There were no significant changes (p>0.05) in the body weight of rats in both control and 25 mg/kg; i.v TQ-NLC treated groups.

Table I: Body weight of control and rats treated with 25 mg/kg of TQ-NLC

Day	Control (g)	25 mg/kg TQ-NLC (g)
1	182. 60 ± 21.76	238.20 ± 14.92
4	180.00 ± 21.75	230.80 ± 14.86
7	192.20 ± 30.68	229.00 ± 17.16
11	193.40 ± 29.17	236.20 ± 14.65
14	191.40 ± 28.47	235.40 ± 12.88

Results are shown as mean values ± S.E.M.; n=6 animals per group

Figure 1 shows that there was no significant difference (p>0.05) in the food intake between the control and 25 mg/kg; i.v TQ-NLC treated groups.

Figure 2 represents the organ-to-body weight ratio between the two groups with no significant differences (p>0.05) in the weight of liver, spleen, lungs, heart and kidneys.

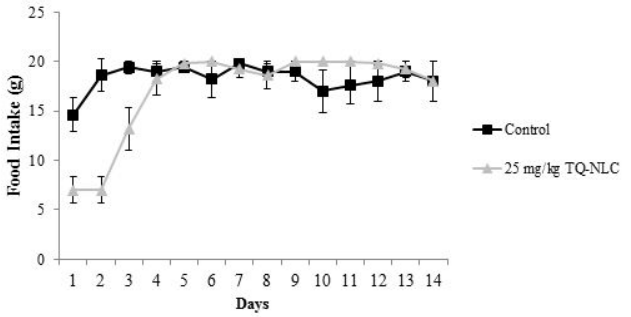

Figure 1: Effect of intravenous administration of TQ-NLC on the food intake of rats. Data are presented as mean ± S.E.M.; n=6 animals per group.

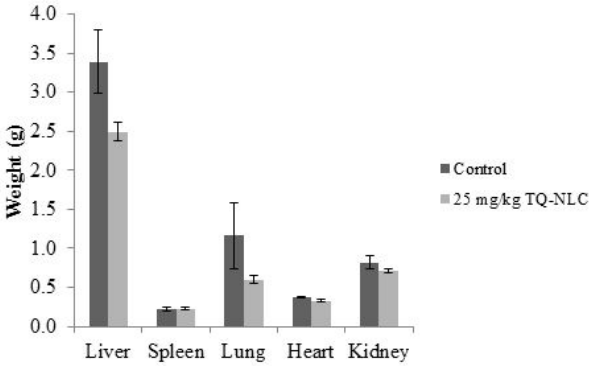

Figure 2: Effect of intravenous administration of 25 mg/kg TQ-NLC on the organ-to-body weight ratio of rats. Data are presented as mean ± S.E.M.; n=6 animals per group.

Figure 3 shows the haematological profile of rats in 25 mg/kg; i.v TQ-NLC treated rats as compared to the control animals. There were no significant differences (p>0.05) in the RBC, Hb, PCV, MCV, WBC and thrombocytes counts.

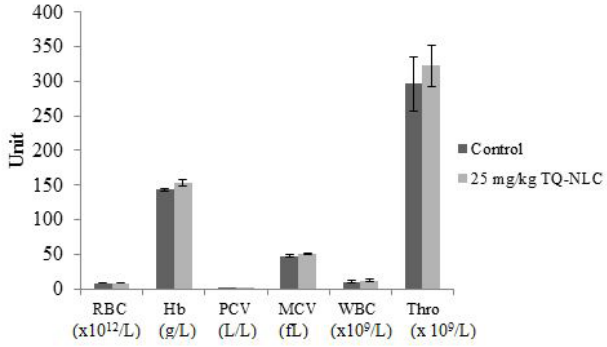

Figure 3: Effect of intravenous administration of TQ-NLC on the haematological profile of rats. Data are presented as mean ± S.E.M.; n=6 animals per group. RBC= red blood cell, Hb= haemoglobin, PCV=packed cell volume haematocrit, MCV=mean corpuscular volume, WBC=white blood cell, Thro.= thrombocytes.

Figure 4 represents the effect of 25 mg/kg; i.v TQ-NLC on the biochemical profile of rats compared to the control group. There were no significant differences (p>0.05) in the serum ALP, ALT, creatinine, urea, total protein, albumin and total bilirubin levels. However, AST level was significantly (p<0.05) reduced in 25 mg/kg; i.v TQ-NLC treated rats compared to control rats.

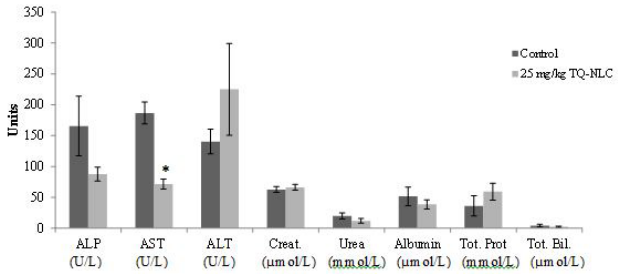

Figure 4: Effect of intravenous administration of TQ-NLC on the biochemical profile of rats. Data are presented as mean ± S.E.M.; n=6 animals per group. *significantly different than control at ($p \leq 0.05$). ALP= alkaline phosphate, AST=aspartate aminotransferase, ALT= serum alanine aminotransferase, Creat.= creatinine, Tot. Prot.= total protein, Tot. Bil.= total bilirubin.

Histological changes in the spleen tissue 14 days after saline and 25 mg/kg; i.v TQ-NLC are shown in Figure 5. The spleen tissues of control rats exhibited normal morphology but the one of 25 mg/kg; i.v TQ-NLC treated rats showed formation of brown spots.

Signs of inflammation at the site of injection at the tail of rats were only noted in the group injected with 25 mg/kg; i.v TQ-NLC as shown in Figure 6.

DISCUSSION

Any form of formulation or substance that is established as drug needs to undergo a few screening tests. This is essential for the identification of the benefits and more

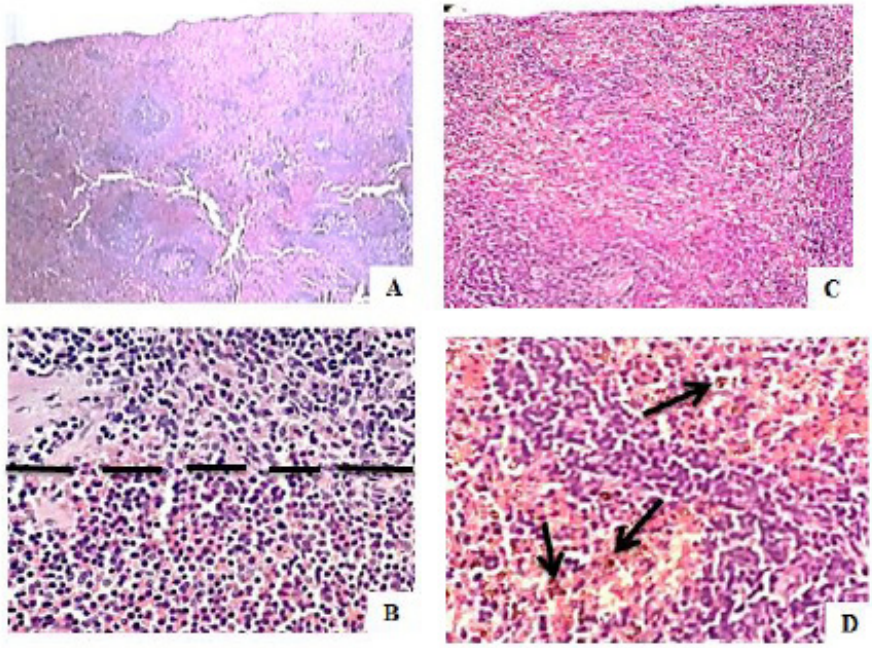

Figure 5: Histology of the spleen after treatment with TQ-NLC. Both groups show normal spleen condition (A and C) (10X magnification). The spleen of the control group has normal white pulp (upper region) and red pulp (lower region) (B). Formation of brown spots (hemosiderin) (arrows) is observed in the rats treated with 25 mg/kg TQ-NLC (D) (40X magnification).

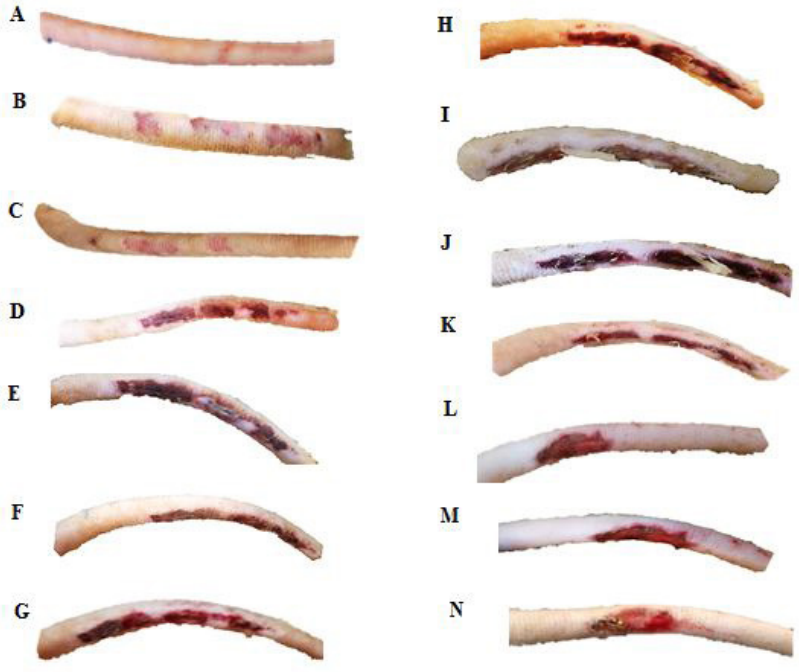

Figure 6: Inflammation at the site of injection on the tail if rats following 25 mg/kg; i.v TQ-NLC from day 1 to day 14 (A-N).

importantly the possible adverse effects that the substance may exhibit. In this study, we carried out the acute toxicity of intravenously administered TQ-NLC to the overall health, major body organs and blood profile.

No mortality was recorded throughout the 14 days of the study period indicating that TQ-NLC at 25 mg/kg was not lethal. Since none of the animals experienced body weight fluctuation of more than 10% from the initial one, it is believed that there was no adverse reaction to the general wellbeing of the rats. The adverse effects of drugs and chemicals can be determined by the changes in body weight (21). Furthermore, TQ-NLC treated rats showed no difference in food intake indicating that the ability to access food and appetite were not affected by the treatment. Adverse reaction of the treatment could cause stress to the animals influencing their eating habits (22). Oral administration of TQ-NLC at a dose of 50 mg/kg also showed no mortality but had 33% mortality rate at a high dose of 300 mg/kg (16).

There was also no apparent organ-to-body weight difference between treatment group and control group indicating that TQ-NLC did not cause toxicity to the body organs. In toxicological studies, analysis of organ-to-body weight is a vital endpoint to identify potential harmful effects of chemicals (23). Furthermore it serves as a good indicator of any changes that has been triggered chemically to the organs.

The blood profile readings fell within the normal range after treatment with 25 mg/kg, i.v TQ-NLC. Many toxicants cause toxicity to the hematopoietic system as it is one of the most sensitive targets for toxic chemicals. Therefore, the screening of chemicals for hematoxicity could highly predict possible risk by the chemical or drug to be tested (24). Moreover, in this experiment, the substance was administered directly into the blood system. We, therefore, conclude that TQ-NLC has no untoward effects to the blood system.

The readings for all biochemical parameters for kidney function inclusive of creatinine, urea, albumin levels and for liver function that includes ALT, ALP, total protein and total bilirubin of rats treated with 25 mg/kg; i.v TQ-NLC were also in the normal range. Lower level of AST in the 25 mg/kg; i.v TQ-NLC group might be reflecting the inflammatory activity in the liver (25) due to the skin inflammation at the site of injection. Histological sections of kidneys and liver in the treated groups showed no abnormality or alterations to the normal structures suggesting that TQ-NLC showed no any undesired effect to the structure and function of kidneys and liver. Toxic substances, especially heavy metals could bioaccumulate in vital organs that causes damages and eventually death. Furthermore, the main site for the metabolism and production of various essential compounds. It also plays a role in the detoxification of toxic materials making it an ideal organ to assess the toxic influence of a drug (26).

Our outcome is consistent when compared with the study by Ong et al. (16) where TQ-NLC was administered orally. Biochemical and haematological parameters indicated no signs of toxicity up to a dose of 100 mg/kg. However, following of long term TQ-NLC consumption, histological evaluations indicated possible liver toxicity but the organ's functions are preserved. As mentioned earlier the drugs administered orally has to bypass the gastrointestinal barrier to get into the blood system but drugs employed intravenously is directly present into the blood stream making the bioavailability and drug effect to be exerted differently.

The spleen of rats treated with 25 mg/kg, i.v TQ-NLC showed formation of brown spots or hemosiderin. Hemosiderin is a by-product of ferritin which is produced from haemoglobin during hemolysis (27). This condition might be due to the inflammation that occurred in the rat's tail following intravenous injection. It is possible that the degenerated red blood cells at the site of inflammation are circulated in the blood. Upon reaching the spleen, the degenerated red blood cells are destroyed (28) as the macrophages in the white pulp of the spleen engulf them. Heme release iron which can be stored as ferritin inside the cells. Subsequently, in cytoplasm, ferritin is oxidised and degraded to form hemosiderin which is observed as brown spots in the spleen (27).

Inflammation occurred at the rat's tails is postulated to be caused by the use of Polysorbate 80 in the formulation of TQ-NLC. Polysorbate 80 was used to synthesis TQ-NLC to improve particle stability (14). Furthermore, previous studies have reported on similar incidence of skin inflammation at the site of injection where the substance was administered (29). Further optimization of this formulation should highly consider a substitute for Polysorbate 80 to prevent unwanted consequences when TQ-NLC is developed into a drug for human use.

CONCLUSION

In conclusion, intravenous administration of 25 mg/kg TQ-NLC did not exhibit acute toxicity in female Sprague dawley rats. However, TQ-NLC caused inflammation at the injection site. The data can be used as a basis to further develop TQ-NLC as a potential therapeutic drug.

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