ORIGINAL ARTICLE

Thymoquinone-Loaded Nanostructured Lipid Carrier Reduces Proliferation of Human Liver Cancer Cells, HepG2

Nurfahima Mustafa Azmy, Aminah Suhaila Haron, Sharifah Sakinah Syed Alwi

Department of Biomedical Science, Faculty of Medicine & Health Sciences, Universiti Putra Malaysia, 43400, UPM Serdang, Selangor Darul Ehsan, Malaysia.

ABSTRACT

Introduction: Hepatocellular carcinoma is one of the most common cancers that affected human in more than half of the world population. Although there is yet any alternatives treatment found for this disease, the antitumor property of thymoquinone has been well studied in most of cancer cell lines. Nonetheless, poor bioavailability of TQ limits its efficiency. The encapsulation form of TQ, TQ-NLC is suggested to enhance its bioavailability as well as cytotoxicity towards cancer cells via increasing resistance time and targeting drug to specified location in the body. Therefore, it is a great advantage to look at the effects of TQ-NLC towards HepG2. This study is design to look at the anti-proliferative effect of TQ-NLC on HepG2 and the changes in the cells morphology. **Methods:** Both cells were bought from ATCC and cultured in supplemented DMEM. Cell viability was determined via MTT assay. Pro-apoptotic effect of TQ-NLC was further confirmed with Annexin V staining. Morphology hallmarks of apoptosis of treated cells were also analysed using inverted microscope. Images were captured at 24, 48 and 72 hours. **Results:** TQ-NLC was very potent towards HepG2 compared to 3T3 with the relative IC_{50} of 25 µM. TQ-NLC was also more potent compared to the non-encapsulated form, TQ. Further analysis confirmed that TQ-NLC capable to increase the percentage of apoptotic cells in time-dependent manner. Qualitatively, all treated cells displayed the apoptosis morphology with increasing concentration and longer time-point. **Conclusion:** TQ-NLC showed greater cytotoxic effects towards HepG2 which was further confirmed with the morphological analysis.

Keywords: TQ-NLC, TQ, HepG2, Apoptotic cells, Necrotic cells, Apoptosis

Corresponding Author:
Sharifah Sakinah Syed Alwi, PhD
Email: sh_sakinah@upm.edu.my
Tel: +603-8947 2302

INTRODUCTION

Cancer occurs when there is mutation or dysregulation in cell structures that lead to the detachment of primary tumor and amplified the distribution potential causing the metastatic spread of cancer cells to the secondary site throughout the body (15). Cancer cells able to develop proliferative signaling by their own producing growth factor ligands which can react via cognate receptors expression that lead to the autocrine proliferative stimulation (20). Notably, these growth factors are also responsible for the angiogenesis that supply sufficient oxygen and nutrients in order to support the tumor growth as well as metastasis (5).

Hepatocellular carcinoma (HCC) is the sixth most common cancer affecting human in the world which is the third leading cause of cancer-related death (28). HCC is the main histological subtype among all types of primary liver cancers, about 70%– 85% of the total liver cancer worldwide (27). The incidence of HCC is arising annually with 75,000 cases worldwide (16). In Asia, there is a shift in the epidemiology of HCC. The age-standardized rates (ASR) of HCC in 2012 for males in Eastern Asia were 31.9 per 100,000 when compared to 2008 with the rates of 35.5 per 100,000 (11, 16).

In a study of natural compounds, herbal plants become valuable sources for new drugs since the conventional medicine carry some side effects to the body. Hence, the usage of natural compound with pharmacological properties as a substitution of the synthetic drugs has been escalated recently (9). The broad application of natural medicine has influenced the scientists to further scrutinize the presence of their active ingredient as well as the effects on human health in protecting cells from abnormalities (2). Amongst the favorable medicinal plants is *Nigella sativa*, the annual flowering plant that contains 5 to 10 petals, pink-purplish in color. The seed from Nigella could produce an essential (volatile) oil that contains bioactive compounds. Chemical composition of the compounds is very diverse and entails a range of dissimilar components comprising carbohydrates,

proteins, fats, oils and alkaloids (9, 12).

The most abundant constituent of an essential (volatile) oil of *N.sativa* seeds is thymoquinone (TQ). Although TQ has been reported with the ability to eradicate cancer cells and hinder any modification in the genetic material of normal cells, it also has been shown to have poor bioavailability for *in vivo* test (12). TQ also has limited bioavailability and the solubility of pure TQ is relatively low in water (4). Therefore, a lipid nano-carrier has been developed to overcome this problem. TQ loaded with nano-structured lipid carrier or TQ NLC has a potential used as an alternatives carrier for TQ compound. In pharmaceutical industry, NLC has been applied for topical purposes, oral and parenteral administration drugs as well as helping in increasing the bio-administration of natural compound (1).

Therefore, this study is design to look at the anti-proliferative effect of TQ-NLC on HepG2 and the changes in the cells morphology upon treatment.

MATERIALS AND METHODS

Chemical and reagents.

Dulbecco's Modified Eagle's Medium (DMEM), trypsin-EDTA, antibiotics (penicillin and streptomycin), 3-(4, 5-dimethylthiazol-2-yl)-2, 5-di-phenyltetrazolium bromide (MTT) powder, and trypan blue dye solution were purchased from Nacalai Tesque (Kyoto, Japan). Dimethyl sulfoxide (DMSO) was purchased from Fisher Sc. (UK) and the phosphate buffer saline tablet was purchased from Oxoid (England). Fetal Bovine Serum (FBS) was purchased from iDNA (South America Origin). Propidium iodide was purchased from Sigma (St. Louis, USA). TQ and TQ-NLC were provided by Assoc. Prof. Dr. Latifah Saiful Yazan, Laboratory of Molecular Biomedicine, Institute of Bioscience (IBS), Universiti Putra Malaysia.

Cell lines

Human liver cancer cells, HepG2 and a normal skin fibroblast cell line (3T3) were purchased from the American Type Culture Collection (ATCC), USA. The cells were cultured in a complete DMEM medium and incubated at 37°C in an atmosphere containing 5%CO_2.

Growth Inhibition Assay

Cytotoxicity effect of TQ-NLC or TQ on HepG2 and 3T3 cells were determined by MTT assay in accordance to Tao et al. (30) with some modifications. Briefly, 4.0 X 10^4 cells were seeded onto a 96-well plate in 0.05 mL completes DMEM medium. After overnight incubation for cell attachment, TQ-NLC at various concentrations (0.78 µM to 50 µM) were added to the culture medium. A control without treatment was also included. TQ was also added as a parental comparison while cisplatin acts as positive control. The assay was later terminated at 24, 48, and 72 hours and the 'relative cell growth' was measured by MTT assay. After incubation, the MTT solution (5 mg/mL) were added into each well. Absorbance was set at 570 nm and the reference wavelength of 630 nm was measured using a micro plate reader (Opsys MR, USA) (21).

Detection of mode of cell death

Cell death induced by TQ-NLC or TQ was detected using the FITC-Annexin V Apoptosis Detection Kits II (BD Biosciences) following the manufacturer's instruction. Briefly, 1.0 X 10^5 cells were seeded with 3 mL media in a 6-well plate. After overnight incubation, the cells were treated with TQ-NLC or TQ and incubated again for 24, 48, and 72 hours. Cisplatin at 25 µM acts as a positive control. The cells were harvested and 100 µL of the cells were transferred into 5 mL FACS tubes. The cells were washed twice with cold PBS, and centrifuged at 486 x g for 5 minutes. A Master Mix containing 300 µL of binding buffer, 2.5 µL of 50 µg/mL PI, and 1.25 µL of Annexin V-FITC for each sample was prepared in the dark and 300 µL of the mixture was then added into each sample. The cells were incubated for 15 minutes at room temperature in the dark prior to flow cytometry analysis using the FL1 channel on a BD Biosciences FACSCalibur.

Cell morphology

Based on previous report by Ng et al. (25) with some modifications, changes in the treated cell morphology were observed under inverted microscope. HepG2 were treated in a 6-well plate with selected concentrations of TQ-NLC obtained from MTT assay. Untreated cell acts as a control. Cells were left to be incubated at 37°C and 5% CO_2 for 24, 48 and 72 hours. Morphological changes of treated cells were observed and recorded.

RESULTS

Cytotoxic effect of TQ-NLC or TQ on human liver cancer, HepG2.

Table I shows the anti-proliferative effects of TQ-NLC on the growth of HepG2 and 3T3 by MTT assay when exposed to various concentrations. DMSO and NLC act as negative control. TQ is added as a comparison of parental compound. Although TQ was less sensitive towards HepG2 compared to 3T3, TQ-NLC effect towards HepG2 was opposite with IC_{50} value of ~25 µM when exposed to various concentrations (Table I). Both compounds also showed non-cytotoxic selective effect towards normal 3T3 with overall IC_{50} values > 35µM. Meanwhile, both of the positive and negative control; cisplatin and NLC was observed to have minimum inhibitory effects in HepG2 and 3T3 with IC_{50} > 50µM.

TQ-NLC induced apoptosis in HepG2

To investigate in more detail the effects of TQ-NLC on HepG2, the percentage of cells death via apoptosis were analyzed using Annexin V staining at 24, 48 and 72 hours of post-treatment with 25 µM of TQ-NLC

Table 1: Cytotoxic effects of TQNLC or TQ on HepG2 and 3T3 reflected by the IC_{50} values determined from the MTT assay.

Cell line	Incubation time (hour)	IC_{50} (μM)				
		TQ-NLC	TQ	DMSO	CISPLATIN	NLC
HepG2	24	25.8±1.82	49.3±2.24	NP	NP	NP
	48	23.8±3.41	41.9±0.44	NP	NP	NP
	72	23.1±6.24	40.7±2.47	>50	>50	>50
3T3	24	41.2±0.25	46.7±1.14	NP	NP	NP
	48	38.7±1.55	38.9±2.88	NP	NP	NP
	72	39.2±0.84	39.1±0.96	>50	>50	>50

(Table II). Annexin V-FITC has high affinity to bind to phosphatidylserine (PS). Exposure of PS at the external surface of plasma membrane enables the binding of Annexin V that has been conjugated with FITC to the negatively charged PS. Data obtained showed that TQ-NLC significantly reduced the percentage of viable HepG2 (~82-85%) with p< 0.01 and increased the percentage of apoptotic cells (~12-13%) after 24, 48 and 72 hours of treatment compared to control (Fig. 1). Although cisplatin also demonstrated a significant reduction in healthy HepG2 (p<0.05), TQ-NLC showed a more potent effect with lesser viable cells compared to the positive control (p<0.01).

Table II: Percentage of viable, apoptotic and necrotic cells as determined via flow cytometry.

	Percentage of cell (%)			
	Viable Cell	Early apoptosis	Late apoptosis	Necrosis
UNTREATED	95.9	1.7	1.2	1.2
NLC	94.7	2.2	1.9	1.3
Cisplatin	87.4	5.5	4.7	2.5
TQ-NLC (24h)	85.2	7.4	4.9	2.3
TQ-NLC (48h)	84.6	7.9	5.3	2.3
TQ-NLC (72h)	82.2	7.5	6.1	4.3

Percentage of viable cell computed in comparison to the untreated cells which has been calculated as 100%. Each value represented as mean ±SEM. NP, not performed. Notes: * compare to control with p<0.05

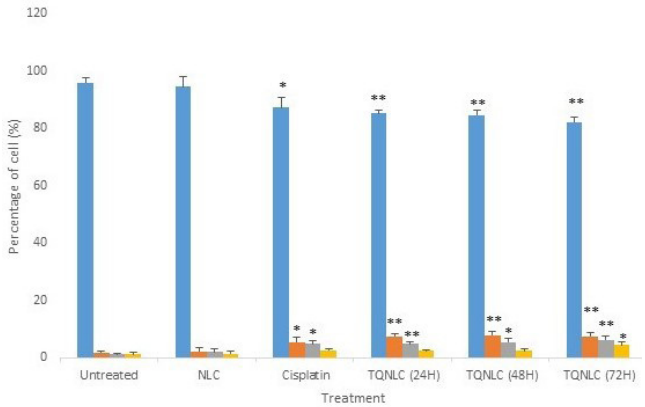

Figure 1: Quantitation of apoptosis in HepG2 cells treated with NLC, and TQNLC for indicated time points (24, 48 and 72 hours). AV-/PI-(BLUE), AV+/PI-(ORANGE), AV+/PI+ (GREY) and AV-/PI+ (YELLO\V). Data shown are the average of three independent experiments. Statistically significant is noted as (*p<0.05), (**p<0.0l) and (***p<0.00l).

Morphological changes of HepG2 upon treatment with TQ-NLC

Treatment with designated concentrations of TQ-NLC (12.5 μM and 25 μM) showed significant morphological changes in HepG2 compared to control after 24, 48 and 72 hours incubation (Fig.2). Concentration of 12.5 μM was chosen to see if half of the concentrated toxicity have any effects on the treated cells morphology. Interestingly, at lower concentration, treated cells remain attached at the substratum after 24 hours treatment. Although some cells had detached and undergoing cell shrinkage at 48 hours, the present of apoptotic bodies were only recorded after 72 hours treatment. Meanwhile, at higher concentrations, more cells underwent membrane blebbing as early as 48 hours followed by apoptotic bodies. At 72 hours, more apoptotic bodies were observed compared to the control. The reduced number of cells population was prominent with increasing concentration of TQ-NLC and longer incubation period. Cells morphology was observed using an inverted microscope.

DISCUSSION

Traditionally, plants or plant extracts are important source of remedies that rich in natural compounds used to treat a wide variety of diseases. However, most of the compounds have not been discovered until today and the biodiversity of plants represent indefinite source of novel chemical compounds. To date, about 60% of the discovered anticancer agents including paclitaxel, vinblastine and vincristine are attained from plants (7). Thymoquinone (TQ), an active lipophilic compound found in Nigella sativa plant has a variation of pharmacological properties such as anticancer effects in diverse human cancer (3, 13).

However, due to the poor bioavailability of TQ, Ng et al, (24) had designated and effectively prepared thymoquinone loaded nanostructured lipid carrier (TQ-NLC) via high-pressure homogenization technique. The encapsulation of TQ in NLC has increased drug efficiency and controlled drug release (24). NLC has been established to transport drugs to several application routes including topical skin delivery, parenteral injection, ocular delivery, oral administration and pulmonary inhalation (10).

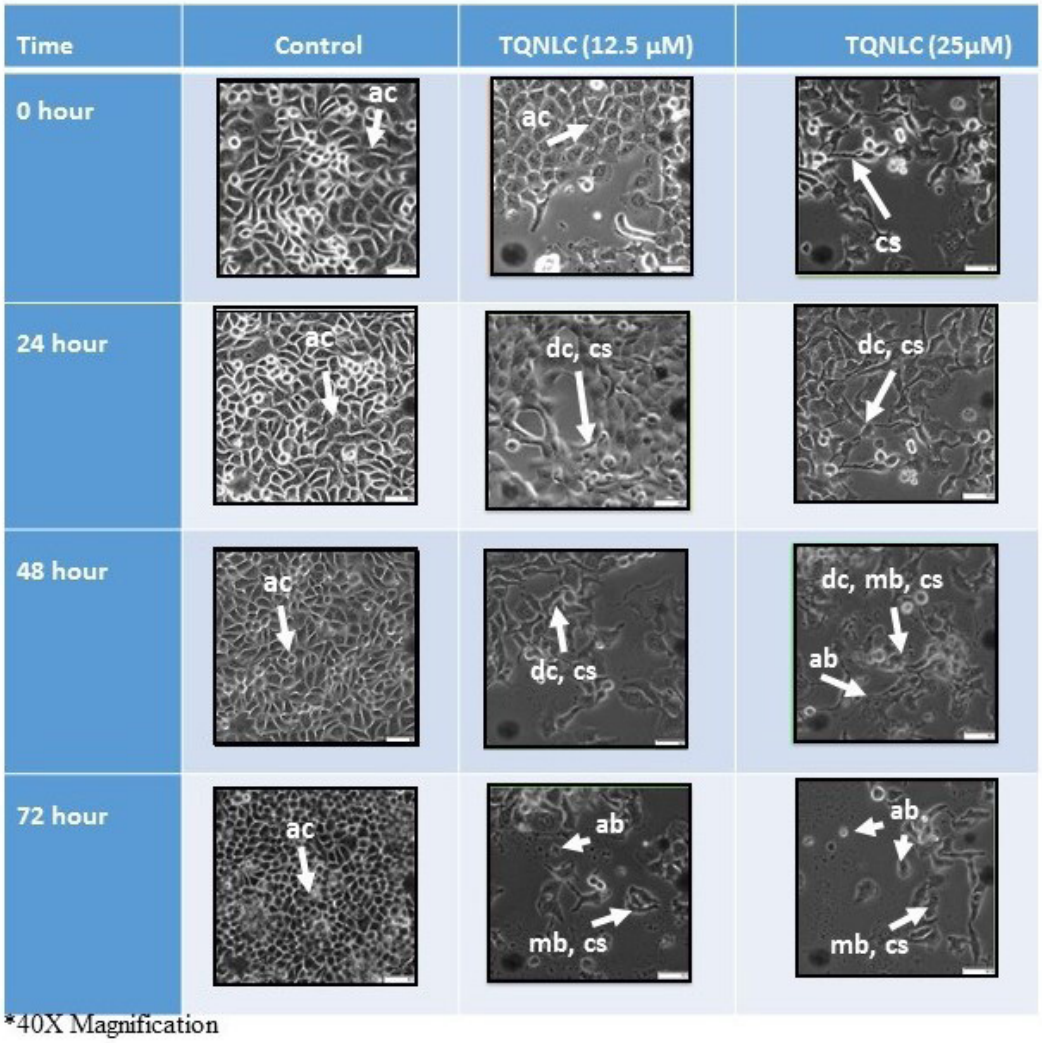

Figure 2: Morphological changes of TQNLC-treated HepG2 observed wider an inverted light microscope (40X magnifications). Cell population decreased in a concentration and time dependent. All treated cells showed apoptotic characteristics such as cellular detached (de), cell shrinkage (cs) and membrane blebbing (mb). Formation of apoptotic bodies is being labelled as ab. Healthy cells which remained attached at the surface of the flask is labelled as attached cells (ac).

It is particularly useful for targeting water-soluble drug administration.

Lipid-based drug delivery systems are promising oral carriers due to their ability to enhance oral bioavailability and increase the solubility of poorly water-soluble and lipophilic drugs (17). NLC comprises of a biocompatible solid lipid matrix entangling a liquid lipid as nano-compartments that enhanced drug loading capacity with greater physical stability and preventing drug leakage during storage (23, 29). Moreover, NLC particles could evade liver metabolism through the formation of micelles with bile salts in the small intestines after they entered the gastrointestinal (GI) tract (19). Based on the previous acute toxicity study (26), encapsulation of TQ in NLC reduces the toxic effect of the compound which provide safety information for TQ-NLC, which is important for further study in clinical use. TQ-NLC has been well demonstrated to be more cytotoxic towards breast cancer, MDA-MB-231 and MCF7 cells as well as cervical cancer SiHa cells compared to TQ (8, 24). Although there was a report on the effect of TQ towards HepG2 cells, no study has been conducted to look at the toxicity effects of TQ-NLC towards liver cancer cells, HepG2. HepG2 is a non-tumorigenic cells with hepatitis B virus negative and express majority of drug-metabolizing enzymes (6). Our data showed that TQ-NLC is more toxic towards HepG2 compared to TQ. Similarly, TQ-NLC was also observed to be more toxic compared to TQ in Hep3B cells (14). This has been suggested due to the encapsulated form of TQ that contribute to the differential effect as it has been improved in bioavailability and cytotoxicity (14). Nevertheless, TQ-NLC is less toxic towards normal 3T3 cells with IC_{50} > 30 µM (15, 24). Similar cytotoxic selective effect of TQ-NLC was also observed towards normal cells 3T3-L1 and Vero cells after 72 hours treatment with IC_{50} values of >50 µM and 32 µM respectively (18, 25).

Further analysis to confirm the vulnerability of HepG2 towards TQ-NLC at selected concentration was performed using Annexin V staining. A concentration was chosen based on the IC_{50} value obtained from MTT assay. The result (Table II) demonstrated significant reduc-

tion in the percentage of healthy cells (~82-85%) and increased in the percentage of apoptotic cells (~12-13%) in time dependent compared to the untreated (Fig. 1). TQ-NLC was also demonstrated to be more cytotoxic compared to cisplatin which acts as a positive control. This confirmed that TQ-NLC caused the sensitivity and increased the vulnerability of HepG2 upon treatment. Previous studies had also demonstrated that TQ-NLC reduced the viability of several other cancer cells such MDA-MB-231 (24) and Hep3B (14). Although exact mechanism has not been fully elucidated, it is suggested that TQ-NLC may trigger cell death via apoptosis.

Cancerous cells are well-known to evade apoptosis. Apoptosis is a physiological response to eliminate unwanted or dysfunctional cells as well as to maintain homeostatic balance. In HepG2 treated TQ-NLC, features of apoptosis such as detachment of cells from substratum, cells shrinkage, membrane blebbing and formation of apoptotic bodies as reported by Zhuang et al. (31) were observed in concentration and time-dependent manner. At higher dosage of 25 µM, more treated cells were deformed and demonstrated morphologic hallmark of apoptosis. Number of apoptotic bodies were increased after 72 hours of treatment. These will later be phagocytosed by the neighboring cells such as parenchymal and macrophages cells. Hence, this shows that TQ-NLC is able to induce apoptosis in HepG2. Similar apoptotic features was also recorded in MDA-MB-231 treated TQ-NLC (24). Although not many studies had been conducted to look at the anti-apoptotic effect of TQ-NLC, the classical sign of apoptosis such as chromatin condensation, translocation of phosphatidyl serine across plasma membrane and fragmentation of DNA have also been observed in TQ-treated cells (4). TQ-NLC is also suggested to have improved pro-apoptotic effect due to the high encapsulation efficiency and drug loading capacity. The improved effect of TQ-NLC has been reported previously by Haron et al. (14) that demonstrated better apoptotic effect promoted by TQ-NLC compared to TQ itself.

CONCLUSION

Our data provide important preliminary insights on the effect of TQ-NLC towards HepG2 which can be an effective anti-proliferative agent of liver cancer. Our finding showed that TQ-NLC able to inhibit the proliferation of HepG2 which was further confirmed with the Annexin V staining and the presence of apoptotic hallmark in the morphology of treated cells. However, further molecular investigations are needed to explicitly map out the mechanism involved in triggering the apoptosis.

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