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OCULAR CONTROLLED DELIVERY OF PREDNISOLONE USING LIPOSOMES AS EFFECTIVE CARRIERS SYSTEM

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ABSTRACT

The potential use of liposomes for ocular delivery of encapsulated prednisolone was evaluated in the rabbit's eye. Liposomes used in this study, multilamellar and unilamellar, were prepared from dipalmitoyl phosphatidylcholine and cholesterol, with or without a charge inducing agent. Stearylamine and dicetyl phosphate have been incorporated so as to import either a positive or a negative surface charge to liposomes. Sequential extrusion of liposomes through polycarbonate membranes was used to improve the homogeneity of the preparations. Results demonstrated that the maximal encapsulation of drug was achieved by using multilamellar liposomal formulations prepared with negatively charged lipid. The in-vivo performance of various formulations of the drug was assessed on the basis of the influence of the drug on the intra-ocular pressure. The data were interpreted in terms of area under the intra-ocular pressure/time curve, duration of action, the maximum response, and time of maximum response which have been taken in consideration as paraeters of drug activity. This study showed that the intra-ocular activiy of the drug using various liposomal form was greater than that of solution form. In addition, regarding the liposomal types, multilamellar liposomes produced significantly higher drug activity than the small unilamellar ones. Also, in both liposomal types, the parameters of drug activity depends to great extent on the liposomal surface charge and its influence was generally greatest with positively harged multilamellar liposomes as compared to other formulations. This may be attributed to electrostatic bindings between corneal surface bears a net negative charge and the positively charged liposomes. Accordingly, positively charged multilamellar liposomes represent an optimal delivery system which appeared to be suitable in modulating drug action for selective targeting in the ocular therapy.

INTRODUCTION

Many previous studies have been made to enhance the corneal absorption of topically applied ophthalmic drugs in order to optimize its therapeutic effectiveness. Most ophthalmic drugs for topical administration were intended to reach the anterior chamber of the eye and to affect the pathological process taking place in the various layers of the cornea[1,2].

However, the ocular bioavailability of topically applied drugs was generally considered to be poor when the drugs were instilled as the commonly used ophthalmic eye drops dosage form. This may be attributed to the short corneal contact time for drug absorption and rapid clearance from the eye[3-5]. Other dosage forms, like suspensions and emulsions suffer from the same problem, and frequent dosing or repeated medications were still required, where the duration of drug action was short. Also, other common ophthalmic dosage forms, using oily bases or viscolizers, like ointments and gel formulations have several therapeutic disadvantages including imprecise dosing and vision impairment. Accordingly, these systems does not provide an optimal drug delivery to the intra-ocular tissues[6-8].

In this regard, numerous studies of coular bioavailability, which have been utilized various drug delivery system such as nanoparticles, liposomes, microemulsion and ocusert, have been recently proposed and investigated by many groups of researchers[7]. Compared with other carrier systems, liposomes can be considered to be one specific type for targeting of drugs to achieve sustained release and prolonged retention at the appropriate site of action[9,10]. A recent reports indicated that liposomal encapsulation improved corneal absorption of both hydrophilic and lipophilic topically applied drugs[11-14].

The manner by which liposomes can alter the behaviour of encapsulated drugs and their affinity for corneal surface of the eye as greatly dependent on some important parameters of liposomes such as lipid composition, surface charge, size, number of lamellae and bilayer rigidity[4,15,16]. Consequently, the optimal design for specific formulations of liposomes can be modulated according to their theraeutic usefulness and depending on the physicochemical properties of liposome-drug combination. For these reasons, it appears that liposomes can be considered to be a promising and versatile system of useful applications for ocular drug delivery[17, 18].

Topically aplied ophthalmic corticosteroids, as prednisolone and hydrocortisone, have been potentially used as an effective means of controlling many ocular inflammatory conditions. Generally, prednisolone was commonly employed as a model or as the drug of choice in the topical testing protocols, and also became the standard drug for all conditions in which routine systemic corticosteroid therapy was indicated[3,8,19].

We compared here the activity of free and liposomal prednisolone on the basis of the ocular hypertensive effect after topical instillation into the rabbit's eyes. In the following experiment, attempts have been made to design a liposomal form that will enable the drug to reach the desired site of action at a controlled rate and duration of action by changing some parameters such as the charge and type of liposomal formulations. The effect of these parameters on the time course of the intra-ocular pressure in response to the drug was investigated.

MATERIALS AND METHODS

1. Equipments:

The following equipments were used: Perkin-Elmer Lamba 3B UV?VIS spectrophotometer, Schiotz Tonometer (Riester, Germany), Büchi Rotary Evaporator Cole-Parmer T-1602-21, polycarbonate membrane and membrane holders (Bucleopore Corp.), and Ultrasonic Bath-Type Sonicator (Ultrasonic Instr. Inc., Model G40 C2 H-T 40 Cl, 80 K cycles/sec.).

2. Materials:

Prednisolone (Sigma Chemical Co., St. Louis, Mo., USA). Mono and dibasic sodium phosphate, propylene glycol, sodium choride and xylocaine hydrochloride were all of pharmaceutical grade. L-α-Dipalmitoyl phosphatidylcholine, dicetyl phosphate, stearylamine, cholesterol, and all other chemicals were all obtained from sigma Chemical Co., St. Louis, MO., USA.

3. Experimental Animals:

Albino rabbits (2.0-2.5 kg) were obtained from Faculty of Veterinary Medicine, Zagazig University, Animal Breeding Center, Egypt. The animals were kept in restraining boxes in normal upright position during the experiments, and all of healthy eyes. The boxes were placed on a swivel-stool which allowed the rabbit to be easily positioned for the measurement of intra-ocular pressure. The animals were kept in a room with standardized illumination and individually housed. During the experiments, extreme care was taken to avoid any intra-ocular pressure responses correlated with light change, sudden noise, disturbance, etc.

4. Methods:

4.1. Liposomal preparation:

Three different lipid compositions were used to procedure either a neutral, positive, or negative surface charge to liposomes. Neutral liposomes were prepared using L-α-dipalmitoyl phosphatidylcholine and cholestered in an 7:3 molar ratio. The positively charged liposomes were composed of L-α-dipalmitoyl phosphatidylcholine, cholesterol and stearylamine in 7:2:1 molar ratio. The negatively charged liposomes were composed of L- α- dipalmitoyl phosphatidylcholine, cholesterol and dicetyl phosphate in 7:2:1 molar ratio.

Both unilamellar and multilamellar liposomal types of each of the above lipid compositions were prepared.

(A) Multilamellar liposomes (MLV):

Essentially, liposomes were prepared by the technique of Bangham et al.[20]. The lipids and drug were dissolved in a minimal amount of chloroform. Chlorform was completely removed on a rotary evaporator under reduce pressure at 25°C until a thin film formed on the wall of the flask. The lipid film was hydrated with the appropriate amount of isotonic phosphate buffer solution (pH 6.8 ± 0.2) containing prednisolone as follows: the drug was dissolved in five drops of propylene glycol and incorporated into the aqueous compartment of liposomes by mixing with the isotonic phosphate buffer to give 0.1% w/v final concentration. The suspension was shaken gently by hand for about 1 hour under nitrogen gas at 25°C. The resulting MLV was adjusted with the same buffer to yield a final concentration of 60 μ mol lipid/ml aqueous phase. In the usual way it is known that, this hand-dispersion technique resulted in a heterogeneous size liposomes. For this reason the final preparation was then sequentially extruded through polycarbonate membranes of defined pore size to produce a reproducible homogeneous extrusions[21,22].

(B) Small Unilamellar Liposomes (SUV):

SUV were produced from MLV by sonication for about 30 minutes at 20°C in a bath-type sonicator (60 seconds sonication sollowed by 30 seconds cooling on ice-water mixture). Clarification of the turbid suspension indicates the conversion of MLV to SUV type[23].

For all liposomal preparations, an empty control liposomal formulations were also prepared following the same procedure mentioned before.

4.2. Sequential extrusion and size distribution of the liposomal preparations:

As it was reported, A homogeneous liposomal preparations with controlled particle size distrubtion was obtained by sequential extrusion through polycarbonate membranes. The liposomal size of this extrusions could be designated by the smallest membrane size through which the suspension was extruded[21,22].

In all liposomal preparations, the total lipid concentration was 60 μ mol/ml and the liposomes were diluted to 12 μ mol/ml in the same buffer prior to extrusion.

For extrustion of MLV, the preparations were forced several times through polycorbonate membrane

filters with 3.0, 2.0, 1.0 and 0.8 µm pores. The process was accomplished at a relatively low pressure (approximately 10 pounds/squre inch) in 25 mm membrane holder. However, for extrusion of SUV, the preparations were forced several times through polycarbonate membranes filters with pore size of 0.6, 0.1 and 0.08 µm at the same previous pressure.

4.3. Surface charge of liposomal preparations:

Initially, as a preliminary test, the surface charge of liposomes (SUV and MLV) was determined by electrophoretic mobility. The sign of the net charge prevailing at the liposome surface was determined from the direction of migration of the particles using Carl Zeiss cytopherometer.

4.4. Encapsulation efficiency of different liposome preparations:

The liposomes were immediately separated from the free drug unentrapped by centrifugation at 8,000 rpm for 15 minutes (4°C). The supernatants, which contained the unencapsulated drug, was carfully decanted and the liposomes were resuspended gently in the same buffer used previously. The procedure was repeated twice and the supernates from each process were collected and assayed spectrophotometrically[24] for determination of free drug concentration.

The encapsulation efficiency was calculated as the ratio of the amount of drug remaining within liposomes after separation from the unencapsulated drug to the amount of drug present for the encapsulation[25].

4.5. In-vivo studies:

Isotonic xylocaine solution (1% w/v) was dropped into the rabbit's eyes (one drop was enough) to anaesthetize the cornea. Ophthalmic drug solution or liposomal formulations were instilled into the right eye, while the non medicated formulations were instilled into the left eye. A single 50 µL dose (0.1% w/v) of each formulation was instilled directly into the corneal surface of the eye. During instillation, the upper eyelid was slightly raised and the lower eyelid was gently pulled away from the globe.

The eyelids were gradually returned to their normal position. At certain time intervals, the intra-ocular pressure was measured before and after administration of both control and test formulations by using Schiotz tonometer. The mean of three consecutive tonometric measurements of each eye was calculated for each sample and a minimum of one weak (washout period) elapsed between tests in the same rabbit. Each preparation was tested in a group of six rabbits.

4.6. Analysis of the data:

The area under the intra-ocular pressure/time curve values were calculated using the trapezoidal method[26]. The data were analyzed statistically using the Student's t-test-.

RESULTS AND DISCUSSION

To investigate the influence of liposomal type and surface charge on the drug encapsulation, multilamellar and unilamellar liposomes were formulated and also different lipid compositions, were used to provide either a neutral, positive, or negative charge. The results of this study demonstrated that the encapsulation efficiency was variable due to changes in lipid composition and preparation techniques:

A. Effect of liposomal types:

The data presented in Table 1 showed that, liposome prepared by hand-shaking method (MLV) provided higher entrapment efficiencies than that prepared by sonication (SUV). The smaller capture or encapsulation efficiency obtained with sonicated liposomes, as compared to MLV, was related to the smaller size or capture volume produced with this method. Thus, liposome exhibit size-dependent efficiency of encapsulation, and this interpretation was in agreement with the suggestion of Szoka and Papahadjopoulos[27].

B. Effect of charged lipids:

Table 1, showed the encapsulation efficiencies of different liposomal types prepared from various lipid composition. The results clearly showed that the encapsulation efficiency was variable due to changes in liposomal lipid composition. Generally, for both types of liposomal preparations, the presence of negatively or positively charged lipids will tend to increase the entrapped volume. However, negatively charged liposomes provided high encapsulation efficiency of the drug as compared to positively charged ones. The greater percentage of drug entrapped within charged liposomes than within neutral liposomes could be explained on the consideration that, the charge density will tend to increase the interlamellar resistance between adjacent bilayers, where the charge repulsion leads to a greater entrapped volume. Furthermore, it was possible that both the charge density and the physiochemical properties of the encapsulated drug will tend to maximize the encapsulation efficiency in presence of negatively charged lipid. This suggestion was consistent with the reports of Riaz, Weiner and Martin[10].

On the basis of the above mentioned results, the overall encapsulation efficiency was affected by the type and lipid composition of the liposomal preparations. Thus, in conclusion, the drug-liposomal lipid interaction can affect the liposomal behavior particularly with respect to the encapsulation efficiency which is an important aspect of liposomal drug delivery.

The ocular activity of free and liposomal-encapsulated prednisolone was investigated in the rabbit's eye. The in-vivo evaluation of the various preparations was assessed on the basis of the influence of the drug on the intra-ocular pressure. Both MLV and

SUV types of neutral, positive, and negative charge were studied. Figures 1 and 2 illustrate the time course of the intra-ocular pressure after instillation of the different formulations of the drug. The data were statistically interpreted in terms of area under the intra-ocular pressure/time curve, duration of action, maximum response, and time of maximum response which have been taken in consideration as parameters of drug activity. Values of these parameters were calculated and summarized in Table 2. It was clearly observed that these parameters were different with different preparations, and was greatly influenced by the liposomal type and also surface charge. However, for all liposomal formulations, instillation of empty liposomes showed no effect on the intra-ocular pressure.

Concerning the area under intra-ocular pressure time/curve, the statistical analysis of the differences between the different formulations indicated that, the differences between the solution and any one of the liposomal preparations were very highly significant ($P < 0.001$). For the same liposomal type, the value of area under curve was greatest for positively charged liposomes, less for neutral, and least for negatively. Moreover, the difference between positively charged MLV and positively charged SUV type was very highly significant ($P < 0.001$). Also, the differences between positively charged liposomes ad negatively charged or neutral of the same type were also very highly significant ($P < 0.001$). Accordingly, compared to other formulations, positively charged MLV showed the largest area under the curve.

The duration of the drug action was also varied depending upon liposomal type and lipids composition. The results revealed that the duration of action could be prolonged up to 24 hours for positively charged MLV, which displayed the most prolonged effect compared to other formulations. Howver, this effect disappeared within 6 hours after instillation of the drug in solution form. Also, statistical analysis of the data, concerning the duration of action, demonstrated that difference between positively charged MLV and positively charged SUV type was very highly significant ($P < 0.001$). On the other hand, it ws found that neutral and negatively charged liposomes of the same type were equally effective in prolonging the duration of the drug action. On the basis of the above results, it seems possible to change the duration of drug action from short or moderate acting to long acting by changing both the type and surface charge of liposome as a selective drug delivery system.

Also, from Table II, it was obvious that the various formulations of the drug were differentially effective in producing maximum response. The maximum response was greater for liposomal formulations comparing to the solution form, and intensity of the drug action could be arranged in the following descending order: positively charged MLV > positively charged SUV > neutral SUV > neutral MLV > negatively charged SUV > negatively charged MLV > solution. Statistical analysis of the data reveaed that the differences in the maximum response were: (1) very highly significant ($P < 0.001$) between solution and any one of the liposomal preparations; (2) very highly significant ($P < 0.001$) between positively charged and negatively or neutral liposomes of the same type; and (3) insignifiant between neutral and negatively charged liposomes of the same type.

In addition, concerning the time of maximum response, it was clearly observed that the time required to reach the maximum response was greatest for positively charged MLV as compared to other formulations. Also, statistical analysis of the data indicated that the difference in the time of maximum response was insignificant between: (1) neutral and negatively charged liposomes of the same type and (2) positively and negatively charged SUV.

According to the above mentioned results, it could be concluded that all liposomal formulations used in this study showed markedly higher levels of the ocular drug activity and produced a greater influence on the in-vivo parameters of drug action compared with he solution form. This influence can be explained on the consideration that the main components of liposomes were materials that are present as naturally occuring constituents in cell membranes, as phospholipids and cholesterol, and therefore they are biocompatible, biodegrable and of good bioacceptability. Phospholipids, for instance, usually form the backbone of the liposomal bilayer structure and the net surface charge of liposomes can be modified by using sveral variety of lipid components. Consequently, the liposomal surface charged will influence the behaviour of the encapsulated drug and its specific ability to interact activity with the biological environment. Also, cholesterol was often included as a regular component of liposomal membranes, and its incorporation into the phospholipid bilayers strongly controlled drug release which, in turn, would help in providing higher drug loading at ocular tissues and also increasing the drug binding affinity for the corneal surface of the eye[4,10,28-30].

On comparing the different formulation, it could be also concluded that MLV with positive surface charge greatly enhanced the drug response, and displayed the greatest increase in the parameters of the drug activity. This enhancement effect was dependent on:

(1) **Mean number of bilayers or lamellarity and liposomal size:** It could be suggested that the delay or prolongation of drug action, in case of MLV encapsulation, may be attributed to the presence of number of lamellae or concentric lipid bilayers which act as a hydrophobic barriers. Also, may

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Table (1): Effect of liposomal types and surface charge on the encapsulation of prednisolone. Multilamellar and small unilamellar liposomes were prepared to contain 12 μ mol lipid/ml aqeous phase.

Liposomal surface charge	% Encapsulation*	
	Multilamellar liposomes	Small unilamellar liposomes
1- Positively charged.	53.6 (0.9)	21.9 (0.5)
2- Negatively charged.	67.9 (0.7)	44.4 (0.9)
3- Neutral.	28.7 (0.8)	17.1 (0.6)

* Each value represents the mean of three separate liposomal preparations.

Table (2): Values for area under intra-ocular pressure/time curve, duration of action, maximum response, and time of maximum response of prednisolone in solution and in different liposomal formulations.

Formulations	Parameters of Activity			
	Area under the curve (mmHg hr.)	Duration of action (hr.)	Maximum response** (mmHg)	Time of maximum response (hr.)
A- Multilamellar Liposomes:				
1- Positively charged.	46.13 (0.89)*	24.00 (0.37)	4.96 (0.29)	4.00 (0.00)
2- Negatively charged.	19.12 (0.75)	18.00 (0.37)	2.69 (0.39)	3.50 (0.37)
3- Neutral.	23.97 (0.88)	18.00 (0.26)	2.99 (0.30)	3.50 (0.26)
B- Small Unilamellar liposomes:				
1- Positively charged.	30.26 (0.69)	15.00 (0.00)	4.17 (0.35)	3.00 (0.37)
2- Negatively charged.	15.96 (0.70)	12.00 (0.26)	2.74 (0.23)	3.00 (0.26)
3- Neutral.	20.56 (0.82)	12.00 (0.37)	3.29 (0.33)	2.83 (0.17)
C- Solution:	5.89 (0.60)	6.00 (0.26)	2.26 (0.30)	1.58 (0.20)

* The values between parantheses represent the standard error of the mean (n = 6).
** The differencce between the maximum intra-ocular pressure of solution or liposomal formulation and control.

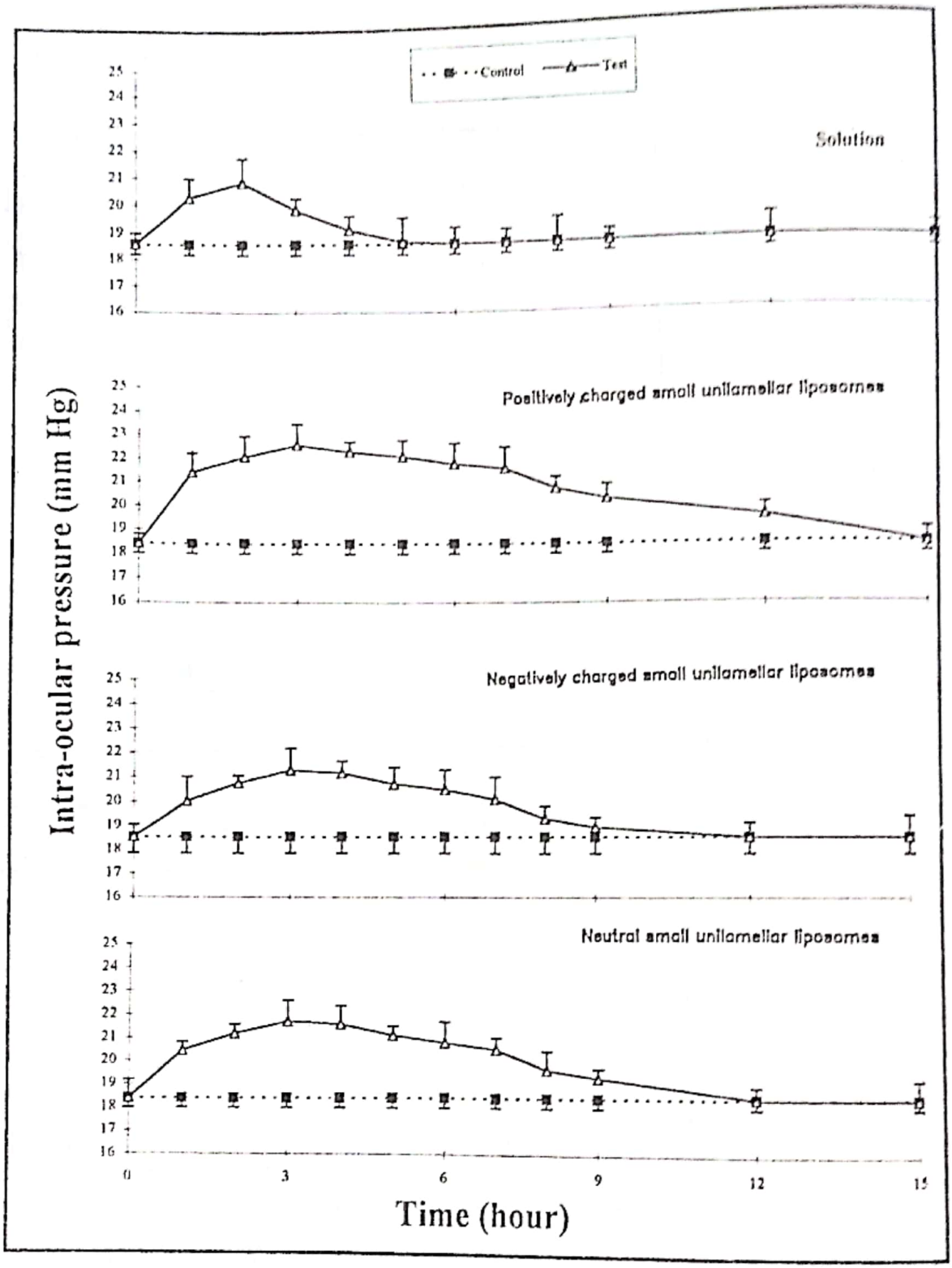

Figure (1): Intra-ocular pressure (mmHg) of rabbit's eye after instillation of 0.1% (w/v) prednisolone in solution and in different small unilamellar liposomal formulations. The values in this figure and subsequent one represent the mean of three consective tonometric measurements of each eye, and each formulation was tested in a group of six rabbits. The vertical bars indicated standard error of the mean.

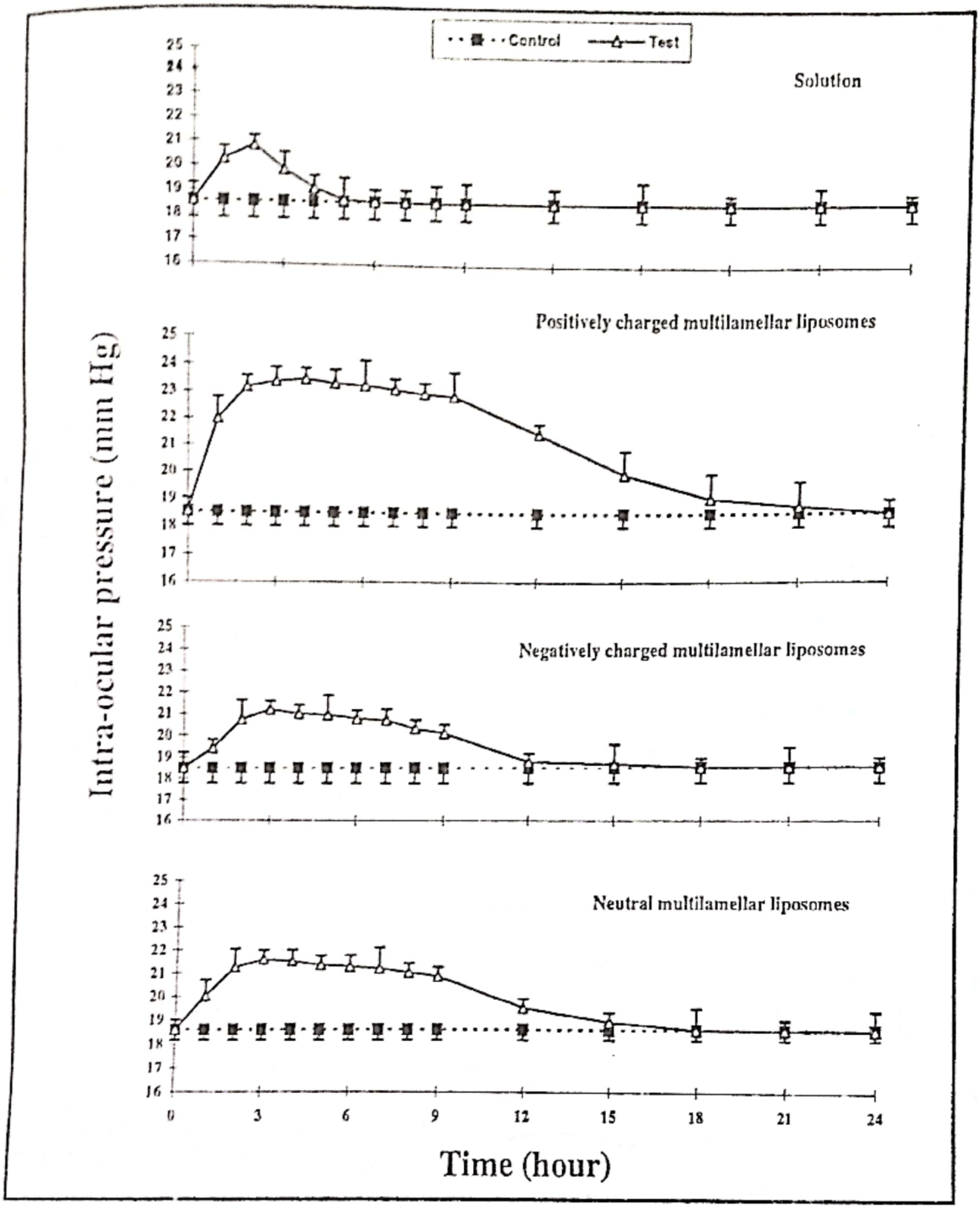

Figure (2): Intra-ocular pressure (mmHg) of rabbit's eye after instillation of 0.1% (w/v) predinsolone in solution and in different multilamellar liposomal formulations. The vertical bars indicated standard error of the mean.

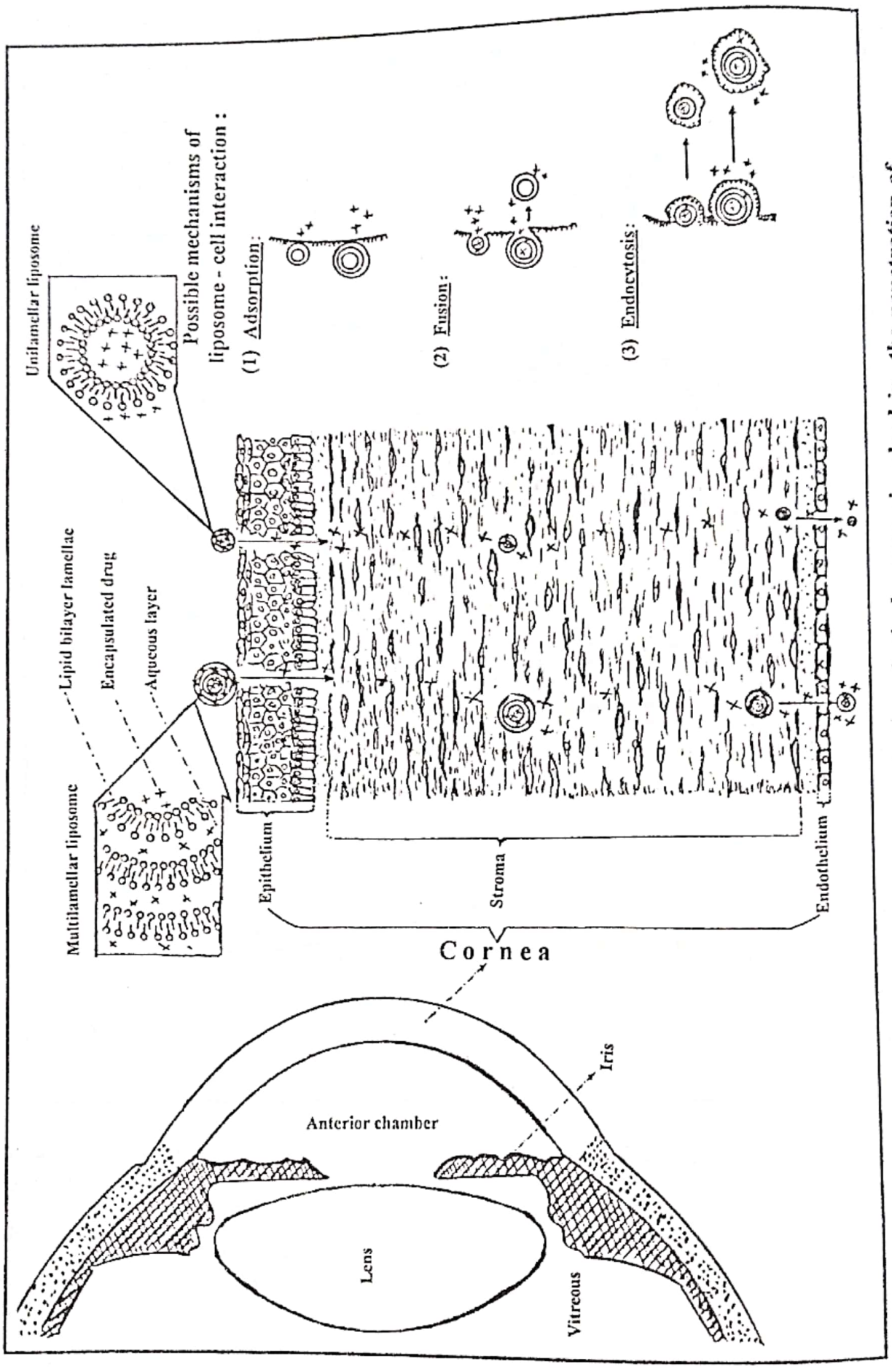

Figure (3): Schematic representation of the hypothetical process involved in the penetration of liposome-encapsulated drug through the cornea of the eye.

studies have shown that the number of bilayers determines the relation between liposomal size and encapsulation volume[10,25,31].

(2) **Liposomal surface charge:** Fundamentally, under normal environmental conditions and at physiologic pH, the mucin layer overlying the corneal epithelium bears a net negative charge[4]. Therefore it seems reasonable to assume that the initial interaction between the corneal surface nd liposome might be attributed to the electrostatic attraction of the negatively charged surface of the corneal epithelial cell membranes and positively charged liposomal surface.

A number of recent studies have been suggested that cholesterol and many positively charged phospholipid were shown to be nontoxic. Also, positively charged emulsion prepared with stearylamine was reported to be well tolerated and did not induce a toxic or inflammatory response[4,32,33].

Many recent investigations confirmed that the transcorneal permeation of instilled drug takes place mainly through the cornea, where the corneal epithelium plays an important role as a reservoir for prolonged the release of the drug to the underlying layers of the cornea. The cornea, which is the portal of entry for ocular administration of drugs, is a multilayer structure consisting of a hydrophilic stromal layer sandwiched between a very lipophilic epithelial layer and a much less lipophilic endothelial layer[1,2,4].

Regarding the above mentioned hypothesis and facts, it seems possible to establish the mechanism of liposome-cell interaction at the cornea in the following steps (Figure 3): (A) The inact liposomes may adhere to the cell walls of the target site by electrostatic adsorption. Upon attachment to liposomes to the epithelial cell the drug released from the outermost liposomal lipid bilayer and penetrated through transcellular or intercellular pathway to cross the corneal barrier, where the drug was carried away by diffusion. The permeability of the epithelial barrier of the human and rabbit corneal to prednisolone and its intraocular pharmacokinetics were studied and estimated by S. Mishima[34]. (B) Hydrophobic interaction was proposed to occur between lipoidal corneal epithelial layer and hydrophobic liposomal lipid bilayers, where liposomes penetrated cells by endocytosis (as phagosomes) or by fusion. Thus, in this manner the liposmal contents were released to the target cell since the liposomal lipid portion becomes part of corneal epithelium cell wall. (C) Deeper penetration of liposomes into the next barrier layer of the cornea, which is the stroma, with its very high water content. (D) Liposmes penetrate further from the stroma into the anterior chamber, and this step is somewhat controlled by the endothelial layer, which is the final layer of the cornea and is one cell thick.

Furthermore, it seems possible to propose that these steps of liposomal penetration were accompanied by perturbation and disruption of its membrane structure with loss of lipid bilayer integrity. Also, as shown in the schematic representation (Fig. 3), the disrupted membrane fragments may anneal forming liposomes of smaller size and the liposomal size decreased during penetration. Thus, it was reasonable to assume that perturbation process proceeds sequentially from the outermost bilayer toward the liposomal center, resulting in a partial and gradual release of the liposome-encapsulated drug, in a stepwise manner.

The above mentioned interpretations were confirmed by the clinical and electron microscopic studies of Foldvari et al.[35] which proposed that the intact liposomes penetrated into the skin and deposited in the dermis where they carry their content into the skin and acted as a slow release depost system.

In conclusion, it would appear that liposomes, as ocular delivery system, can be used to improve the ocular bioavailability and to alter the behaviour or the pharmacokinetics of the encapsulated drug by modifying the drug action and maximizing the ratio of drug concentrations in the target tissues. Thus, the significance of this study is the possibility of designing a liposomal system for offering a means of achieving sustained/controlled release and targeting of drug to the selected site of action for enhancement of the corneal penetration or transcorneal drug flux.

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استخدام الليبوزوم كحامل للتحكم فى تأثير البردنيزولون فى العين

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تم تقييم فاعلية البردنيزولون المحمول داخل الليبوزوم فى عين الأرنب. حضر الليبوزوم سواء المتعدد أو الوحيد الطبقات فى داى فوسفاتيديل كولين والكولستيرول مع إضافة الاستياريل أمين لإعطائه شحنه موجبه أو داى ستيل فوسفات لإعطائه شحنه سالبة. ورشح الليبوزوم خلال أغشية البولى كربونات لتحسين تجانس المستحضر. ودلت النتائج على أن أعلى درجة لاحتواء العقار داخل الليبوزوم كان للمستحضر ذو الشحنه السالبة.

ولقد تم تقييم فاعليه المستحضرات المختلفة على أساس قياس تأثير العقار على الضغط الداخلى للعين وترجمت النتائج على هيئة المساحة تحت منحنى الضغط الداخلى للعين مع الزمن - زمن الفاعلية - أعلى تأثير - وأعلى زمن تأثير - وأعتبرت هذه القيم كمقاييس لفاعلية العقار. وأظهرت هذه الدراسة أن كل المستحضرات الليبوزومية كان لها فاعلية أكبر من فاعلية المستحضر عند وجوده على هيئة محلول. وكذلك فأن الليبوزوم المتعد الطبقات كان له تأثير واضح وأعلى من الليبوزوم الاحادى الطبقات. ودلت كل النتائج على أن فاعلية المستحضرات الليبوزومية كانت تعتمد على نوع الشحنه الموجودة على سطحه وكانت اعلاها فى حالة الشحنه الموجبة. وعلل ذلك على أساس التجاذب الكهروستاتيكى بين الشحنه الموجبة على سطح الليبوزوم والشحنة السالبة على سطح قرنية العين. ولذلك فأن أفضل هذه المستحضرات من الناحية العلاجية للعين هو الليبوزوم ذو الشحنة الموجبة.