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Research Article

Toluidine blue loaded transferosomes for topical photodynamic therapy: formulation and characterization

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ABSTRACT

Targeted drug delivery systems have distinct advantages over conventional dosage forms. In the present approach, flexible liposomal drug carriers (transferosomes) were formulated and their physicochemical properties were studied compared with conventional liposomal and niosomal formulae. Lipid vesicles composed of lecithin, cholesterol, span 60, and sodium deoxycholate (SDC) were prepared by thin film hydration method. Photodynamic treatment of subcutaneous Ehrlich tumor using prepared and pharmaceutically studied hydrogel containing 1% hydrophilic toluidine blue (TB) loaded transferosomes was evaluated. The results showed that transferosomes containing both span 60 and SDC were of the highest entrapment efficiency (> 82%), particle size (1.36 µm) and released 52% of its content within 2 hours. The *in vivo* study on BALB/C mice showed that there was a marked increase in the survival time and decrease in the tumor size in mice group treated with transferosomal (span 60 and SDC) hydrogel for 1h followed by irradiation with 90J/cm^2 from 650 nm diode laser twice weekly compared with animals treated with free TB and irradiated only. Furthermore, there was a marked deep necrosis of tumor cells, which may be attributed to the deep penetration of TB through skin layers. These findings suggest the use of TB loaded transferosomes in topical targeted photodynamic treatment of skin tumors.

Keywords: In vivo studies; Photodynamic therapy; Subcutaneous Ehrlich tumor; Toluidine blue; transferosomes.

INTRODUCTION

Photodynamic therapy (PDT) is one of the most promising new techniques being explored for use in a variety of medical applications and is known as a well-recognized treatment for the destruction of tumors (Dougherty, 2002). The use of PDT for the treatment of various types of disease is limited due to the inherent features of photosensitizers. These include their high cost, extended retention in the host organism, skin photo toxicity, low solubility in physiological solutions, and low targeting effectiveness. These disadvantages generally lead to the administration of very high doses of a photosensitizer, which dramatically increase the possibility of accumulation of the photosensitizer in non-damaged tissues and the accompanying risks of affecting non-damaged sites (Torchilin, 2001). However, some phenothiazinium photosensitizers, such as toluidine blue (TB) and methylene blue (MB), have the promising applications in photodynamic therapy of tumor (Harris et al., 2005; Rohs et al., 2000; Tremblay et al., 2002). These potential anti-tumor drugs can kill cancer cells through indirect or direct reactions with DNA (Tuite et al., 1993).

Since the application of photodynamic therapy is used in treating many dermatological problems and also in cancerous and precancerous skin conditions it is necessary to develop an effective drug delivery system which can be applied topically (Montanari et al., 2007). Topical application reduces side effects like skin photosensitivity in normal skin cells and targets the abnormal cells in the skin and beneath.

In topical delivery, penetration depth and selectivity depend on drug's physicochemical properties and not on the excipients (creams, gels, lotions) (Weiner & Lieb, 1998). The permeability of the drug molecule is directly related to its lipophilicity and inversely proportional to molecular size. Attempts have been made to overcome this limitation. Different drug delivery systems are described in prior research to overcome the skin barrier (El Maghraby et al., 2008). Liposomes have been much investigated as a system for dermal application (El Maghraby et al., 2006; Meizei & Gulasekharam, 1982). But most studies have shown that liposomal vesicles are not very efficient in penetrating the physical skin barrier due to their rather rigid structure

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(El Maghraby et al., 1999). On the other hand, transfersomes are lipid vesicles that incorporate molecular edge activators, which can deform and squeeze through microspores in stratum corneum (Cevc & Blume, 1992; Cevc & Blume, 2004; Honeywell-Nguyen & Bouwstra, 2005). Similarly, niosomes (Balasubramaniam et al., 2002; Collins et al., 1993; Gianasi et al., 1997; Lu et al., 2003) are other carrier systems used for the dermal application of various drugs. Niosomes are based upon nonionic surfactants. Transferosomes combine the advantages of liposomes and that of niosomes, possessing high penetration ability as well as flexibility. The success of such carrier systems depends on the ability of the drug loaded carrier to penetrate through the skin in sufficient quantities to achieve its desired therapeutic effect.

The present work aims to overcome the impermeability of intact human skin to penetration in a reversible and non-damaging manner as well as to design therapeutically effective topical drug delivery systems for transporting hydrophilic low cost photosensitizer TB to be administered through photodynamic therapy. Hence, this work describes the formulation and characterization of highly flexible and penetrating liposomal carrier systems for topical application and the use of this delivery system for efficient transport of the photosensitizer across stratum corneum to deeper layers within the skin for treatment of subcutaneous Ehrlich tumor in albino mice skin.

MATERIALS & METHODS

Reagents and chemicals

Cholesterol (CHOL), phosphatidylcholine (Lecithin), sodium deoxycholate (SDC), sorbitan monostearate (Span 60) were purchased from Sigma Co. (St. Louis, MO). Toluidine blue (TB), carboxymethylcellulose sodium (CMC Na), propyl paraben and methyl paraben were purchased from Sigma-Aldrich, Buchs. All other materials used in the study were of analytical grade. For in vivo study Ehrlich ascitis carcinoma cells were supplied kindly from National Institute of Cancer research, Cairo University

Preparation of TB loaded vesicles

Transferosomes composed of Lecithin/CHOL/Span 60/SDC at different concentrations were prepared by thin film hydration method (Bangham et al., 1965) and named T1 to T4 in addition to conventional liposomal sample (L) and niosomal sample (N) as shown in Table 1.

In brief, phospholipids and CHOL were dissolved in chloroform: ethanol (1:1 v/v) solution. The solution was then evaporated to dryness using a rotary evaporator (Heidolph-Eleksto GmbH+CoKG, Germany), rotated under vacuum at a speed of 90 rpm at 45°C in order to produce a thin dry film. The flask was left in a vacuum desiccator overnight and hydration of the dry lipid film was accomplished by 10 ml of TB solution (0.1 mg/ml) nitrogen purged PBS pH 5.5 for 1 h. Liposomal suspension was sonicated in ultrasonic water bath (Retsch, Germany) for 30 min. The suspension was left 4 h at room temperature (RT) to complete hydration and then stored at 4°C overnight before use. Free TB was removed by gel chromatography using sephadex-G75 gel column (2 cm in diameter and 30 cm in length). Phosphate buffer pH 5.5 was used for elution. The vesicles were eluted first from the column according to their sizes; free TB was retained in the column for long time sufficient for collecting all the vesicles. The obtained dispersion was used for characterization studies.

Table 1: Characterization and composition of the lipid vesicles prepared by thin film evaporation method

F code	Lecithin (mg)	CHOL (mg)	Span 60 (mg)	SDC (mg)
L	100	50	-----	------
N	------	50	100	------
T1	50	35	25	-----
T2	50	35	25	25
T3	50	35	-------	50
T4	50	-------	-------	50

L: liposome, N: niosome, T: transferosome

Determination of entrapment efficiency (EE %)

One ml of each prepared samples was subjected to high speed centrifugation (KONTRON Centrikon, type 42K, Italy) at 10 000 rpm for 10 min. The precipitated vesicles were mixed with 2 ml 1% Triton X-100 in PBS pH5.5 and filtered through 0.2 µm polycarbonate filter (Whatman International Ltd. Springfield mill, Maidston, Kent, UK). The clear solution was measured for encapsulated TB spectrophotometrically by using Shimadzu UV-1650 UV–VIS double beam spectrophotometer (Shimadzu, Japan) at λ_{max} 630 nm. A base line correction was done using a placebo dissolved empty vesicle dispersion diluted suitably with Triton X 100 to nullify any possible absorption arising from the soluble lipid (Chang et al., 2008).

For initial TB concentration 1 ml of the freshly prepared MLV (before gel filtration) dispersion was diluted with 2 ml 1 % Triton X-100, filtered and TB concentration determined: results were the mean of three determinations (n=3)

EE% = (drug amount in precipitated fraction/ total drug amount initially used) x 100

Vesicle size distribution

Size distribution was measured through the use of optical microscope type CKX41, Olympus, U.K attached to color CCTV Camera Panasonic WV-CP 240/G Manufactured by Panasonic System solution Suzhou. Co., Ltd. Suzhou China. MLV formulations (10µL) were diluted 10 times with PBS and the number of vesicles/mm^3 was examined for the size through the use of Microme-

trics SE/CMOS Version 2.6, ACCU-SCOPE INC., using a hemocytometer (Feinoptik, Blakenburg, Germany).

Leakage test of TB from lipid vesicles

The drug release profile of MLV encapsulating TB was studied by directly mixing 1 volume of the MLV suspension (0.5 mL) with 10 volumes of PBS (4.5 mL). The samples were incubated at 37°C with gentle agitation and 1mL aliquots were withdrawn at different time intervals up till 5 h. For quantification of TB, the aliquots were centrifuged at 10 000 x *g* for 15 minutes and their supernatant containing the released TB was quantified by UV spectrophotometry as described above. The vesicles were resuspended in fresh PBS and returned back to the samples. Correction dilution factor was done for the release data.

Preparation and characterization of hydrogel

Based on the results of entrapment efficiency and size distribution, only formula T2 was selected to be formulated in 5 % carboxymethylcellulose sodium (CMC Na) hydrogel. Propyl and methyl paraben were dissolved in 20 ml distilled water in aluminum foil covered beaker. TB (0.1%) in freshly prepared sample of T2 (higher EC and smaller size) was added with gentle agitation. Na CMC (5%) was then added to the solution with continuous agitation at 50 rpm till complete swelling. The gel was left overnight in vacuum desiccators. Similar gel formula containing an equivalent amount of free TB (0.1 % TB) was prepared applying the previous procedure.

The prepared hydrogels were evaluated for some pharmaceutical properties such as percent drug content, uniformity, pH, viscosities, drug release and some physical characters such as color, texture and transparency. For determination of percentage dye content, 1 g samples were taken from different places in the gel formulations and analyzed by dissolving each sample in 100 ml PBS pH 5.5 then measured spectrophotometrically at λ_{max} 630 nm. Viscosities of the prepared gels were determined using Brookfield Viscometer, spindle 52 (DV-III, USA); the flow behavior was also studied by measuring the viscosity at different rates of shear. The release of TB from free and transferosome gel preparations was studied by injecting 2 g samples into dialysis bags (Visking 20/32, made of cupropban) with an effective diffusion area of 9.6 cm^2 and previously kept overnight in the dissolution medium. The dialysis bags were placed in 400 ml phosphate buffer pH 5.5 in dissolution apparatus (Hanson research, SR 8, USA) at 36°C. Aliquots were taken at regular time intervals and were replaced by an equal volume of fresh phosphate buffer. Withdrawn aliquots were analyzed spectrophotometrically. The experiments were conducted independently in triplicate.

In vivo studies

All animal experiments were performed following 'Principles of laboratory animal care' (NIH publication No. 85-23, revised in 1985), as well as specific institutional laws on 'Protection of Animals' under the prevision of authorized investigators. Forty female BALB/C mice of average weight 20 ± 3 gm were used in the study. Mice were housed at room temperature with regular light/dark cycle with free access to diet and water. Eight animals were kept healthy in a group and the other thirty two animals were injected in the thigh of the hind limb, each with 2×106 Ehrlich ascites's carcinoma cells in 0.2 ml normal saline. Mice were housed at room temperature with regular light/dark cycle with free access to diet and water.

Photodynamic therapy (PDT) protocol

Seven days after tumor implantation, solid tumor was observed. Animal's treatment protocol started at the 8^{th} day after tumor implantation. All animals injected with tumor cells were subjected to hair shaving with electrical shaver at the site of the tumor and subdivided into 4 separate groups each comprised 10 animals as follows:

Control group (C): animals which did not receive any treatment;

Group (L): animals treated with 90 J/ cm^2 laser energy delivered from Diode laser of 650 nm continuous wave (type Intelite R 650-500 with maximum output of power 500 mW made in USA), twice weekly;

Group (FTB): animals treated with 1g 1% free TB gel applied topically on shaved skin for 1 hour, followed by 90 J/cm^2 laser irradiation twice weekly;

Group (TTB): animals treated with 1g gel containing 1% TB loaded in T2 for 1hour followed by 90 J/cm^2 laser irradiation twice weekly.

The assessment and evaluation of the effect of PDT on the tumor in different animal groups was done through measurement of the tumor volume, survival assay, and histopathological examination of tumor specimen.

The tumor growth was followed by measuring the three mutually orthogonal tumor diameters with a caliper. The tumor volume was calculated from the equation:

$$V = \pi/6\ abc\ cm3 \quad (1)$$

Where V is the tumor volume, a, b and c are the orthogonal dimensions of the tumor. The tumors size was measured weekly and the changes in the volume of the tumor were represented as the mean ± S.D.

The survival assay was evaluated as follows: the day of death of each mouse from each group was recorded and the percent of surviving animals was calculated. The experiment was terminated 78 days post tumor implantation.

A histopathological examination was performed for all groups. Three mice of the control group were sacrificed with overdose of a mixture of 90 mg/kg of keta-

mine and 9 mg/kg of xylazine (i.m.) 7 days post tumor implantation. Three mice of groups L, FTB and TTB were sacrificed at 7, 15 and 21 days post PDT respectively. The tumor tissues were excised, fixed in 10% formalin and embedded in paraffin. The blocks were cut 3µm thick, stained with heamatoxylin and eosin and pathologically examined by optical microscope type BML 2200 (Bio-med, Labrgerate Ges.m.b.H made in Japan attached to color CCTV Camera Panasonic WV-CP 240/G Manufactured by Panasonic System solution Suzhou. Co., Ltd. Suzhou China.

One way analysis of variance ANOVA followed by Turkey-Kramer Multiple comparison test using GraphPad Instat software v.2.05 to determine the statistical significance for encapsulation efficiency, release data and animal's tumor volume. All p-values were two tailed, and differences were considered significant when the p -value is less than 0.05 and highly significant when less than 0.01.

RESULTS AND DISCUSSION

Preparation and characterization of TB vesicles

All of the vesicles formed showed mean size diameters ranging from 0.3 to 4.1 µm (Table 2). Smaller vesicles with higher entrapment efficiency may have resulted from the incorporation of SDC in the transferosomes. The results reveal that although niosomes were the largest in size, they showed the lowest entrapment efficiency (Table 2). The loading of TB was higher for transferosomes than liposomes of approximately the same particle diameter and size distribution. Also, the presence of cholesterol in the prepared transferosomes with the same composition had no significant effect on the vesicles size or their entrapment efficiency.

Table 2: Entrapment efficiency and particle size of the prepared vesicle

Type	Entrapment%± SD	Size µm ± SD
T1	39 ± 2.5	2.2 ± 0.11
T2	82.30 ± 3.6	1.5 ± 0.075
T3	61 ± 3.4	0.4 ± 0.02
T4	56.70 ± 3.1	0.3 ± 0.015
L	36 ± 2.4	1.1 ± 0.05
N	15 ±1	4.1 ± 0.21

SD= standard deviation, T=transferosome, L= liposome, N=noisome

Furthermore, the use of chloroform: ethanol (1:1 v/v) mixture and the rotational speed of the flask demonstrated discernible influence on the thickness and uniformity of the lipid film. Although using the solvent mixture showed slower evaporation than using chloroform alone, it yielded a uniform thin, lipid film yielding vesicular preparation of desired characteristics on hydration. Lower and higher rates of rotation resulted in preparation with noticeable aggregated non-vesicular lipid artifacts.

Incorporation of cholesterol was known to influence vesicle stability and permeability. Cholesterol makes the membrane more ordered and abolishes the gel to liquid phase transition of the vesicle system hence; it was able to effectively prevent leakage of drug from vesicles. On the other hand, higher amounts of cholesterol may compete with the drug for packing space within the bilayer, hence excluding the drug as the amphiphiles assemble into vesicles. Moreover, the decrease in the entrapment efficiency with increasing cholesterol ratio above a certain limit may be due to the fact that cholesterol beyond a certain concentration can disrupt the regular linear structure of vesicular membranes (Agarwal et al., 2001; Mokhtar et al., 2008). The high entrapment efficiency of T2 may be attributed to the presence of two types of surfactants rendering the membrane more flexible to engulf large amounts of drug, which may be verified from the results of entrapment efficiency of T1 and T 3. The use of either span 60 (T 1) or SDC (T 3) resulted in the decrease of entrapment efficiency.

The cumulative amounts of TB released from the vesicles in PBS pH 5.5 after incubation at 37^{o}C for different periods of time up to 6 h are presented in Fig. 1. All lipid vesicles release more than 95% of its dye content after 6 h. Statistical evaluation for percent dye released showed that there is no significant difference between all preparations in release (GraphPad Prism version 5.0).

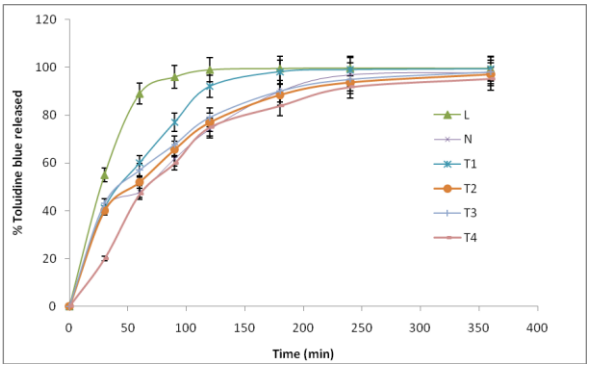

Figure 1: Percentage Toluidine blue released from different formulations as a function of incubation period

The release profiles of TB from lipid vesicles of different compositions were apparently biphasic release processes. Rapid dye leakage was observed during the initial phase where about 47–89% of the entrapped dye was released from various formulations in the first hour. However, during the following five hours slow release occurred in which only further 10–28% of TB was lost from different vesicle preparations. Similar results were obtained from the study of flurbiprofen release from different niosomal preparations (Mokhtar et al., 2008).

It was found that all preparations release their contents according to first-order model. Furthermore, the

use of mixed lipids enhances the entrapment efficiency of the prepared transferosomes.

Characterization of TB loaded vesicular hydrogel

Formula T2 was chosen to be incorporated in 5% CMC Na gel based upon the high entrapment efficiency, reasonable particle size as well as good release properties. All gel formulae were elegant with blue color. Analyzed samples show that there is homogeneity by about 97-103 % in the T2 gel and 98-102 % in free TB gel with no significant difference ($p > 0.05$). All of the prepared gel preparations exhibited pseudoplastic flow with a thixotropic behavior which is a desirable character in pharmaceutical gels (Figure 2) with viscosities of 4675 cp and 5060 cp for FTB gel and TTB gel respectively showing no significant difference between their values ($p>0.05$).

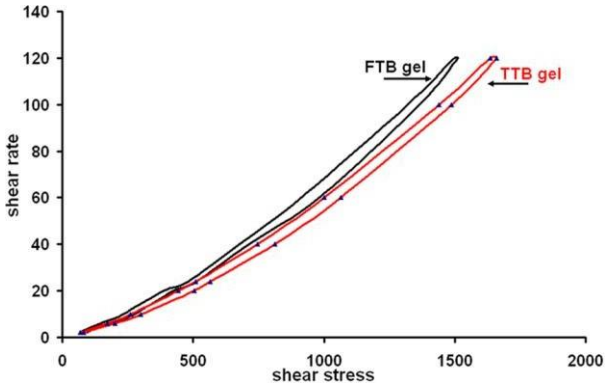

Figure 2: Shear stress as a function of shear rate for 5% CMC hydrogel. Showing the exhibited pseudoplastic with thexotropic property which was identical for hydrogel containing transfersomes loaded with TB

From Fig. 2, it could be observed that for each formula the η_{max} is less than η_{min}. So, there is a structural breakdown of the polymeric chains during shear. Fig. 3 shows percentage TB released from the two gel formulations that containing free TB (TB gel) and gel containing T2 formula (T TB gel). From the figure and from the statistical analysis of release data, there was a significant difference ($p<0.05$) in release rate between the two gel formulae. Both had the first-order model for release.

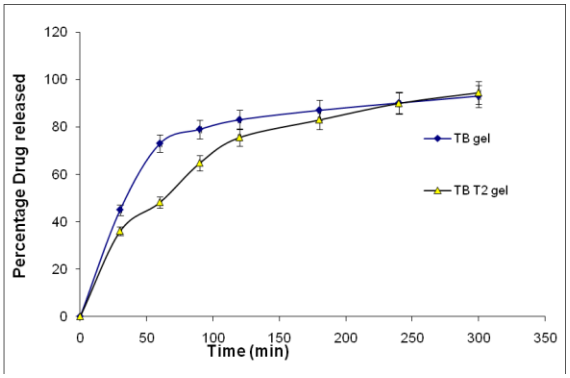

Figure 3: Percentage drug released from hydrogel formulation as a function of incubation periods

CMC Na was used in the preparation for its biocompatibility as well as being reported as non toxic. In most of the studies conducted up to date, lipid vesicle incorporation within hydrogels did not result in significant modifications of their rheological properties. Indeed, it has been reported that liposome addition in pluronic-alginate gel systems at low lipid concentrations (70 µg/ml) has a slight effect on the structural buildup, but no effect on the final system properties (Mourtas et al., 2008a). Gel rheological properties may influence drug release kinetics. Thereby, especially in cases in which the formulation of complex gels is used as a mean to load a depot amount of drug in gels (intended for slow release over a prolonged time period), where high vesicle concentrations will be needed, the implications of the vesicles on the final product rheological profile should be seriously considered as it may affect drug release profile (Mourtas et al., 2008b).

The release of TB was modified when transferosomes were loaded in the gel; this may be attributed to the presence of cholesterol which impacts rigidity in the vesicle membrane. It has been previously proven that in the case of hydrophilic drugs, vesicle-membrane rigidity is the most important parameter that determines the release rate of the drug from liposomal gels (Spyridon et al., 2007).

In vivo study

Regarding the tumor growth studies (Fig. 4), animals from control group (C) did not survive more than 22 days post tumor injection. The tumor growth and size was markedly reduced for photodynamic treated animals in group (TTB). This indicates that transferosomes encapsulating TB delivered most of their drug in the target tumor and the animals had significantly ($p > 0.05$) longer survival period (Fig. 5), to about 2.5 months, as compared with animals in control group (C), (L) and (FTB). The number of animals surviving from each group after implantation of the tumor was considered as a function of incubation period.

Fig. 6 (A, B, C and D) shows histopathological sections of the studied groups in which the subcutaneous neoplastic cells with hyperchromatic and pleomorphic nuclei and scant eosinophilic cytoplasm are arranged in sheets as shown in scanning power. Higher magnification (400 X) shows the histological section of a tumor from group (L) animals, islands of aggregated neoplastic cells with hyperchromatic and pleomorphic nuclei and scant eosinophilic cytoplasm were clearly observed as in control untreated group. The atypical cells vary in size and shape and islands of necrotic cells are also seen. A partial cure of the tumor cells with residual tumor cells at the periphery of the subcutaneous tissue is observed from the light microscopic image of the histological section of the tumor from group (FTB) animals (Fig. 6 C). While, there is a nearly complete cure of the aggregates of neoplastic cells with normal subcutaneous tissue in the histological section in the

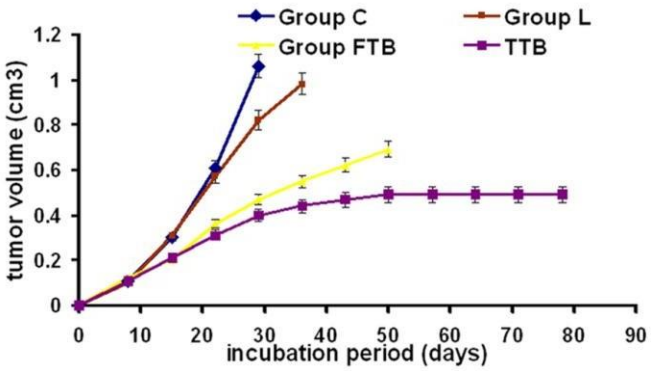

Figure 4: Variation in the average tumor size as a function of the incubation period for different mice groups

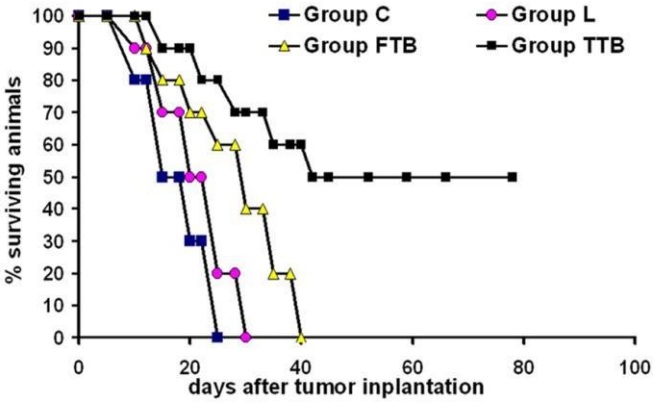

Figure 5: Percentage survival animals in different groups as a function of days post tumor inoculation

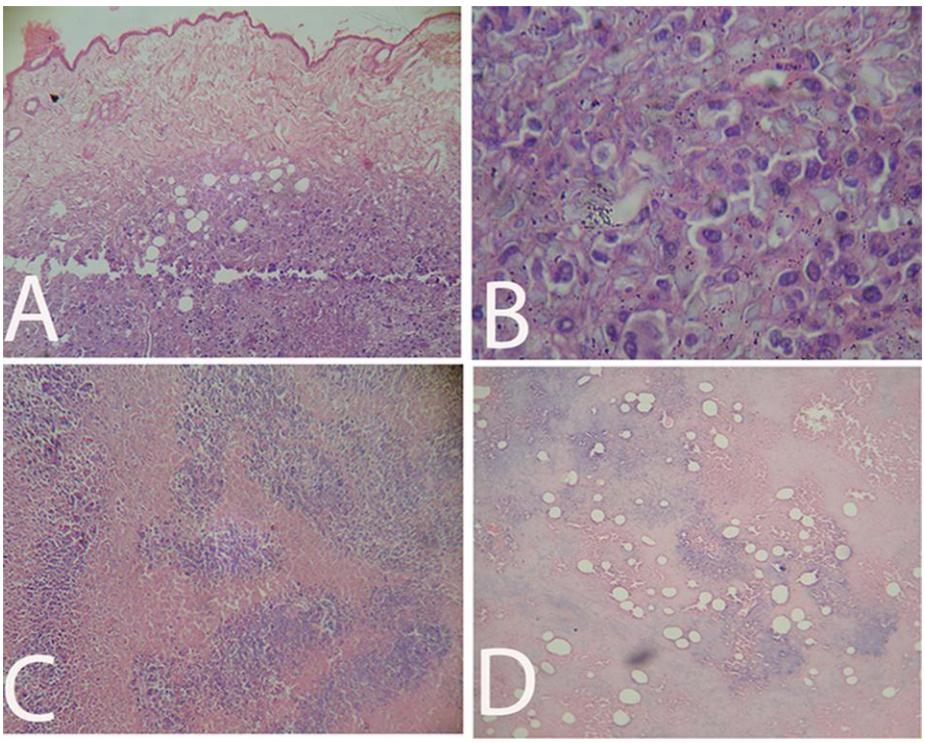

Figure 6: Histopathological skin sections of mice

Control group C (A) showing with scanning power (200 X) the subcutaneous tumor cells beneath the dermal layer, group treated with L (B), showing neoplastic cells with hyperchromatic and pleomorphic nuclei and scant eosinophilic cytoplasm. Group treated with free TB (C) is showing islands of cytoplasm of some tumor cells and group treated with TTB (D) 30 days post-injection is showing vanished tumor cells

tumor of group (TTB) animals (Fig. 6 D). It may be persumed that apoptotic cancer cells were eliminated. These apoptotic cells were rapidly recognized by the phagocytic cells and removed from the treated organ. However the mechanism of recognition of the phagocytic cells to the apoptotic cells when treated chemically is still unclear.

Several studies investigated possible mechanisms by which deformable vesicles could improve skin delivery of drugs. Two mechanisms were proposed (Honeywell-Nguyen & Bouwstra, 2003).

First, vesicles can act as drug carrier systems, whereby intact vesicles enter the stratum corneum carrying vesicle-bound dye molecules into the skin (mechanism 1). Second, vesicles can act as penetration enhancers, whereby vesicle bilayers enter the stratum corneum and subsequently modify the intercellular lipid lamellae. This will facilitate penetration of free dye molecules into and across the stratum corneum (mechanism 2). For hydrophilic drugs, the penetration enhancing effect seems to play a more important role in the enhanced skin delivery than in case of lipophilic drugs (as for many penetration enhancers), since permeation of hydrophilic molecules tends to be relatively slower and hence more enhanceable (Williams & Barry, 1991; Williams, 2003). Results of the current study support the existence of the two proposed mechanisms as the chosen formula expressed a good release of TB from vesicles which form a concentration gradient to facilitate the penetration with the enhancing effect of the phospholipids (mechanism 2). The intact vesicle permeation mechanism will have also an important role specially in improving skin deposition. However, as previously mentioned, drug release from vesicles in the stratum corneum is an important step that affects transdermal flux (Honeywell-Nguyen & Bouwstra, 2003).

Note worthy, after laser treatment, sensitizer embedded into vesicles can isomerizes and/or induce photodamage to intrinsic membrane components such as unsaturated lipids, leading to the destabilization of the structure and release of trapped materials (Khairutdinov & Hurst, 2004).

CONCLUSION

TB loaded liposomal, niosomal and transferosomal vesicles were successfully prepared and evaluated invitro. Based upon the in vitro evaluation a transferosomal formulation was further investigated by incorporation in a final topical dosage form; hydrogel. The in vivo characterization revealed that loading TB in transferosomes composed of phospholipids phosphatidyl choline and span 60 and the incorporation in a carboxymethyl cellulose based hydrogel could be considered as a promising delivery system for topical photodynamic therapy in coetaneous and subcutaneous skin diseases.

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