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Full Length Research Paper

Antitumor activity of tamoxifen loaded solid lipid nanoparticles on induced mammary tumor gland in Sprague-Dawley rats

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An *in vivo* study was conducted to determine the effect of tamoxifen-loaded solid lipid nanoparticle on LA7 cell-induced rat mammary tumor. After the first tumors appearance, the thirty rats were divided into five groups. Group I served as normal control animal. The other four groups were mammary gland tumor-bearing animals treated with soy oil (Group II), tamoxifen (TAM) dissolved in soy oil (Group III), tamoxifen-loaded solid lipid nanoparticle (Group IV) and solid lipid nanoparticle (SLN) (Group V) dispersed in soy oil. The effect of TAM-loaded SLN in the reduction of tumor size was greater than that of free TAM. The transmission electron microscope analysis showed apoptosis in TAM-loaded SLN and free TAM treatment groups. After treatment, the rats treated with TAM-loaded SLN showed decreases in concentration of serum enzymes. In conclusion, the study suggests that TAM-loaded SLN is very effective for treatment of rat mammary gland tumors and breast cancers.

Key words: Mammary tumor gland, Sprague-Dawley rat, tamoxifen loaded solid lipid nanoparticle.

INTRODUCTION

In chemotherapy, the aims of the treatment are to contract the primary tumors, slow tumor growth, destroy cancer cells that might have been metastasized and control malignancy (Padmavathi et al., 2006). Tamoxifen (TAM) has long been the only option for the treatment of hormone dependent breast cancer. The antitumor effect of tamoxifen is related to growth arrest and induction of apoptosis (Manlekar and Kong, 2001) mediated by linkage to the intracellular estrogen receptor on breast cancer cells, blocking of steroid hormone action (Jordan, 1993), inhibition of protein kinase C (O'Brian et al., 1985) and its binding to calmoduline (Lam, 1984). There are side-effects to the use of tamoxifen; among them are development of liver cancers, increasing blood clothing, retinopathy and corneal opacities (Han and Liehr, 1992; Fisher et al., 1994; Memisoglu-Bilensoy et al., 2005). Due to these side-effects, the colloidal delivery systems were suggested to be the best way of delivery of tamoxifen for long-term chemotherapy of breast cancers. Solid lipid nanoparticles (SLNs) have been recommended as the carrier in these drug delivery systems. The main benefit of SLN is that it is non toxic, has good bioavailability, not easily degraded and can be produced on a large scale (Jores et al., 2005; Yuan et al., 2007). SLNs also have been recommended to substitute as drug delivery systems to approved polymeric nanoparticles (Luo et al., 2006). Carcinogenicity in animal models has been widely used in development of new anticancer therapeutic methods (Kim et al., 2006). The aim of this study is to determine the effect of TAM loaded SLN on the size and ultrastructure of tumor and biochemical serum factors of the rat mammary gland tumor.

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Abbreviations: TAM, Tamoxifen; **SLNs,** solid lipid nanoparticles; **DMEM,** Dulbecco's modified eagle's medium; **FBS,** fetal bovine serum; **H&E,** Hematoxylin-Eosin; **TEM,** transmission electron microscope; **LDH,** lactate dehydrogenase; **ALP,** alkaline phosphatise; **ALT,** alanine aminotransferase; **TAM-SLN,** tamoxifen loaded solid lipid nanoparticles.

MATERIALS AND METHODS

Cells, chemicals and animals

LA7, rat mammary gland tumor cells was a gift from Dr Teo Guan Young (Institute Bioscience, UPM). The cells were maintained at 37°C in a humidified atmosphere of 5% CO_2 in Dulbecco's Modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 µg/ml streptomycin and 100 IU/ml penicillin. Softisan® 154 (S154) or hydrogenated palm oil, was a gift from CONDEA (Witten, Germany). Lipoid S100 (soy lecithin) was a gift from Lipoid KG (Ludwigshafen, Germany). Thimerosal, sorbitol and tamoxifen were purchased from Sigma.

Thirty virgin female Sprague-Dawley rats aged 6 to 8 weeks, weighing 180 - 200 g were purchased from Sapphire Enterprise, Malaysia. The animals were housed at two rats per plastic cages and allowed to acclimate in standard conditions (under a 12 h light/dark cycle) for one week. The rats were given free access to distilled water and commercialized food throughout the experiment.

Preparation of cancer cells for injection

When cells reached 90% confluence, the medium was replaced with fresh medium to remove dead and detached cells. The next day, the medium was removed and cells were washed with phosphate buffered saline (PBS). A minimum amount of trypsin-ethylenediaminetetraacetic acid (EDTA) was added to detach the cells. The cells were obtained immediately by centrifuging at 100 g for 10 min at 4°C, washed twice with PBS, dispersed in PBS and counted using a hemocytometer. Trypan blue staining was used to exclude dead cells. Eventually, cells were suspended in a volume of 300 µl PBS. All harvested cells were used within one hour of preparation.

Preparation of SLN and TAM-loaded SLN

SLN was prepared using the high pressure homogenization (HPH) technique (Schubert and Müller-Goymann, 2005). Briefly, 70 g palm oil (S154) and 30 g soy lecithin (S100) were weighed, mixed and ground in a ceramic crucible and then heated up to 65 - 70°C until a clear yellowish solution was obtained. A solution consisting of 1ml oleyl alcohol, 0.005 g thimerosal, 4.75 g sorbitol and 89.25 ml bidistilled water was added to each of the lipid matrices. The mixtures were stirred on a magnetic stirrer using a teflon coated magnet, for 30 min at room temperature.

The lipophilic drug model, TAM, with concentration of 10 mg was dissolved in 1 ml oleyl alcohol and mixed with 50 mg SLN using an Ultra Turrax® (Ika, Staufen Germany) at 13000 rpm for 10 min. The mixture of TAM-SLN was then incubated at 50 - 60°C while stirring overnight with a teflon coated magnet at 500 rpm and then exposed to air until solidification.

Mammary tumor induction

Twenty-four rats were anesthetized using an intraperitoneal injection with a mixture of ketamine-HCl (150 mg/Kg body weight) and xylazine (10 mg/Kg body weight). The LA7 cells (300 µl containing 6×10^6 cells) were inoculated subcutaneously into the mammary fat pad (right flank) of each rat using a tuberculin (TB) syringe and 26G needle.

Experimental design

The animals were divided into five groups of six animals. The first group comprised untreated normal healthy rats and served as the negative control group, while the second group comprised rats induced to develop mammary gland tumor and served as the positive control. This group of rats received a single dose of 1 ml of soy oil and was ascribed as untreated. The third group of mammary gland tumor-bearing rats was treated weekly with 10 mg TAM dissolved in 1 ml soy oil and assigned the group TAM. The fourth group also comprised the mammary gland tumor-bearing rats and each rat received 10 mg TAM-loaded SLN (a total of 10 mg TAM loaded in 50 mg SLN dispersed in 1 ml soy oil). As a result, this group was assigned the group TAM-SLN. Also, the fifth group comprised rats with mammary gland tumor that received 50 mg TAM-free SLN dispersed in 1 ml soy oil and assigned the group SLN. The treatments were given orally for 4 consecutive weeks to the animals using gastric intubations. The experimental procedure was approved by the Institutional Animal Care and Use Committee (IAUC) of the Faculty of Veterinary Medicine at University Putra Malaysia (UPM), and research was conducted according to the guidelines for the care and use of laboratory animal.

Tumor

All animals were monitored for the development of mammary gland tumor mass by palpation, rolling up the skin and pinching the mass between the fingers (Perumal et al., 2005). The presence of tumor was confirmed by X-ray imaging. The animals were weighed twice weekly and the tumors measured using a digital caliper. The radius of individual palpable tumor was horizontally and vertically measured and averaged. The two dimensional tumor areas were calculated as an ellipse. The volume of tumor (V) was calculated by the formula given by Carlsson et al. (1983): $V = ab^2/2$. Where, 'a' is the longest diameter and 'b' is the shortest diameter of the tumor. At sacrifice, the tumors were removed for histopathological and ultrastructural examination.

Histopathology

At the end of the study, the rats were scarified and examined for tissue abnormalities. Samples of tumor mass from all groups were immediately fixed in 10% formalin overnight, embedded in paraffin, cut into 5 µm sections, placed on slides and stained with Hematoxylin-Eosin (H&E). The tissues sections were viewed under a light microscope (Nikon ECLIPSE TS 100, Japan). The number of apoptotic cells was determined in the sections. The apoptotic cells were recognized among them, as cells with nuclei containing marginated chromatin, homogeneously staining chromatin, dense staining nuclear fragments and apoptotic bodies (membrane-bound structures containing chromatin). Ten fields were randomly chosen from each slide and the number of apoptotic cells were enumerated and expressed as percentage of total cell number in the respective field.

Transmission electron microscope

The ultrastructure of apoptotic cells was examined through transmission electron microscope (TEM). Briefly, the mammary gland tissue sections were fixed in 2.5% glutaraldehyde in 0.1 M PBS, pH = 7.4 for 1 h at 4°C. After fixation, the sections were washed in the same buffer and post-fixed with 1% OsO_4 in 0.1 M PBS for 2 h. The fixed sections were dehydrated in a graded series of acetone, embedded in resin (100%) and polymerized in oven at 60°C for 48 h. Ultrathin sections were cut with a diatom knife on ultra-microtome (Leica UCT), collected on copper grids and stained with uranyl acetate and lead citrate. The sections were viewed under a transmission electron microscope (Hitachi H-7100, Japan).

Table 1. Effect of treatment with TAM, TAM loaded SLN and SLN on animal body weight (g).

Group	Drug treated days			
	0	7	14	28
Control	184.00±14.5	195.00±16.7	204.33±18.86	211.00±21.51
Untreated	164.68±21.2	174.10±20.7	181.34±21.72	184.57±23.93
SLN	165.22±25.9	180.02±22.24	189.90±22.60	194.27±22.69
TAM	177.97±22.6	183.65±11.00	190.26±11.17	196.33±11.00
TAM-SLN	174.02±20.0	181.75±17.80	188.42±17.94	193.88±19.83

All values are expressed as mean ± standard deviation.

Serum biochemical parameters

Blood samples were collected from healthy control and tumor-bearing rats by cardiac puncture using 23 or 26 G needles before and after tumor development. The blood samples were allowed to clot at room temperature and centrifuged at 1000 *g* for 10 min, and serum were separated and analyzed for lactate dehydrogenase (LDH), alkaline phosphatase (ALP) and alanine aminotransferase (ALT) by a chemistry analyzer (HITACHI, 902, Japan) using standard diagnostic kits (Roche).

Statistical analysis

The data obtained were subjected to statistical analysis. The differences in means among the groups were expressed as mean ± standard deviation. All the data were subjected to one-way analysis of variance (ANOVA) followed by Post Hoc multiple comparison and Duncan test after verification of the normal distribution of the data. The Statistical Package for the Social Sciences (SPSS) version 15.0 (SPSS 2006) was used to perform all statistical tests and p-value less than 0.05 were considered significant.

RESULTS

Tumor development

Palpation of mammary gland was performed by rolling up the skin and pinching the mass between the fingers (Perumal et al., 2005) in 6 - 8 early days after injection of LA7 cells. The tumors that developed in 80% of the rats were soft, rubbery and as they grew they become irregular, lobulated and more adhesive to the skin than to the body wall (data not shown).

Body weight, tumor size and volume

The results showed that treatment with SLN, TAM and TAM-loaded SLN did not negatively affect the body weights of these animals (Table 1). The body weight of all animals increased during the study period and there was no significant ($p > 0.05$) difference among treatment groups.

The tumor growth was measured weekly using a caliper. The tumors in the control and SLN groups grew rapidly, reaching an average volume of 1564 ± 540 mm^3 and 2024 ± 708 mm^3, respectively by day 28. Groups TAM and TAM-loaded SLN showed a significant ($P < 0.05$) reduction in their tumor volume when compared with the untreated as positive control and SLN treated animals. The TAM-loaded SLN treatment also had greater reduction (87%) in the tumor volume than free TAM (74%) treatment (Table 2).

Blood biochemical parameters

In this study, blood samples were obtained from the rats at days 0 and 28 of treatment and were used to determine biochemical parameters. As it is shown in Table 3 before treatment at day 0, there was significant increase ($P < 0.05$) in ALP concentrations in experimental groups. In our study, the serum ALP concentration decreased after 28 days of treatment, and still remained significantly higher than the controls. In our study, the serum LDH concentrations in all treated rats increased significantly ($P < 0.05$). After 28 days of treatment, only rats treated with TAM-loaded SLN showed a decrease in serum LDH concentration for the pretreatment period.

Histopathology

The histopathological features of tumors that developed in rats with mammary gland tumors untreated (Figure 1) and treated TAM-free SLN (Figure 2) were reactive lymphatic follicular hyperplasia, typical appearance of neoplastic cell with invasive features, high mitotic activity and necrosis consistent with adenocarcinomas. Histological analysis of tumors from tumor-bearing rats showed mitosis and treatment with TAM-loaded SLN (Figure 3) showed 25 - 50% apoptosis and the mitotic activity was low.

Transmission electron microscopy

The TEM examination showed the presence of tumor cell populations with variable electron density levels termed “light” and “dark” cells. The typical morphological features of apoptosis include margination and condensation of chromatin, fragmentation of the nucleus and formation of apoptotic bodies (Figures 4 - 7).

Table 2. Effect of treatment with TAM, TAM loaded SLN and SLN on animal tumor volume (mm^3).

Group	Drug treated days			
	0	7	14	28
Untreated	657±209	1090±177	1445[a]±549	1564[a]±540
SLN	441±159	874±210	1813[a]±717	2024[a]±708
TAM	384±217	914±316	350[b]±207	99[b]±49
TAM-SLN	467±340	807±351	448[b]±218	60[b]±29

All values are expressed as mean ± standard deviation; [ab]Means with different superscripts are significantly different; $p < 0.05$.

Table 3. Statues of serum biochemical parameters of animal before and after treatment of animals with TAM, TAM loaded SLN and SLN.

Group	Serum biomarker		
	ALT (U/L)	ALP (U/L)	LDH (U/L)
Control			
(Healthy rats)	49.0[a]±2.5	62.0[a]±12.8	584[a]±117.25
Untreated			
Pre treatment	82.8[a]±44.7	236[b]±45.6	1952.0[b]±820
Post treatment	52.7[ab]±9.5	203[b]±24	2431.0a[b]±1471.2
SLN			
Pre treatment	63.3[a]±43.4	289.0[b]±150.7	2464.7[b]±403.23
Post treatment	43.0[a]±4.21	273.0[b]±63.5	3885.8[b]±660.65
TAM			
Pre treatment	64.7[a]±46.90	237[b]±78.17	1349.0[ab]±339.0
Post treatment	95.5[b*]±34.5	229.8[b]±60.4	2441.7[ab]±1126.8
TAM-SLN			
Pre treatment	62.3[a]±20.1	231[b]±3	2290.9[b]±782.5
Post treatment	57.1[ab]±16.5	187[b]±68.6	2161[ab]±1163.54

All values are expressed as mean ± standard deviation; [ab]Means with the different superscripts are significantly different; $p < 0.05$; *shows significant difference in column at $p < 0.05$ as compared to before treatment; ALT = alanine transaminase; ALP = alkaline phosphatase; LDH = lactic acid dehydrogenase; SLN = solid lipid nanopartivle; TAM = tamoxifen; TAM-SLN = tamoxifen loaded SLN; Pretreatment = Day 0; Posttreatment = day 28.

DISCUSSION

Tumors in the control and SLN groups grew rapidly, while groups treated with TAM and TAM loaded SLN showed a significant ($P < 0.05$) reduction in tumor volume. This finding is similar to those reported earlier, when TAM alone and TAM plus riboflavin, niacin and CoQ_{10} were used (Perumal et al., 2005).

Blood biochemical parameters can be useful in the diagnosis of breast cancers because it can be easily done in most diagnostic laboratories. In one study, significantly increased ALP was observed in bone and liver metastases of breast cancers (Sandhya and Sharma, 2004). During the treatment period, although the serum ALP concentration decreased, it still remained significantly higher than the controls. This study suggests that TAM treatment did not significantly affect serum ALP concentration in tumor bearing rats concurring with a previous report (Abdel-Kader and Hemieda, 2007). The ALP concentration of TAM-loaded-SLN-treated rats with tumor decreased by 20% from the control while that of the free TAM-treated group decreased by 4%. It should be noted that ALP is not an organ specific enzyme. The enzyme is present in many tissues of the body. Thus ALP may not be useful in the diagnosis of mammary gland tumors. Liver disorders can be determined by serological markers such as alanine transaminase. The abnormal increase of serum ALT concentrations, may suggest liver metastasis (Wang et al., 2006) and hepatotoxicity (Lynch et al., 2005). The lack of change in ALT in this study suggests that there is no liver metastasis in these rats with mammary gland tumor. By the end of the study, the level of ALT in the free TAM-treated rats only showed significantly increased ($P < 0.05$) serum ALT. It is reported that treatment with TAM can significantly increase serum activity of ALT which is correlated with hepatotoxicity (Abdel-Kader and Hemieda, 2007). How-

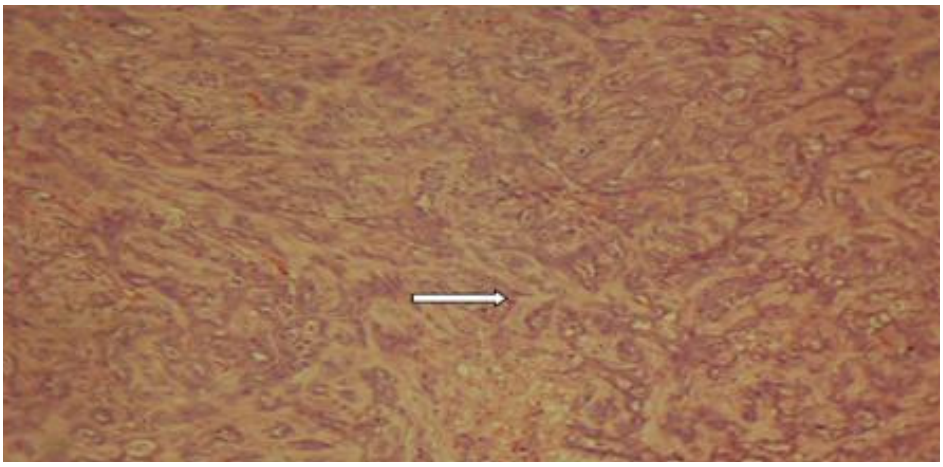

Figure 1. Rat mammary gland tumor section showing adenocarcinoma (arrow) (H&E, x25).

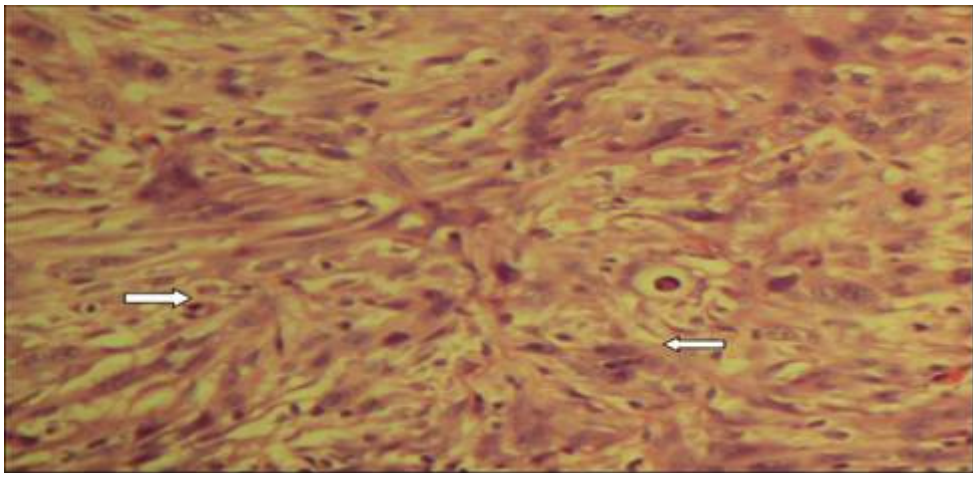

Figure 2. Rat mammary gland tumor after oral treatment with solid lipid nanoparticle showing high mitotic activity (arrow) (H&E, x40).

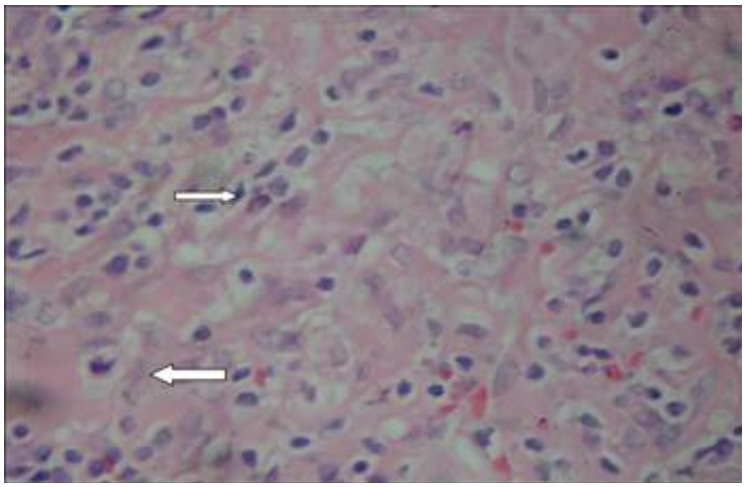

Figure 3. Rat mammary gland tumor treated with tamoxifen showing apoptosis (arrow) (H&E, x40).

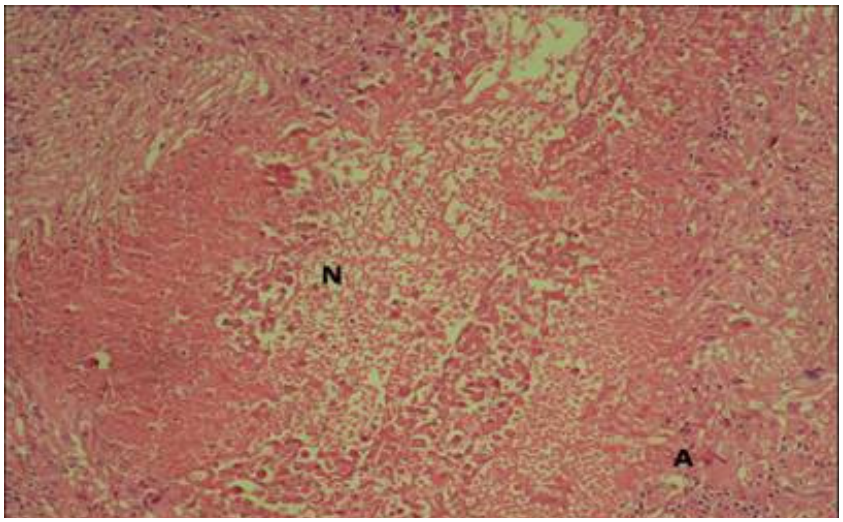

Figure 4. Rat mammary gland tumor treated with tamoxifen-loaded SLN showing 50% apoptosis (A) and 50% necrosis (N) (H&E, x25).

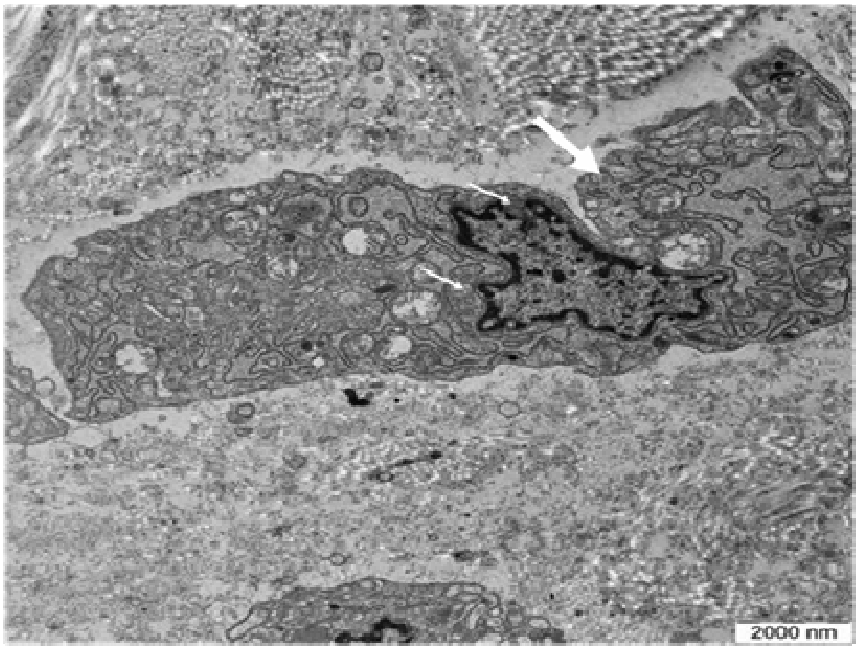

Figure 5. Untreated mammary tumor cells of female Sprague-Dawley rat induced with subcutaneous injection of LA7 cells, showing normal structure (thick arrow) and chromatin condensation at the perinuclear margin (thin arrow).

ever, when TAM is encapsulated in SLN, the same effect was not seen, suggesting that loading TAM in SLN had eliminated the hepatotoxic effect of the drug. The serum LDH concentrations significantly increased in patients with endometrial adenocarcinoma, ovarian adenocarcinomas and breast cancers (Koukourakis et al., 2008). However, this enzyme has been suggested not to be useful in the diagnosis of metastatic cancers (Sandhya and Sharma, 2004). In our study, the rats treated with TAM-loaded SLN only showed decrease in serum LDH that reflects lower tumor volume resulting in decreased release of cytoplasmic LDH when compared to the TAM-treated group. Therefore this novel drug delivery may represent a useful strategy for cancer treatment.

The histopathological examination revealed adenocarcinomas feature of the mammary gland tumor. In adenocarcinoma, malignant tumors are derived glandular epithelium and have an adenomatous appearance. The

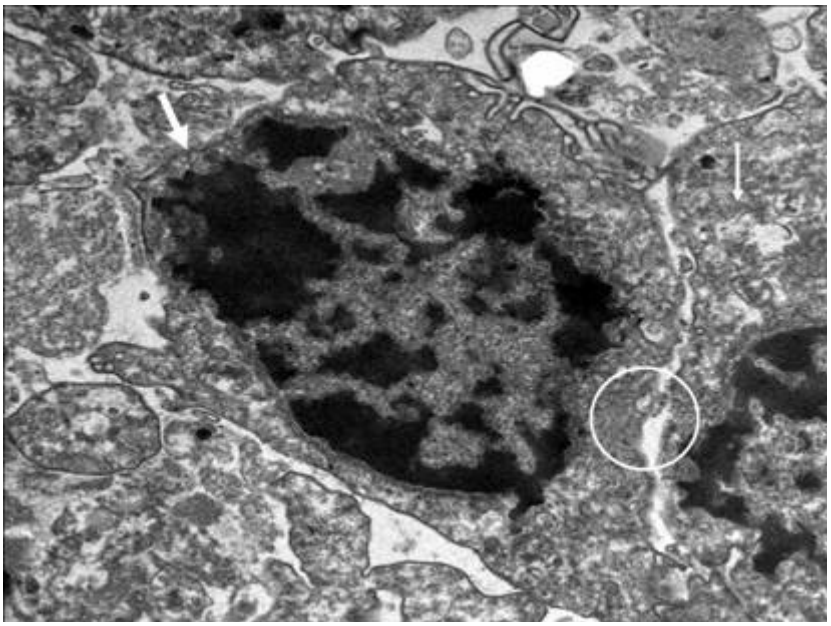

Figure 6. Rat mammary tumor treated with tamoxifen-loaded solid lipid nanoparticle, showing early apoptosis (cell shrinkage, chromatin condensation (thick arrow) and disrupted cells (thin arrow) with wide tight junction (circle).

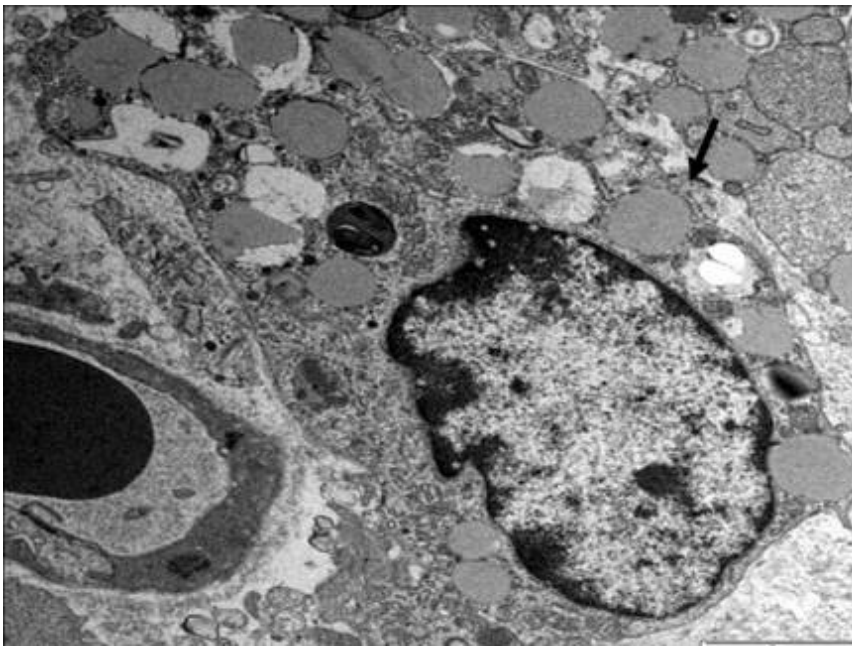

Figure 7. Rat mammary tumor treated with tamoxifen showing apoptosis (amorphous cytoplasm) and intact plasma membrane (arrow) of the cell.

epithelium cells are arranged in gland-like structures surrounding a lumen. Nucleoli are often prominent and mitotic figures are abundant. Necrosis is a common feature of adenocarcinomas and the necrotic tumors will be softer and possibly fluctuant (Young and Hallowes, 1973) as shown in Figure 3. Although routine light microscopy examination has been established as an adequate assessment in the diagnosis of the tumor mass, TEM allows the view of the cell ultrastructures, which could provide detailed information on the cellular

changes. Through TEM, programmed cell death or apoptosis in the mammary tumor gland of the rats treated with TAM (Figure 5) and TAM loaded SLN (Figure 6) may be demonstrated. The high percentage of apoptosis in group treated with TAM-loaded SLN showed that the antitumoral efficacy of tamoxifen is still high even when TAM is encapsulated in SLN.

Apoptosis is 'silent death' and happens without inflammation, because dying cells are phagocytosed by intraepithelial macrophages and alveolar epithelial cells (Tatarczuch et al., 1997). Both cell proliferation and apoptosis have been used clinically for assessment of tumor prognosis because there is a relationship between proliferation index and malignancy in many tumors (including breast cancer) and for the analysis of the response of cancer cells to clinical interventions (Beresford et al., 2006; Railo et al., 2006). Loading of tamoxifen inside SLN enhanced the treatment efficacy of tamoxifen and decreased its side effect on liver and biochemical serum parameters. However, further studies using clinical trials will be needed to determine if the results obtained in this study can be extrapolated to humans.

Conclusion

The study showed that mammary gland tumors can easily be induced by subcutaneous injection of LA7 cells into the mammary pad of the female Sprague-Dawley rats. This method of tumor induction is safe, quick, economical and practical with tumor development occurring within 7 to 10 days of injection. This method eliminates the hazards associated with the use of carcinogens and immunosuppressive compounds. The histopathological and ultrastructural feature of the induced tumor resembled that of the spontaneous mammary gland tumors and showed similarities to human breast cancers, particularly in morphological feature and cellular components. One of the main advantages of this tumor model is that it is easily reproducible. However, a previous study suggested that the rat mammary gland tumor model rarely develop metastasis and expressed cell surface molecules different from the original tissue (Jacob et al., 2004). However, this may just be an evidence of mutation that could be a characteristic of tumors. In conclusion, this rat model can be applied for the study of breast cancers. This study also determined the effect of free TAM, TAM loaded-SLN and TAM-free SLN on the induced rat tumors. The result suggests that TAM-loaded SLN has similar effect on the tumor as free TAM, which promoted apoptosis in the rat mammary gland tumor. The efficacy of free-TAM and TAM-loaded SLN was similar. However, the TAM-loaded SLN showed a more prolonged effect suggesting that incorporation of TAM in SLN is suitable for delayed drug release in the chemotherapy of breast cancers, while decreasing the hepatotoxic effects. This study suggests that TAM-loaded SLN is suitable for used as a new drug delivery system in the treatment of breast cancers. Thus, further studies are warranted to further develop and optimize this drug delivery system in the treatment of cancers.

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