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IN VIVO EVALUATION OF THE ANTICANCER ACTIVITY OF THE DOCETAXEL INCORPORATED INTO NANOEMULSION BASED ON ORANGE OIL

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ABSTRACT

Docetaxel (DOC), an antimicrotubule, is used to inhibit the proliferation of various kinds of cancer cells. However, it has serious adverse side effects that restrict its clinical application. The aim of the present study was to examine the antitumor activity of the DOC-loaded nanoemulsions based on orange oil (DOC-NEOO) and to assess its cardiotoxicity in mice bearing Ehrlich tumor in their ascetic fluid (EAC). One hundred twenty female Swiss Albino mice were divided into six groups (n=20). Groups I and II were the untreated mice (Control (-)) and the mice administered orally with 0.1ml of NEOO (NEOO (-)), respectively. Group III was the mice bearing EAC that served as the control (+). Groups IV-VI were mice bearing EAC treated with 0.1ml of DOC-NEOO, NEOO and DOC-water, respectively. The mean survival times of both of DOC-NEOO and NEOO (+) groups have enhanced, while the tumor volumes in their ascetic fluid have decreased when compared to the DOC-water group. The administrations of the formulas incorporating NEOO into the mice have enhanced the amount of high density lipoproteins and reduced the amount of cholesterol and triglyceride in their serum. They have also improved the antioxidant activities of glutathione peroxidase and superoxide dismutase in the heart tissues of the mice. Additionally, they decreased the creatine kinase in serum. In conclusion, incorporating the DOC into the NEOO has enhanced its efficacy and reduced its effect on the heart.

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Introduction

Cancer, one of the most fatal disease worldwide, is caused by the uncontrolled proliferation of the abnormal cells [1]. Cancer therapy can be implemented by surgery, chemotherapy and/or radiotherapy [2]. In spite of all of the medical advances in oncology, cancer treatment is still challenging with the clinical use of the chemotherapeutic agents due to their toxicity on the vital organs. Therefore, many research studies in nanomedicine have proposed many solutions to overcome the serious issues associated with the cancer therapy [3]. Docetaxel (DOC) is an antiproliferative agent derived from yew tree that impede the growth of the cancer cells by inhibiting the depolymerisation of microtubule [4]. Although DOC is used against numerous cancers, e.g. prostate, breast, ovarian and non-small cell lung cancers, it may cause serious and long-term side effects [5].

One of the recent proposed deliveries of the anticancer drugs to overcome their side effects is the essential oils based nanoemulsions, which are colloidal systems that consist of oil, water, surfactant and/or cosurfactant. Although nanoemulsions include a small amount of surfactant like the emulsions, they require input of energy to decrease the size of the dispersed droplets and to convert the milky appearance of the emulsion to a transparent or slightly cloudy nanoemulsions [6]. Orange oil is one of the essential oils that is very hydrophobic and needs to be emulsified in order to increase its intestinal absorption [7]. Orange oil, extracted from the citrus peel, contains more than 200 constituents, mainly linalool, limonene and vitamin C. Many research studies have demonstrated the cardioprotective property of the orange oil [8, 9, 10].

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Mixing the DOC with a nanoemulsion-based orange oil (DOC-NEOO) would improve the antiproliferative effect of DOC while eliminating its side effect. The objective of the present study was to evaluate the antitumor activity and the cardioprotective effect of DOC-NEOO in mice bearing tumor in their ascetic fluid.

Materials And Subjects

Orange oil was purchased from i herb, one of the world's largest online stores for natural products. Sorbitan laurate (span 20), polysorbate (tween 80) and distilled water were purchased from Al-Rowad modern establishment for the supply of medical equipment (Jeddah, KSA). Docetaxel (DOC) was obtained from Elezaby Pharmacy (Cairo, Egypt). The QuantiChrome thiobarbituric acid reactive substances (TBARS) assay kit and EnzyChrom assay kits of superoxide dismutase (SOD), catalase kit, and Glutathione peroxidase (GPX) were obtained from Bioassays for diagnostic and research reagents (Hayward, USA). Serum analysis kits were obtained from the Crescent diagnostics Company (Jeddah, KSA) and Human Biochemical and Diagnostic (Wiesbaden, Germany). Commercial pelleted mice food, was obtained from Saudi Grains Organization (Jeddah, KSA).

One hundred twenty female Swiss Albino mice, weighing between 22-30 g, were acclimatized in accordance with King Abdulaziz University's policy and the International Ethical Guidelines for the care and use of laboratory animals [11]. The ethical approval was obtained from the research ethics committee in the Faculty of Medicine at King Abdulaziz University.

Methods

Preparation and characterization of NEOO formulations

The drug-free NE based on orange oil (NEOO) was prepared by mixing 9.4% (v/v) of surfactant mixtures of span 20 and tween 80 at a ratio of 1:2, respectively, 89.7% (v/v) of orange oil and 3.6% (v/v) of distilled water. Then, the mixture was vortexed with continuous heating in a water bath for one week at 100°C. The amount of DOC used for the drug administration was according to [12]. The DOC-loaded-NE (DOC-NEOO) was prepared by dissolving directly a 24 mg of DOC/ kg of mouse body weight in 0.1 ml of NEOO. Another formula for the DOC solution (DOC-water) was produced by dissolving 24 mg/kg of mouse in 0.1ml of distilled water. The physical characteristics of the nanodroplets of the NEOO formulations were determined by using Zetasizer Nano ZS (version no MAN0487-2-0, Malvern Instruments, UK).

Transplantation of the Ehrlish tumors in the ascetic fluid of the mice

The mice were divided into six groups (n =20), as illustrated in Table 1, and their body weights were recorded. As described elsewhere, all of the mice in groups III-VI were injected intraperitoneally with 2.5×10^6 EAC cells/mouse for 48h incubation [13, 14]. Groups IV -VI were administered with the desired formulas at four doses every four days as shown in Table 1. The average amount of food consumed by the mice in each group per day was recorded for 21 days determined by subtracting the amount of the remaining food from the initial amount of the served food which was a hundred grams.

Table 1. The tested animal groups with their administered treatment

Group No.	Group name	Treatment	Doses
I	Control (-)	-	
II	NEOO (-)	NEOO	0.1 ml
III	Control (+)	-	
IV	DOC-NEOO	DOC-NEOO	24mg/kg of mouse dissolved in0.1 ml NEOO
V	NEOO (+)	NEOO (+)	0.1ml NEOO
VI	DOC-water	DOC-water	24mg/kg of mouse dissolved in 0.1 ml distilled water

On the 21th day, half of the mice in each group (n=10) were set aside fasting for 12h followed by recording their body weight and collecting their ascetic fluid for the detection of cancer cell death. After that, they were slaughtered and their hearts were resected followed by recording their weights in order to calculate the heart weights relative to the body weight of the mice recorded on the 21th day.

For the antioxidant assays, a small part of each lobe of the excised heart was cut off and rinsed in ice-cold normal saline followed by deep freezing at - 80 ° C in the freezer (Revco™ CxF Series Ultra-Low Temperature Chest Freezers) in order to be stored for utmost 3 months before performing the experiment. The other ten mice in each group were left to determine their mean survival time.

***In vivo* anticancer activity of the tested drug formulations**

The ascetic fluid from the tested mice was assembled to record its volume followed by the centrifugation of the fluid at 800 rpm for 5 min at 4°C. The supernatant was isolated to determine the lactate dehydrogenase (LDH) activity as described by the protocol of LDH LR (SCE MOD, Cat.No. CZ 908 L) kit [15]. The mice survival was monitored for 30 days by observing the mortality of the mice. The average mortality of the mice was expressed as the mean survival time (MST).

Detection of drug toxicity on the hearts of the tested mice

The serum of the blood was collected after blood clotting and centrifugation at 3000 RPM for 15 min. The creatine phosphokinase (CK), creatine phosphokinase-MB (CK-MB) and LDH activities were determined. In addition, the lipid profile, including cholesterol (CHO), high density lipoprotein (HDL) and triglyceride (TG), were measured.

For the oxidative stress analysis, the lobes of the heart tissue (10mg) of each group were homogenized in 200 µl of cold PBS at pH 7.0 per gram tissue. Then, the homogenized tissue was centrifuged at 14000 rpm for 10 min at 4°C immediately before the assay. The collected supernatant was used immediately for the estimation of superoxide dismutase (SOD), lipid peroxide (Malondialdehyde, MDA), catalase activity and glutathione peroxidase (GPx). All the antioxidant assays were detected by colorimetric methods using bioassays kit.

Statistical analysis

Statistical analysis was determined with one-factor analysis of variance (ANOVA) test and identifying the p-values for the pairwise t-test using the MegaStat Excel (version 10.3, Butler University). The variations between the individual samples were significant when $p < 0.05$.

Results

Physical characteristics of the NEOO formulations

According to the Zetasizer measurements illustrated in Table 2, it has been found that loading DOC in the NEOO formulation has significantly increased the z-average diameter of the droplets of NEOO formula (P-value = 0.0244). However, the zeta potential of both formulas did not considerably vary (P-value = 0.5530). Additionally, the sizes of both formulas were homogeneously distributed as the polydispersity indexes (PDI's) were less than 0.30.

Table 2. The physical characteristics of the nanoparticles of the NEOO formulations. Data were expressed as $\overline{X} \pm$ SD.

Formulation	Z-Average diameter (nm)	PDI	Zeta Potential (mV)
NEOO	15.616 ± 3.12	0.200	-12.02 ± 3.93
DOC-NEOO	34.52 ± 8.753	0.254	-13.7 ± 2.19

Anticancer activity of NEOO formulation

The anticancer effect of the NEOO formulations in terms of the percentage change in body weight of the tested mice and the average amount of food consumption of the mice per day (g) within 21 days (Figure 1), the volume of the ascetic fluid, the LDH activity in the ascetic fluid (U/L) and MSTs (Table 3) were determined. Regarding the body weight change among all of the tested groups, DOC-water group has got the maximum gain of weight, whereas NEOO (-) group has lost weight. Relative to the Control (+) group, the percentage change in body weight of NEOO (+) group did not significantly differ while DOC-NEOO group has got a considerable decrease in the body weight. It should be noted that the percentage change in body weight of the Control (-) group has significantly differed from the rest of the tested groups.

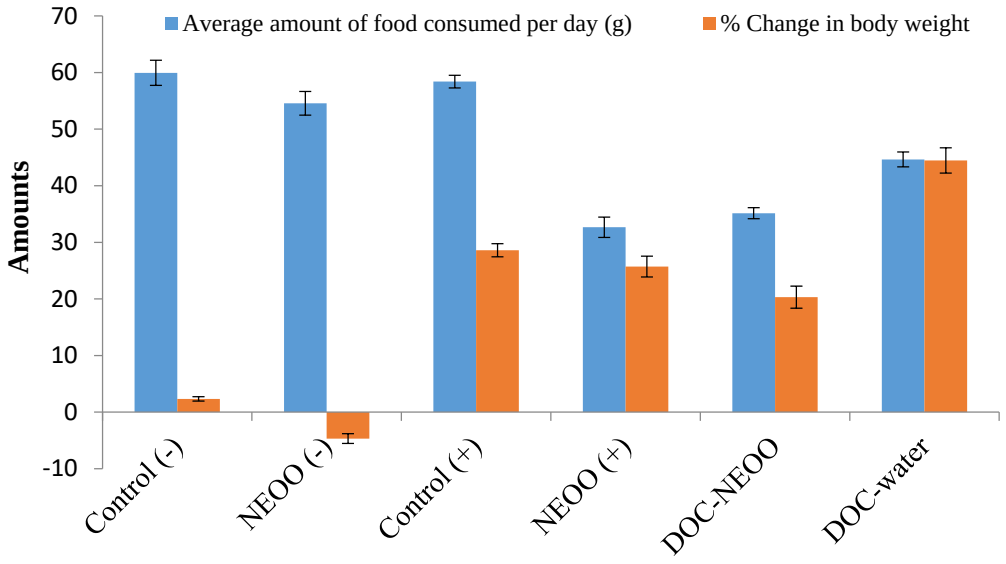

Figure 1. The average amount of food consumed by the tested mice per day and the % change in the body weight of the mice within 21 days. *Error bars* represent the standard error of the mean (n=10).

Table 3. The anticancer effect of the drug formulations on the tested mice. Data were expressed as $\bar{X} \pm$ SD.

Group	Volume of ascetic fluid (ml)	LDH activity in the ascetic fluid (U/L)	MST (days)
Control (-)	----------	----------	30.0 ± 2.00
NEOO (-)	----------	----------	30.0 ± 2.00
Control (+)	9.00 ± 0.354	172.45±1.44	21.7 ± 3.46[ae]
NEOO (+)	7.80 ± 2.05[d]	187.74±2.23[bdf]	23 ± 5.56[ae]
DOC-NEOO	6.43 ± 1.69[bc]	205.33± 1.58[bcg]	25.38 ± 4.69
DOC-water	11.33 ± 2.75[cd]	212.71±2.37[bcd]	22.00 ± 4.40[ae]

[a] There is a significant difference between the desired group and the control (-); [b] There is a significant difference between the desired group and the control (+); [c] There is a significant difference between the DOC-water and DOC-NEOO groups; [d] There is a significant difference between the DOC-water and the NEOO (+) groups. [e] There is a significant difference between the desired group and the NEOO (-).[f] There is a significant difference between the desired group and the DOC-NEOO.[g] There is a significant difference between the desired group and the NEOO (+).

In terms of the average amount of food consumption per day (g) by the tested mice, both of Control (-) and Control (+) groups have got the maximum amount of food. Interestingly, when compared to the DOC-water group and the other tested groups, the administration of NEOO formulations into the EAC-bearing mice, NEOO (+) and DOC-NEOO, has significantly reduced the amount of food consumed. In fact, mice treated with NEOO (NEOO (-)) have got less amount food than the untreated mice, Control (-).

In addition to the least amount of food consumed by NEOO (+) and DOC-NEOO groups, the amounts of ascetic fluid collected from their peritoneal cavity were the least compared to the Control (+) and DOC-water groups (Table 3). According to the measurements of the LDH activity in the ascetic fluid, there were very highly significant differences between the tested EAC-bearing mice. Moreover, the MSTs of NEOO (+) and DOC-NEOO were greater than the MSTs of Control (+) and DOC-water groups.

Effect of drug formulations on the heart function

Serum analysis

Table 4 illustrates the effect of the drug formulations on the heart function of the tested mice in terms of heart –to-body weight ratio and serum analysis. The heart-to-body weight ratios of all of the treated groups were comparable to the Control (-) group. The levels of CK and CK-MB of all of the tested groups did not significantly differ. Similarly, the levels of LDH of all of the tested groups were comparable except NEOO (-) group has an elevated level.

In terms of the lipid profile, NEOO (-), NEOO (+) and DOC-NEOO groups have less amount of CHO than the other tested groups (Table 5). Compared to the Control (+) group, all of the tested groups have increased the amount of HDL. The amount of TG was elevated in NEOO (-), DOC-NEOO and Control (+) groups when compared to the Control (-) group which was comparable with NEOO (+) and DOC-water groups.

Table 4. The serum analysis of the tested mice treated with drug formulations in order to detect the heart function. Data were expressed as $\bar{X} \pm$SD.

Group	Heart/body weight ratio	CK (U/L)	CK-MB (U/L)	LDH (U/L)
Control (-)	0.005±0.0003[b]	39.72±15.43	6.96±4.66	159.82±0.79
NEOO (-)	0.005±0.0007[b]	29.21±12.14	6.71±2.20	598.72±192.48[abfg]
Control (+)	0.002±0.0007[a]	32.45±5.7	6.56±4.49	256.28±73.76
NEOO (+)	0.003±0.0007	35.36±18.40	9.17±2.27	247.20±79.11[e]
DOC-NEOO	0.003±0.0009	27.65±7.64	8.44±2.28	298.86±11.75
DOC-water	0.005±0.005[b]	39.10±16.73	14.27±7.97	222.76±2.84[e]

Table 5. The lipid profile of the tested mice treated with the drug formulations. Data were expressed as $\bar{X}$±SD.

Group	CHO (mg/dL)	HDL (mg/dL)	TG (mg/dL)
Control (-)	142.10±33.6^{egf}	99.04±28.96^{b}	138.06±3.4
NEOO (-)	99.54±3.89^{ab}	116±46.24^{b}	176.13±10.7^{abeg}
Control (+)	183.75±5.26^{agf}	32.32±7.68^{ae}	203.97±19.8^{afb}
NEOO (+)	106.17±27.45^{abd}	71.2±36	135.79±10.7^{bfeg}
DOC-NEOO	103.203±6.63^{abc}	76.16±25.28	157.67±2.55^{abg}
DOC-water	147.13±18.07^{bcde}	113.44±44.96^{b}	138.92±9.3^{be}

[a] There is a significant difference between the desired group and the control (-); [b] There is a significant difference between the desired group and the control (+); [c] There is a significant difference between the DOC-water and DOC-NEOO groups; [d] There is a significant difference between the DOC-water and the NEOO (+) groups. [e] There is a significant difference between the desired group and the NEOO (-).[f] There is a significant difference between the desired group and the DOC-NEOO.[g] There is a significant difference between the desired group and the NEOO (+).

Oxidative stress analysis

As exhibited in Table 6, the oxidative stress analyses were detected in the heart's tissues of the tested mice. The GPX activity was raised in all of the tested groups when compared to the Control (-). In fact, the GPX activity of the DOC-NEOO was the highest among all of the groups. In contrast, the catalase activity did not significantly differ in all of the tested groups. The SOD activity of the Control (+) was significantly less than all of the other tested groups. In addition, TBARS amount was lowered in the Control (+) and DOC-NEOO groups.

Table 6. The ROS analysis of the heart tissues of the tested mice. Data were expressed as $\bar{X}$±SD.

Group	GPX (U/L)	Catalase (U/L)	SOD (U/mL)	TBARS (µM)
Control (-)	177.42±9.47	2.82±0.92	2.91±0.04^{b}	12.52±1.19^{b}
NEOO (-)	206.50±4.87^{a}	2.50±0.28	2.64±0.12^{b}	11.68±5.7^{b}
Control (+)	203.79±6.87^{a}	2.12±0.28	1.97±0.71	3.89±1.48^{a}
NEOO (+)	209.03±7.12^{a}	2.84±0.33	2.82±0.02^{b}	18.01±1.09^{abe}
DOC-NEOO	301.76±6.16^{abe}	2.84±0.85	2.85±0.04^{b}	4.31±0.87^{aceg}
DOC-water	219.09±7.74^{ab}	2.94±0.06	2.95±0.10^{b}	14.90±2.41^{bc}

[a] There is a significant difference between the desired group and the control (-); [b] There is a significant difference between the desired group and the control (+); [c] There is a significant difference between the DOC-water and DOC-NEOO groups; [d] There is a significant difference between the DOC-water and the NEOO (+) groups. [e] There is a significant difference between the desired group and the NEOO (-).[f] There is a significant difference between the desired group and the DOC-NEOO.[g] There is a significant difference between the desired group and the NEOO (+).

Discussion

The MSTs of both of DOC-NEOO and NEOO (+) groups have enhanced, whereas the tumor volumes in their ascetic fluid, their food consumption and the percentage change in their body weight have decreased when compared to the DOC-water group. The antitumor effect of the NEOO formulas is due to the presence of the orange oil which contains the radical scavengers, phytochemical antioxidants, and thereby holds a potential in reducing the risk of cancer [16]. In addition, the nanodroplet size of the NEOO would help in the permeation of the orange oil into the cells and improve its intestinal absorption [17]. Interestingly, the % change in the body weight of NEOO (-) group have considerably decreased compared to all of the other tested groups, although their food consumption was comparable to the Control (-) group. It could be attributed to the presence of ascorbic acid in the orange oil which is a cofactor in the biosynthesis of the fatty acid oxidation stimulator (carnitine) [18, 19].

The cardiotoxicity of both of NEOO (-) and DOC-NEOO groups were similar in terms of the reduction in CK and the increase in the GPX and SOD in their serums when compared to the Contorl (+). It has been demonstrated previously that the ascorbic acid, included in the orange oil, was cardioprotective and reduced the cardiotoxicity of the doxorubicin in the heart tissues of the rats [9, 20]. In addition, all of the groups, subjected into the formulas incorporating NEOO, have increased the amount of HDL and reduced the amount of CHO and TG when compared to the Control (+). Many research

studies have demonstrated the beneficial effect of the orange juice in lowering the CHO and LDL while preventing the reduction in the HDL [8, 10].

Conclusion

It has been found that loading the DOC in NEOO has improved the efficacy of DOC. NEOO has a cardioprotective property in the mice bearing tumors since it ameliorated the lipid profile in serum of the mice and stimulated the activity of GPX and SOD in the mice heart tissues. Further studies have to be implemented on the NEOO effect on the other organs in mice bearing tumors.

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