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LIPOSOMAL GEL AS OCULAR DELIVERY SYSTEM FOR DICLOFENAC SODIUM: *IN- VITRO* AND *IN-VIVO* STUDIES

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ABSTRACT

The aim of this work is to formulate topically effective controlled release ophthalmic liposomal gel for targeting diclofenac sodium to the eye in an attempt to heal the inflamed tissue of ocular ulcerative area. Large unilamellar vesicles (LUVs) and multilamellar vesicles (MLVs) gel formulations composing of phosphatidylcholine (PC) and cholesterol (CH) in the molar ratios of (7:2; 7:4 and 7:7) with or without stearylamine (SA) or dicetylphosphate (DP) were prepared using reversed-phase evaporation and lipid film hydration methods respectively. The prepared liposomal systems were evaluated for their entrapment efficiency, morphological characters, physical stability, particle size and drug release rate .LUVs entrapped greater amount of drug than MLVs. Drug loading was increased by increasing CH content as well as by inclusion of SA into the lipid bilayer. Drug release rate showed an order of negatively > neutral > positively charged liposomes, which is the reverse of results of drug loading efficiency. Physical stability study indicated that 92.56%, 84.11%, 76.41% and 91.1%, 82.19% and 75.54% of diclofenac sodium was retained in positive, negative, and neutral MLVs and LUVs respectively after storing for 120 days at refrigeration temperature. The in vivo anti-inflammatory activity was evaluated using a thermal technique , results showed that the percentage of healed ulcers were 12.5%, 35%, 67.5%, 82.5%, 85%, 87.5% and 95% for negative control, positive control, 0.5% carbopol 934 gel, LUVs liposomes suspension, MLVs liposomes suspension, LUVs and MLVs gels, respectively.

INTRODUCTION: Drug delivery in ocular therapeutics is a challenging problem and is a subject of interest of researchers. The anatomy, physiology and biochemistry of the eye render this organ exquisitely impervious to foreign substances.

The challenge to the formulator is to circumvent the protective barriers of the eye without causing permanent tissue damage [1].

The bioavailability of ophthalmic drugs is however, very poor due to efficient protective mechanisms of the eye. Blinking, baseline and reflex lachrymation, and drainage remove rapidly foreign substances including drugs from the surface of the eye. Many ophthalmic preparations are available. These are classified as conventional and non-conventional (newer) drug delivery systems.

The most commonly available ophthalmic preparations are eye drops and ointments; they represent about 70% of the eye dosage formulations in market. Unfortunately, these preparations when instilled into the cul de-sac are rapidly drained away from the ocular cavity due to tear flow and lachrymal nasal drainage. Only a small amount is available for its therapeutic effect resulting in frequent dosing.

Diclofenac sodium (DFS) is the most commonly used anti-inflammatory drug for inflammation treatments. The efficacy of DFS depends greatly on the capacity of the preparation to allow drug penetration through the applied tissues. The two main problems that hinder the topical effectiveness of any drug are its poor solubility and low permeability coefficient. Topical formulations of diclofenac solution were initially unsuccessful because of its limited ocular penetration, which caused an insufficient amount of drug to reach the internal tissues [2].

Other significant attempts have been made to formulate effective diclofenac topical preparations. Various drug delivery systems offer numerous advantages over conventional drug therapy, yet they are not devoid of pitfalls, including poor patient compliance and difficulty of insertion, as in contact lenses, and tissue irritation, as well as damage and toxicological complications caused by penetration enhacers[2]. A liposome offers advantage over most ophthalmic delivery system in being completely biodegradable and relatively non-toxic.

A potential advantage of liposomes is their ability to intimately contact the corneal and conjunctival surfaces, thereby increasing the probability of ocular drug absorption. Liposomes offer a promising avenue to fulfill the need of ophthalmic drug delivery system that not only has the convenience of drops solutions but also can localize and maintain drug activity at its sites of action for a longer period, thus allowing a sustained or controlled action. Moreover, the liposomal systems are used to protect drugs from the metabolic enzymes present at tear/corneal epithelium interface [1].

The objective of this study was to formulate DFS in large unilamellar (LUVs) and multilamellar (MLVs) liposomal formulations in different molar ratios.

A comparative study was performed between LUVs and MLVs liposomal suspension and liposomal gel to evaluate the *in-vitro* and *in-vivo* performances of these formulations. The factors affecting drug encapsulation into liposomes were investigated. Characterization of formed liposomes regarding physical morphology, particle size, and *in-vitro* drug release were studied. Stability study was performed to investigate the leakage of the drug from liposomes during the mentioned storage conditions. The intra-ocular anti-inflammatory effect of selected DFS (LUVs and MLVs) liposomal formulations was also evaluated.

MATERIAL AND METHODS:

Materials: Diclofenac sodium, absolute Ethyl alcohol, Chloroform, Potassium dihydrogen phosphate, Disodium hydrogen phosphate and Sodium chloride (El Nasr Company, Abou Zabal, Egypt). L-α-phosphatidyl choline soybean 99%, Cholesterol , Stearylamine (SA), Dicetylphosphate (DP), carbopol 934 and uranyl acetate (Sigma- Aldrich, Germany), Cellulose nitrate filter membrane, {diameter pore 0.45µm} (Albet Company, Spain). Ciprofloxacin eye drops (0.3%), Ciprocin, Benzoxinate HCL (0.4%) and Benox, (Egyptian International Pharmaceutical Industries Company, Egypt). DFS eye drops (Volatren 0.1%) (Ciba vision, Egypt) Fluroscein sodium ampoule 10% (Chemical Industries Development Company, Cairo, Egypt).

Methods:

1. **Preparation of large unilamellar vesicles (LUVs):** Large unilamellar vesicles (LUVs) were prepared by the reverse-phase evaporation technique[3]. Lipid components 200 mg (soybean phosphatidylcholine and cholesterol with or without charge inducing agent) in molar ratios (7:2), (7:4), and (7:7) were dissolved in chloroform: diethyl ether (1:1). DFS in PBS pH (7.4) was added and the resulting two phases system was sonicated for four minutes (sonicator, Model 275 T, Crest Ultrasonic Corp., Trenton, USA). A stable white emulsion was produced, from which organic solvent was slowly evaporated at 45 °C with rotary evaporator, (Rotavapor, Type R 110, Büchi, Switzerland), until a viscous aqueous liposomal suspension was produced. The liposomal suspension was mentained at 45 °C in a thermostatically controlled

water bath for one hour to anneal liposome structure.

2. **Preparation of multilamellar vesicles (MLVs):** The required amount of lipids was weighed into a quick- fit round bottom flask and dissolved in a small volume of chloroform. The solvent was the evaporated at 40 ºC under reduced pressure, leaving a thin film of lipids distributed over the wall of the flask. Aqueous phase DFS in PBS pH(7.4) was added at a temperature of 40 ºC. The flask was maintained at that temperature for 1h, then shaken on a vortex mixer for 2min to produce the liposomes.

3. **Separation of liposomes from the non-encapsulated DFS:** Liposomes were separated from the non-entrapped material by three successive centrifugation cycle at 14,000 rpm for 1 hr at 2°C (Refrigerated centrifuge, Model 8880, Centurion Scientific Ltd., W. Susses, and UK). The supernatant was removed and the pellet was resuspended in PBS (pH 7.4). The process was repeated three times to ensure that all free DFS was completely removed.

4. **Determination of the Entrapment Efficiency:** The percentage of drug entrapped was determined after complete lyses of the formed liposomes with absolute alcohol and Sonication for 10 min[4]. The concentration of DFS in absolute alcohol was determined spectrophotometrically at 284 nm using UV-visible spectrophotometer (Shimadzu UV- 1601, Double beam spectrophotometer, Kyoto, Japan.). Unloaded liposomes produced insignificant absorbance values at the same wavelength. The entrapment efficiency was expressed as the percentage drug entrapped using the following relationship[5]:

Entrapment efficiency= Entrapped drug/Total drug × 100 (I).

Characterization of DFS liposomes:

1. **Electron microscopy:** Saturated solution of uranyl acetate was used as a negative stain for transmission electron microscopy. 0.2 ml liposome preparation and 0.2 ml negative stain were mixed, one drop was placed on a Carbon coated grid and allowed to dry. Grids were rinsed with water to remove excess stain and examined with electron microscope (Panasonic, Tokyo, Japan).

2. **Particle size measurements:** The mean particle size of freshly prepared neutral, positively charged, and negatively charged LUVs and MLVs liposomal dispersions was determined using (Laser diffraction particle size analyzer, Master sizer X, Model MAMSOOO, Malvern Instrument Limited, Worcester shire, UK.)

3. ***In vitro* drug release:** The release of DFS from LUVs and MLVs liposomal formulations was determined using the membrane diffusion technique[6]. DFS liposomal suspension equivalent to 0.1% DFS was suspended in 1 ml PBS (pH 7.4) in a glass cylinder having a length of 7 cm and diameter of 2.5 cm. This cylinder was fitted, before addition of liposomal suspension, with a presoaked dialysis membrane (diameter pore 0.45µm) and was suspended in the dissolution flask of the United States Pharmacopeia (USP) dissolution tester, (Apparatus I, SR8 plus dissolution test station, Hanson research, USA.), containing 75 ml PBS (pH 7.4) and maintained at temperature of 37 ± 0.5°C and allowed to stir at 50 rpm. Samples were withdrawn at different time intervals and assayed spectrophotometrically at 276 nm, using samples collected from dialysis of the drug free liposomal systems as blank.

4. **Preparation of DFS Carbopol Liposomal Gels:** Liposomes either MLVs or LUVs after removal of free un-encapsulated drug were mixed into 0.5% carbopol 934 by using a magnetic stirrer. The concentration of drug in final preparation was 0.1% w/v.

5. **Physical Stability Study:** This study was performed to investigate the drug leakage out from liposomes under storage conditions. Positively charged DFS (LUVs and MLVs) and their liposomal gel form with lipid components in the molar ratio of (7:7:1) PC : CH: SA were sealed in 20 ml glass vials and stored in a refrigerator at 2-8 °C and at room temperature for a period of 120 days. Samples from each liposomal formulation were withdrawn at definite

time intervals, the residual amount of the drug in the vesicles was determined after separation of un-encapsulated drug as previously described .

6. **Sterilization:** The liposomal formulations (LUVs and MLVs) that have been mixed with carbopol 934 were autoclaved at 121˚C under pressure value 2 bars for 15 minutes according to standard procedure[7]. After autoclaving process has been finished, the tested formulations were evaluated with respect to entrapment efficiency, particle size and drug release rate.

7. ***In vivo* studies:**

a. **Induction of inflammation to rabbit's eye:** A group of five adult male albino rabbits weighting 1.5-2 kg were used for the *in vivo* study of each formula. The rabbits were fed balanced diet pellets and maintained on 12 hrs/12 hrs light/dark cycle in a temperature-controlled room at 20- 24°C before the experiments [8]. The experimental procedures were conformed to the ethical principles of the Egyptian Research Institute of Ophthalmology (Giza, Egypt) on the use of animals [9]. Two drops of 0.4% solution of benzoxinate HCl were instilled onto each eye as a local anesthetic.

Five inflammatory areas (ulcers) were induced in the epithelium of the cornea of each eye, away from the pupil by application of electrocautery to the cornea, which is a standard experimental method of inducing corneal inflammation[10]. Using the tip of a handheld thermal cautery (Aaron Medical Industries), five light burns were applied to the central 50% of the cornea. Immediately after the procedure, one drop of sterile ciprofloxacin eye drops (0.3%) as an antibiotic was applied.

The ulcer had a circular shape (2 mm in diameter) and reached in the depth of the corneal epithelium. This was ascertained by instillation of fluroescin sodium solution (0.2%). As, fluroescin dose not stain tissue by green florescence unless the epithelium is disrupted. The absence of green color was considered as a sign of ulcer healing [11].

b. ***In-vivo* performance of DFS formulations on the inflamed eye of rabbits:** For each rabbit, one eye was considered as a test and the other eye as a control. DFS anti-inflammatory effect was determined by the rate and extent of healing of inflamed tissues in the ulcerative areas in the rabbit's cornea. The used formula were negative control (0.3% ciprofloxacin solution), positive control DFS solution (Voltaren eye drop), mucoadhesive gel 0.5% carbopol 934, liposomal suspensions of LUVs and MLVs composed of phosphatidylcholine: cholesterol: SA in molar ratio (7:7:1) and 0.5% carbopol 934 liposomal gel containing either LUVs or MLVs. All previous formulations contained 0.1% DFS except negative control. The period of observation was 10 days. Preliminary tests were done to all formulations to ensure that no irritation occurred from each formula

RESULTS AND DISCUSSION:

Entrapment efficiency: Table 1 shows the dependence of DFS entrapment on the lipid composition, method of preparation, and the type of charge inducer used in the preparation of liposomes.

Concerning the effect of cholesterol content on the entrapment efficiency of the drug in the prepared liposomes, the results showed that the percentage entrapment efficiency of DFS increased by increasing cholesterol content. The percentage entrapment efficiencies of DFS into reverse-phase evaporation liposomes (LUVs) were 33.61% , 38.93% and 43.16% for formulations of lipid composition of 7:2, 7:4, 7:7 (PC: CH) molar ratios respectively.

Multilamellar liposomes (MLVs) showed the same trend with entrapment efficiencies of 31.32%, 35.37% and 38.31% by increasing the cholesterol content from 7:2, 7:4, 7:7 (PC: CH) respectively. The (ANOVA) test showed a significant difference between all pairs at $p < 0.05$.Incorporation of cholesterol into the lipid bilayers is known to influence vesicles stability and permeability [14]. Cholesterol is one of the common additives incorporated in the lipid bilayers to impact the rigidity of the membrane and hence increasing the drug retention [6].

Producing of liposomes by the reverse phase evaporation technique (LUVs) resulted in an increase in the entrapment of DFS compared with MLVs. Each of the LUVs has a large internal aqueous core relative to its diameter and this is more likely to be responsible for the more efficient entrapment of aqueous volume than in the MLVs liposomes [12].

Concerning the effect of charge-inducing agent on the encapsulation efficiency of DFS in formulated LUVs and MLVs liposomes, results showed that positively charged liposomes exhibited the highest encapsulation efficiency followed by neutral then negatively charged liposomes, using the same PC:CH molar ratio. This order of entrapment efficiency would result because DFS is a weak acid, so electrostatic attractions would occur between the ionic moiety of drug and positively charged SA. On the other hand, in case of negatively charged liposomes, it is likely that repulsion may occur between drug molecules and the negatively charged DP, thus suppressing the loading efficiency.

TABLE 1: ENCAPMENT EFFICIENCY OF DICLOFENAC SODIUM IN LARGE UNILAMELLAR VESICLES (LUVS) AND MULTILAMELLAR VESICLES (MLVS) LIPOSOMES

Liposomal formulation composition (molar ratio)	Liposomal formulation charge	Entrapment Efficiency	
		LUVs (%) ± SD	MLVs (%) ± SD
PC : CH (7:2)	neutral	33.61 ± 1.82	31.32 ± 1.62
PC : CH (7:4)	neutral	38.93 ± 2.51	35.37 ± 0.82
PC : CH (7:7)	neutral	43.16 ± 1.71	38.31 ± 1.86
PC : CH: SA (7:7:0.5)	positive	50.32 ± 1.26	43.85 ± 2.36
PC : CH: SA (7:7:1)	positive	56.73 ± 1.45	51.68 ± 2.61
PC : CH: SA (7:7:2)	positive	59.42 ± 2.31	53.24 ± 1.73
PC : CH: DP (7:7:0.5)	negative	30.69 ± 1.08	25.12 ± 1.92
PC : CH: DP (7:7:1)	negative	29.75 ± 0.95	21.76 ± 1.63
PC : CH: DP (7:7:2)	negative	28.19 ± 1.17	20.23 ± 0.61

PC: L-α-phosphatidylcholine soybean, CH: cholesterol, SA: stearyl amine and DP: dicetylphosphate

Characterization of DFS liposomes:

Photomicroscopic analysis: The transmission electron micrographs of LUVs and MLVs positively charged liposomes of the molar ratio (7:7:1) are shown in **fig. 1.** Producing liposomes by the reverse phase evaporation technique revealed the presence of unilamellar vesicles with phospholipids bilayer (**fig. 1a**). The prepared liposomes are well-identified spheres with a large internal aqueous core relative to its diameter, and this more likely to be responsible for the more efficient entrapment of aqueous volume than in MLVs. In the contrast MLVs liposomes showed a heterogeneous population with the presence of well-identified spheres of multilamellar vesicles that consist of many concentric phospholipids bilayers (**fig. 1b**).

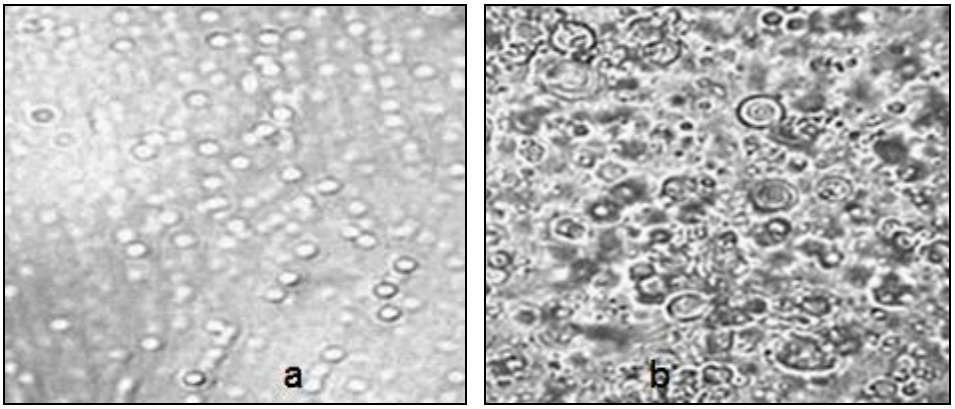

FIGURE 1: TRANSMISSION ELECTRON MICROGRAPHS OF POSITIVE LUVS (A) AND MLVS (B) LIPOSOMES CONTAINING PHOSPHOLIPIDS : CHOLESTEROL: STEARYLAMINE IN A MOLAR RATIO (7:7:1).

Particle size analysis: Table 2 shows the mean particle diameter of freshly prepared neutral, positive and negative charged liposomes with lipid component 7:2, 7:4 and 7:7 molar ratios.

Incorporation of a negative charge DP into the bilayers, resulted in repulsion between similar charges of lipid layers and an increasing in the distance between adjacent bilayers. While, incorporation of SA into liposomal bilayer membrane rendered the surface electrically charged, however the positive charges of lipid bilayers were opposite to the charges of encapsulated DFS leading to a decrease the distance between bilayers because of charges neutralization[13].

By comparing the mean particle diameter of LUVs and MLVs liposomal formulations from previous data, it detected that LUVs liposomes are larger than MLVs liposomes prepared with the same lipid ratio, which would account for their higher entrapment efficiency.

After autoclaving process no significant change at P< 0.05 in the particle size of liposomes.

TABLE 2 : PARTICLE SIZE OF DICLOFENAC SODIUM LARGE UNILAMELLAR VESICLES (LUVS) AND MULTILAMELLAR VESICLES (MLVS) LIPOSOMES.

Liposomal formulation composition (molar ratio)	Liposomal formulation charge	Mean diameter of LUVs (µm) ± SD	Mean diameter of MLVs (µm) ± SD
PC : CH (7:2)	neutral	0.697 ± 0.73	0.723 ± 0.67
PC : CH (7:4)	neutral	0.699 ± 0.34	0.746 ± 0.48
PC : CH (7:7)	neutral	0.787 ± 0.52	0.796 ± 0.31
PC : CH: SA (7:7:0.5)	positive	0.687 ± 0.41	0.709 ± 0.67
PC : CH: SA (7:7:1)	positive	0.658 ± 0.37	0.697 ± 0.58
PC : CH: SA (7:7:2)	positive	0.636 ± 0.66	0.686 ±0.19
PC : CH: DP (7:7:0.5)	negative	0.697 ± 0.26	0.734 ± 0.81
PC : CH: DP (7:7:1)	negative	0.812 ± 0.57	0.872 ± 0.57
PC : CH: DP (7:7:2)	negative	0.816 ± 0.32	0.883 ± 0.26

***In vitro* drug release:** The effect of cholesterol content on DFS release from neutral LUVs and MLVs liposomal formulations could be depicted from **figures 2-3**, the results indicated that the increase of cholesterol molar ratio progressively decrease the DFS release from the liposomal formulations. After 24hrs, 72.32%, 80.61%, and 89.13% were released from MLVs and 80.15%, 83.43%, and 94.11% were released from LUVs liposomal formulations composed of PC: CH 7;7, 7:4 and 7:2 molar ratios respectively. Differences were significant at $p < 0.05$.

These obtained results can be explained by the ability of cholesterol to modulate the membrane fluidity by restricting the movement of the relative mobile hydraocarbone chains , reducing bilayer permeability [14] and decreasing the efflux of the encapsulated drug, resulting in prolonged drug retention [15].

Figures 4-5 show the effect of charge on the release of DFS from LUVs and MLVs, with (7:7) PC: CH molar ratio. The percent released from LUVs containing different charge inducing agent displayed significant difference (P = 0.03) during the first 30 min. The obtained data showed that negatively charged liposomes gave the highest rate and extent of drug release followed by neutral and positively charged ones. After ***24*** hrs, the percentages of DFS released were ***83.21*** %, ***72.32***%, and ***60.12***% for MLVs liposome and ***85.11***%, ***80.15***% and ***70.41***% for LUVs liposomes charged with negative, neutral and positive charge respectively.

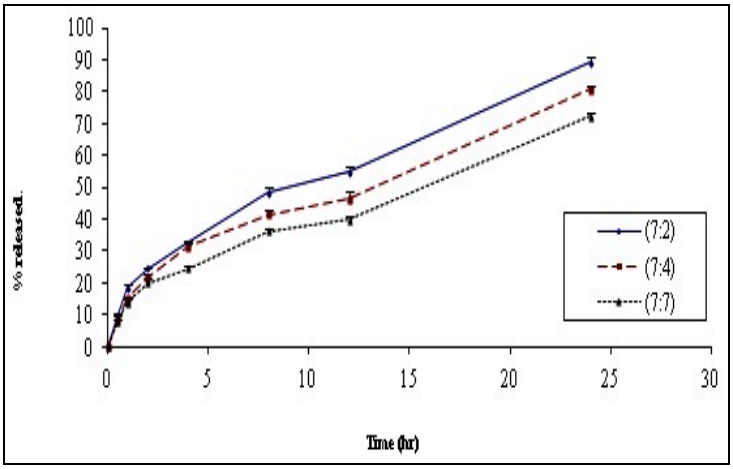

FIGURE 2: *IN-VITRO* RELEASE OF DICLOFENAC SODIUM FROM MLVS LIPOSOMES COMPOSED OF (PC: CH) IN DIFFERENT MOLAR RATIO IN PHOSPHATE BUFFER SALINE (N=3)

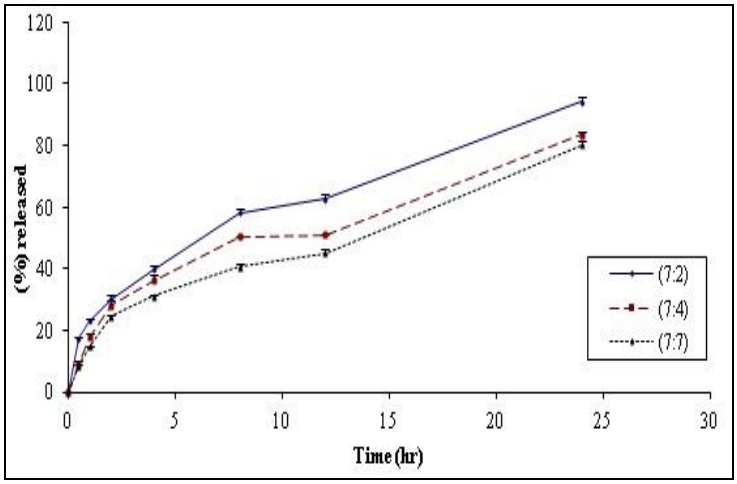

FIGURE 3: *IN-VITRO* RELEASE OF DICLOFENAC SOD. FROM LUVS LIPOSOMES COMPOSED OF (PC:CH) IN DIFFERENT MOLAR RATIO IN PHOSPHATE BUFFER SALINE (N=3)

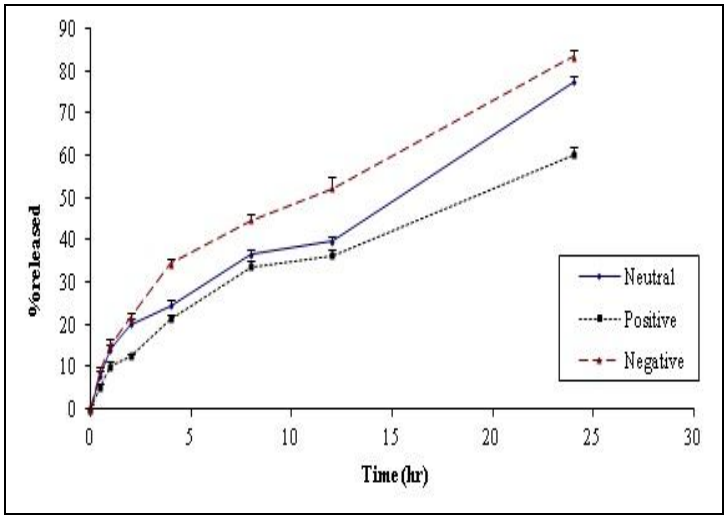

FIGURE 4: *IN-VITRO* RELEASE OF DICLOFENAC SODIUM FROM MLVS LIPOSOMES COMPOSED OF PHOSPHATIDYLCHOLINE: CHOLESTEROL (7:7) MOLAR RATIO IN PHOSPHATE BUFFER SALINE SHOWING DIFFERENT CHARGES (N=3)

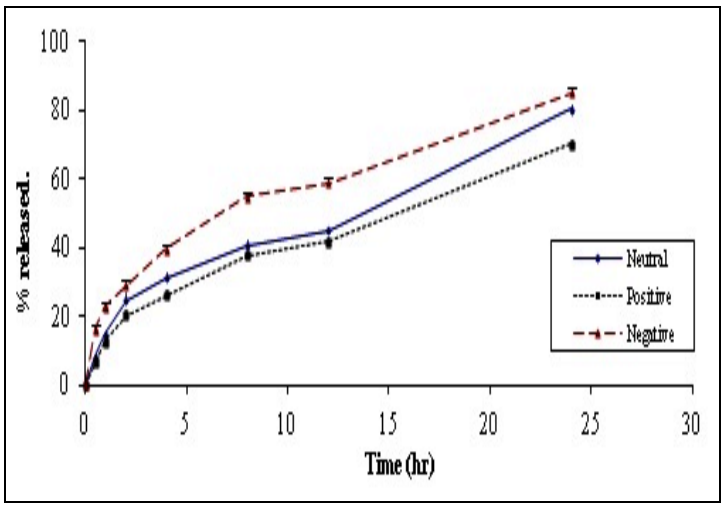

FIGURE 5: *IN-VITRO* RELEASE OF DICLOFENAC SODIUM FROM LUVS LIPOSOMES COMPOSED OF PHOSPHATIDYLCHOLINE: CHOLESTEROL (7:7) MOLAR RATIO IN PHOSPHATE BUFFER SALINE SHOWING DIFFERENT CHARGES (N=3)

The difference in the release pattern of drug may be attributed to the electrostatic attraction forces that exist between the acid moiety of the drug and the amines moiety of positive lipid, in addition, the charged lipids serve to tighten the molecular packaging of the vesicle bilayer [16]. In negatively charged liposomes, an electrostatic repulsion may occur between the drug and liposomes bilayers resulting in a higher percentage of drug release. Meanwhile electrostatic attraction forces may exist between the drug and positively charged liposomes resulting in slow release.

Figure 6 demonstrate drug release profiles from positively charged LUVs and MLVs liposomes and liposomal gels with (7:7:1) PC: CH: SA molar ratio. From the obtained data, DFS release was retarded by liposomal incubation in the gel form. Lower release rate from liposome gel systems compared to basic liposome dispersion could be a result of the influence of the viscosity of the gel matrix followed by slower drug penetration [17].

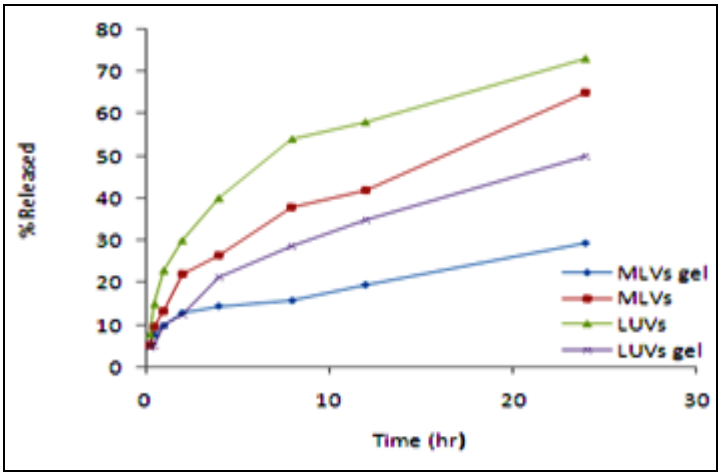

FIGURE 6: *IN-VITRO* RELEASE OF DICLOFENAC SODIUM FROM LUVS, MLVS AND GEL LIPOSOMES COMPOSED OF PC: CH: SA (7:7:1) MOLAR RATIO DISPERSED IN PHOSPHATE BUFFER SALINE.

Table 3 illustrates the kinetic studies of the release data, the data fitted Higuchi release kinetics, suggesting that, the drug transport occurred mainly by diffusion controlled mechanism. The results are in good agreements with the studies of many research coworkers who found that, many drugs were released from liposomal systems by the same mechanism[18-19].

TABLE 3: KINETIC ANALYSIS OF RELEASE DATA OF DICLOFENAC SODIUM FROM DIFFERENT LIPOSOMAL SYSTEMS

Liposomal type	Order of release		
	Zero order	First order	Diffusion order
LUVs			
PC:CH. (7:2)	0.938	0.971	0.995*
PC:CH. (7:4)	0.904	0.939	0.990*
PC:CH. (7:7)	0.915	0.940	0.989*
MLVs			
PC:CH. (7:2)	0.938	0.967	0.988*
PC:CH. (7:4)	0.817	0.862	0.968*
PC:CH. (7:7)	0.869	0.912	0.981*
LUVs			
Neutral (7:**7**:0)	0.916	0.953	0.988*
Positive (7:**7**:1)	0.923	0.960	0.993*
Negative (7:**7**:1)	0.945	0.979	0.993*
MLVs			
Neutral (7:**7**:0)	0.826	0.889	0.951*
Positive (7:**7**:1)	0.920	0.954	0.988*
Negative (7:**7**:1)	0.870	0.938	0.955*
LUVs			
PC:CH:SA (7:**7**:1)	0.952	0.983	0.993
PC:CH:SA hydrogel (7:**7**:1)	0857	0.872	0.920
MLVs			
PC:CH:SA (7:**7**:1)	0.890	0.942	0.984
PC:CH:SA hydrogel (7:**7**:1)	0.789	0.820	0.894

Physical Stability Study: The physical stability study of DFS entrapped in LUVs and MLVs liposomal gel was conducted at 2-8 °C and 25 °C for a period of 120 days. Drug leakage from the liposomal formulations with (7:7:1) PC: CH: SA molar ratio, was evaluated at definite time intervals. After 120 days, the percentage of drug retained in the LUVs liposomal formulations was 91.16%, 82.19%, and 75.54% at 2-8 °C, while it was 71.75%, 64.83%, and 63.35% at 25 °C for positively, negatively charged and neutral liposomes respectively.

The percent of DFS retained in MLVs was 92.56%, 84.11%, and 76.41% at 2-8 °C, while it was 73.87%, 67.18%, and 66.38% at 25 °C, for positively, negatively charged, and neutral liposomes respectively. Student t test showed that there was no significant differences between LUVs and MLVs liposomes prepared with the same lipid molar ratio at $\alpha = 0.05$. From the obtained data, positively charged liposomes showed the highest stability as manifested by the highest drug retention, followed by negatively charged liposomes, then neutral liposomes. Surface charge is one of the important factors that enhance the stability by reducing the aggregation rate and fusion of liposomal system during the storage [17].

Sterilization: **Table 4** summarizes the effect of autoclaving on entrapment efficiency of DFS in LUVs and MLVs composed of PC: CH :SA (7:7:1) molar ratios. The obtained result showed no remarkable difference between entrapment efficiency before and after autoclaving process for either LUVs or MLVs liposomal gel . In case of LUVs liposomal gel, no significant leakage of DFS observed during autoclaving process because the electrostatic attraction between DFS and SA was strong enough to overcome dissipative energy at high temperature[20]. In addition, no significant change ($P<0.05$) in the release rate was observed.

Table 4: Effect of autoclaving on entrapment efficiency of diclofenac sodium liposomal gel.

Liposomal formulations	Entrapment efficiency	
	Before autoclaving	After autoclaving
Positively charged* LUVs	56.73±1.45	55.83±2.03
Positively charged* MLVs	51.68±2.61	51.02±2.96

*PC : CH: SA (7:**7**:1)

***In vivo* Studies:** Positively charged liposomes at molar ratio (7:7:1) was chosen because the presence of electrostatic attraction between it and the corneal epithelium (negatively charged) at physiological pH, may enhance absorption of drug from liposomes [21]. Liposomal gels were chosen to prolong the precorneal residence time and enhance bioavailability.

Figure 7 depicts the healing rate of the ulcers in the rabbit's eyes. After 10 days, the percentage of healed ulcers were 12.5%, 35%, 67.5%, 82.5%, 85%, 87.5% and 95% for negative control, Voltaren eye drops, 0.5% carbopol 934 gel containing DFS, LUVs liposomes suspension, MLVs liposomes suspension, LUVs liposomal gel and MLVs liposomal gel respectively.

There was a significant difference between all formulations under test and the positive control ($P < 0.05$). The liposomal formulations provided higher healing level than the conventional eye drops due to the weak penetration of drug to the ocular tissue and if the drug is adsorbed its effect ends in a short time. On the contrast, the enhancing effect of carbopol 934 on healing of ulcer attributed to the increased ocular contact time of carbopol as a viscosity modifier (**fig. 8**) which in turn drastically enhances drug absorption, bioavailability, and then ulcer healing.

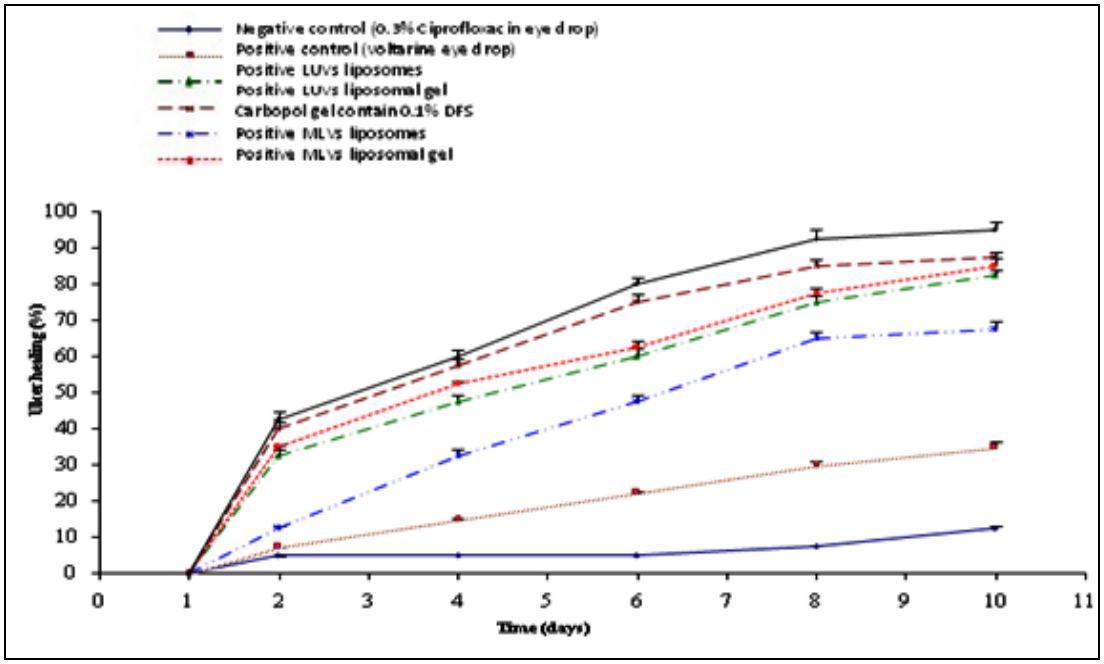

FIGURE 7: EFFECT OF DIFFERENT DICLOFENAC SODIUM FORMULATIONS ON THE HEALING RATE OF ULCERATIVE RABBIT'S EYES (N=3)

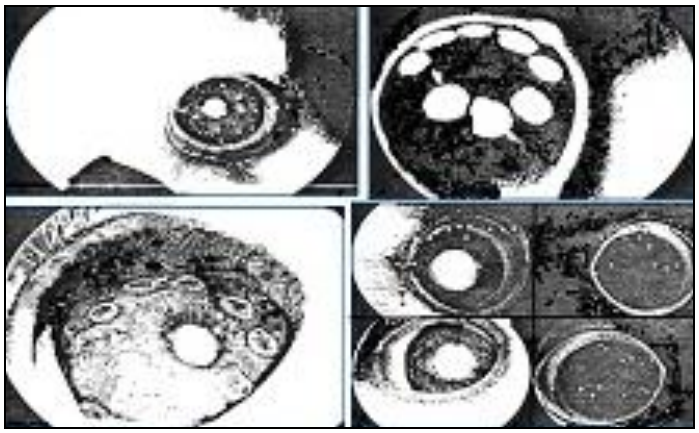

FIGURE 8: PHOTOGRAPHS SHOWING GRADUAL HEALING AND DISAPPEARANCE OF ULCERS

CONCLUSION: Liposomal gels were found to increase the ocular tissue permeation and deposition compared to control. Hence from results obtained it can be concluded that liposomal gels containing DFS have potential effects in treatment ophthalmic inflammation.

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