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PREPARATION AND EVALUATION OF THE ANTIVIRAL ACTIVITY OF ACYCLOVIR LOADED NANO-NIOSOMES AGAINST HERPES SIMPLEX VIRUS TYPE 1

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ABSTRACT

Acyclovir (ACV), analog of 2′-deoxyguanosine, is known for its antiviral activity against Herpes simplex virus (HSV). A major limitation of treatment with acyclovir is high distribution and low half-life that leads to taking high doses of acyclovir. Recent studies have shown that entrapment of acyclovir in nano-carriers can increase effectiveness and also decrease side effects of the drug. Therefore, in the present study the preparation and characterization of acyclovir loaded nano-niosomes was investigated. The non-ionic surfactant vesicles were prepared by thin film hydration method. The lipid composition in optimal formulation consisted of Span, cholesterol and D-α-tocopheryl polyethylene glycol succinate (TPGS) in the molar ratio of 55 : 30 : 15, respectively. Physical characteristics of optimized niosomes such as particle size, encapsulation efficiency (EE) and in vitro drug release were evaluated. Furthermore, the in vitro cytotoxicity study of empty niosomes (E-N), acyclovir loaded niosomes (ACV-N) and ACV as a free drug against Hela cell line was performed by MTT assay. The average of particle size and EE for optimized niosomes were 122.6 ± 0.2 nm and 24 % respectively. The drug release profiles proved the efficacy of optimized niosomes in prolonged release of ACV, so that the percent drug release for 1h was recorded as approximately 11.7 %. The prepared niosomes also showed significant stability with regard to particle size and EE when stored at least for seven days at 5˚C. The results of this study revealed ACV-N (F5) have a higher antiviral activity compared with free drug, and could be a suitable carrier for delivery of acyclovir in the treatment of HSV-1 infections.

Keywords: Acyclovir, Nano-niosomes, Herpes simplex virus.

INTRODUCTION

Herpes simplex virus (HSV) is one of the most common viruses in human populations, and is responsible for a broad spectrum of diseases, including gingivostomatitis, keratoconjunctivitis, encephalitis, neonatal infections, and genital diseases.[1,2] Herpes simplex virus is capable of causing a widespread spectrum of mild to severe disorders.[3,4] Several antiviral drugs which are effective against HSV infections have been

developed since the mid 1950s. Treatment of HSV infections with ACV has been considered as the gold standard.[1] Acyclovir or 9-[(2-hydroxyethoxy)methyl] guanine, an antiviral drug, is active against Herpes simplex virus (HSV-1 and HSV-2), Varicella zoster virus (VZV), Epstein-Barr virus (EBV) and Cytomegalovirus (CMV).[5] ACV is the drug of choice against HSV disease that act as an inhibitors of viral DNA polymerase.[3] HSV-1 DNA polymerase (pol) is known as a target for antiviral drugs.[1] However, this antiviral drug has a number of limitations, the oral bioavailability of acyclovir is poor and variable ranging from 15% to 30% , probably due to the reasonably low lipophilicity of the drug.[6] In intravenous administration, most of the drug molecules are excreted through urine without change.[7] Phlebitis is the most common adverse effect of acyclovir sodium during administration at the injection site, also precipitation of ACV crystals in renal tubules can occur.[8] The mean plasma half-life of acyclovir is 2.5 h.[4] Hence, repeated administration of high doses (200 mg five times daily for 10 days) is needed for treatment of HSV infections.[2] To overcome the mentioned limitations, we used niosomes, which are non-ionic surfactant vesicles that can entrap a number of solutes as drug carriers.[6,9] During the last two decades, niosomes have been suggested for a lot of potential therapeutic approaches, i.e. as the tumor targeting agent, diagnostic imaging agents, drug carriers of anticancer drugs, antifungal drugs, anti-inflammatory drugs and so on.[10] Niosomes are constructed from the self-assembly of non-ionic amphiphiles in aqueous media effecting in closed bilayer structures.[11] Niosomes or non-ionic surfactant vesicles generally consisted of non-ionic surfactants combined with cholesterol and dicetyl phosphate or stearylamine as charged lipids.[12] Cholesterol can have significant effects on bilayer rigidity, EE and generate less leaky vesicles.[13] Niosomes like liposomes are capable to entrap both hydrophilic and lipophilic drugs either in an aqueous layer or in vesicular membrane.[14] Niosomes are preferred Compared to traditional liposomes because they are biodegradable, biocompatible and also have more chemical and physical stability, low toxicity and cost.[15,16] These advantages make niosomes as alternatives to liposomes.[14] The aim of this study is to prepare and characterize niosomes for delivery of ACV and investigate cytotoxicity of formulations on Hela cell line. In addition the antiviral activity of ACV-N was evaluated against herpes simplex type 1 (HSV-1) infections in this type of cells.

MATERIAL AND METHODS

Materials

Acyclovir (ACV) was from Roozdarou (Tehran, Iran). Chloroform, 2-propanol, Sorbitanmonostearate (Span 60), Sorbitanmonolaurate (Span 20) were purchased from Merck (Germany). Cholesterol (CH)was from Northern Lipids, Inc. (Vancouver, Canada). Dicetyl phosphate (DCP), D-α-tocopheryl polyethylene glycol succinate (TPGS) was obtained from Sigma-Aldrich (Steinheim, Germany). Cellulose dialysis tubing (molecular weight cutoff 12000 Da) was from BioGene (USA). Dulbecco's modified Eagle's medium (DMEM) was from Invitrogen (USA), 3-(4, 5-dimethyltiazol-2-ly)-2, 5-diphenyltetrazolium bromide (MTT) was purchased from Sigma (USA). Fetal bovine serum (FBS), penicillin and streptomycin were supplied by Gibco (USA).

Preparation of Niosomes

The non-ionic surfactant vesicles were prepared by thin film hydration method, in which appropriate amounts of surfactant / CH and either DCP or TPGS were dissolved in chloroform according to the formula (Table 1). The mixture was placed in a rotary vacuum evaporator (150 rpm) and dried under reduced pressure at 65 ˚C to form a thin lipid film. The lipid film was then hydrated and sonicated simultaneously with solution of acyclovir (0.5 mg/ml) in phosphate buffer

saline (pH 7.4) at 65 ˚C for 1 h. The prepared niosomes were kept at room temperature for 60 min to form the vesicles. The formulations were optimized for various parameters, such as type of surfactant, surfactant : cholesterol ratio, freeze-thawing, DCP and TPGS in order to get maximum drug entrapment. Additionally, stability studies such as particle size, encapsulation efficiency and in-vitro drugs release showed that ACV-N were stable for at least 1 week at 5 ˚C (data not shown).

Size Distribution

Size and population distribution of niosomes were determined by dynamic light scattering (DLS) measurements using a Malvern Zetasizer Nano ZS (Malvern Instruments, London, England). The analysis was performed at 25 ˚C and after the dispersion was diluted to an appropriate volume with deionized water. The measurement was repeated 3 times for each sample.

Determination of Encapsulation Efficiency (EE)

The non-ionic surfactant vesicles were separated from the free (un entrapped) drug by dialysis bag. The amounts of un entrapped ACV were removed by dialysis for 5 h against 200 mL magnetically stirred PBS at 4˚C. Entrapted acyclovir in niosomes was determined spectrophotometrically (UV mini 1240, Shimadzu, Japan) at 251 nm using 2-propanol as blank. The concentration of entrapped acyclovir was determined after lysis of the niosomes with 2-propanol. A 50 µL aliquot of niosomal preparation was diluted with 950 µL of 2-propanol, so that a clear solution was observed.

The encapsulation efficiency percentage could be calculated as follows

$$\% \text{ EE} = \frac{\text{Amount of incorporated drug (µg)}}{\text{Total amount of drug added (µg)}} \times 100$$

In Vitro Drug Release

The in vitro release of acyclovir from niosomal formulations was performed and compared with free drug solution using cellulose dialysis tubing (molecular weight cutoff 12 kDa). Respective niosomal formulations (F2, F5) and free drug was taken in dialysis bag and placed in a beaker containing 30 ml of PBS for achieving sink condition. The beaker was placed on a magnetic stirrer under 100 rpm. The temperature was kept at 37 ˚C. The samples were withdrawn at a particular time and replaced with fresh dissolution medium. The samples were analyzed by HPLC. The HPLC system was equipped with an Ultra-plus II pump with a UVIS 200 detector that was set at 251 nm. The Nucleosil, C18 column (25 cm × 1 mm) was used. The mobile phase consisting of methanol and water (10:90 v/v) and flow rate was adjusted 100 µl/min.

Effect of Freeze-Thaw Cycling

Routine freeze-thawing cycles are often claimed to multilamellar vesicles (MLVs) for disposition homogenize their lipid composition.[17] The freeze-thaw experiments were studied on the MLV niosomes. The MLV suspensions were frozen and thawed for ten cycles. In each cycle, 1 ml sample of prepared niosomes (F-2) were frozen for 5 min in liquid nitrogen at −196 ˚C and then immediately thawed for 5 min in a water bath at 65 ˚C. The EE was calculated as mentioned before.

Cell Culture and Viruses

Hela cells were obtained from Tehran University Cell Bank of Iran (Tehran, Iran) and were cultured in culture flasks. This cell line was grown as monolayers in DMEM medium with 2% FBS, 1% penicillin–streptomycin. The strain of Herpes simplex type 1 (HSV-1) were prepared and adapted by laboratory from Tehran University, Tehran,

Iran. Virus titer was studied by 50% tissue culture infectious doses ($TCID_{50}$) and examined on Hela cell lines.

Cell Viability Assay

The in vitro cytotoxicity effect of ACV-N, E-N and ACV against Hela cells was investigated by MTT assay, according to the standard method described by Mosmann.[18] Hela cells were seeded in 96-well plates at a density of 5×10^3/well, were incubated with ACV-N, E-N and ACV for 48 h at 37 ˚C. After that 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was added to the wells and incubated for 2 h at 37 ˚C to allow the conversion of MTT to formazan by mitochondrial dehydrogenase. Formazan crystals were dissolved in DMSO solution and determined at 570 nm by using a 96-well plates reader (MRX, Dynex, USA). The viability of each formulation was considered as a percentage, by comparing treated cells (ACV-N, E-N and ACV) with cells incubated with the culture medium.

Evaluation of Antiviral Activity

The titer of the virus was 1×10^6 / ml, determined by $TCID_{50}$, according to Reed and Munch formula. 1×10^5 Hela cells were seeded in 96-well plates and infected with 1000 pfu of HVS-1 (MOI 0.01 pfu / cell) and then incubated at 37 ˚C for 1 h to allow viral adsorbed. After adsorption the medium of plates collected and replaced with fresh medium containing different concentrations of ACV, ACV-N and E-N. After 24 h incubation, the antiviral activity of ACV-N, E-N and acyclovir against HSV-1 were studied. The end-point of the test was the inhibitory concentration of drug that decrease virus yield by 50% in Hela cells.

RESULTS AND DISCUSSION

Determination of Particle Size

The mean particle size of niosomes, composed of span 20 and 60, are shown in Table 1. It was obvious that niosomes using span60 / CH / TPGS were smaller in size than other niosomes formula. As shown in Figure 1, the average size of these vesicles were 122.6 ± 0.2 nm with an acceptably good polydispersity. Size of vesicles depend on the hydrophilic - lipophilic balance (HLB) of the surfactant. The HLB of span 20 and span 60 are 8.6 and 4.7 respectively. The results revealed that, niosomes prepared with span 60, have smaller particle size than those niosomes prepared with span 20. It is reported that the size of vesicles reduced by increasing hydrophobicity of the surfactant, since surface free energy decreases with increasing hydrophobicity [19,20]. This observation is consistent with the results of Ruckmani et al, 2000.

The Effect of Charged Lipids

Usually charged molecules like DCP as negatively and Stearylamine (STR) as positively charged molecules are used for preventing aggregation of niosomes. Normally 2.5–5 mol % of charged molecule is added in niosomal formulation.[13] Electrostatic stability of charged molecule led to prevention of sedimentation of niosomes.[21] Some recent reports indicate, incorporation of charged molecule to niosomal formulation may result in size reduction of vesicles. In contrast, some studies show efficient enhancement in the particle size.[13] Jia-You Fang mentioned using DCP, makes vesicles larger than those without DCP due to high degree of hydrophilicity.[22] However, in our study, inclusion of DCP in niosomal formulations led to decrease of vesiclessize .

The Effect of Freeze-Thaw

The effect of freeze-thawing on EE was investigated. In this study 10 freeze-thaw cycles were used to be sure that increasing in number of freeze-thaw cycle will not able to

increase the EE any more . As illustrated in Figure 2, when the freeze-thawing process were used the EE of vesicles increased after four freeze-thaw cycles (p=0.003). Subsequent increases in the number of freeze-thaw cycles resulted in a further increase of the EE up to 6 cycles and then began to decrease .(p=0.037). Although freezing and thawing is a simple technique to enhance the trapped volume, but the exact mechanism by which this procedure could enhance the trapped volume is not clear. [23] However, breaking of MLVs by freeze-thawing procedures produce more homogeneous vesicles, unilamellar vesicles, and lead to increased encapsulation efficiency.[17,24] It has revealed subsequent freeze-thaw cycles caused undesired broadening of the size distribution, and this resulted in a ~5% increase in EE.[25]

The Effect of TPGS

TPGS (α-Alpha-tocopheryl polyethylene glycol 1000 succinate) has been extensively used in the food and drug industry. TPGS is an amphiphilic vitamin E, remarkably high water solubility, with HLB value between 15 and 19, can act as a suitable surfactant to emulsify hydrophobic molecules.[26] TPGS has a lot of advantages which make it excellent molecule to improve different drug delivery systems such as prodrugs, micelles, liposomes and nanoparticles.[27] TPGS has been approved by US FDA and used in drug formulation as a safe pharmaceutical adjuvant. TPGS can be used as a surfactant and / or component in liposomal formulation, resulting in sustained and controlled drug delivery. TPGS is a suitable emulsifier, resulting in high drug entrapment, and cellular uptake on many cells.[27] In our study, we used TPGS in niosomal structure to develop a new niosomal formulation loaded with acyclovir to enhance the EE. The profiles of drug loading into the niosomes presented in Table 1. As it show the highest EE were observed for niosomes with TPGS component in which maximum EE was about 24 % with a mean particle size of 122.6 ± 0.2 nm. It can be concluded that TPGS can be a suitable surfactant to formation of niosomes, and considering as a potential drug delivery carriers.

The Effect of Surfactant Structure

To study the effect of surfactant structure on acyclovir entrapment, niosomal formulations of span 20 and 60 were prepared with the same total lipid concentration (33 mmol/L). Results showed that the niosomes made from Span 60 has higher EE in contrast to niosomes with span 20 ($P < 0.05$). This can be attributed to some facts. The length of alkyl chain of surfactant is an important parameter on EE. Among these spans, Span 60 has a longer saturated alkyl chain (C_{16}) as compared to span 20 (C_{12}) and exhibits the higher entrapment [28]. Another reason may be due to the phase transition temperature (T_C) of the surfactant. Span 60 has the highest transition temperature (T_C = 53 ˚C) among other spans. The Span with the highest phase transition temperature allows the highest entrapment.[19,29]

The Effect of Cholesterol

Cholesterol is one of the influencing parameters on niosomal system. Cholesterol is known to eliminate the gel to liquid phase transition of niosomal systems. Moreover, cholesterol can increase the membrane stability resulting in less leaky through the membrane out of the vesicles.[30] Incorporation of cholesterol is effective on stability and permeability of vesicles.[29] The effect of cholesterol on EE was investigated by different cholesterol molar ratio (see Table 2). It was found that the EE of niosomes with Span 60 decreased by increasing the amount of cholesterol. This was similar to the findings of GhadaAbdelbary and Himmat Singh et al which showed that the presence of cholesterol decreased the encapsulation efficiency of the prepared niosomes. This reduction maybe due to the fact that excess cholesterol in certain cases disrupt the regular bilayered structure of niosomes.[11,31] It was observed that niosomes prepared using Span 20, the EE increased by increasing the amount of cholesterol, which is in accordance with the results obtained by

Mukherjee *et al.* [32] We note that niosomal formulations using span 20 (F1–F3) are larger in size than niosomes prepared using span 60 (F2–F4). Span 20 have higher HLB compared to span 60 as mentioned before and it was reported that surfactants with higher HLB give larger vesicles.[6,19] In our study niosomes with span 60/ CH at the molar ratio of 70 / 30 respectively, was selected as a highest EE. The amount of cholesterol to be added to niosomal structure is determined by HLB value of the non-ionic surfactant. Span 60 have low HLB value (4.7) and because of this can form niosomes without addition of cholesterol, Therefore only small amount of cholesterol is needed to maintain membrane rigidity.[33]

In Vitro Release

The release profile of acyclovir from niosomal formulations and its free solution in PBS are shown in Figure 3. As a result, the niosomal formulation showed a lower release rate of drug than free drug solution. According to the results of in vitro release studies, the release pattern of F5 was 11.7 % after 1 h. But in case of F2 and free drug solution, the amount of release were 25 % and 80 % respectively. Therefore F5 was an optimized formulations and low cumulative release at specified time interval. It can be considered that release of drug from niosomes with TPGS component showing a slow and sustained released. The physico-chemical properties of the material, drug and surfactant might be effective on the release from nanoparticles.[34] Although TPGS is amphiphilic but its hydrophobicity is more than its hydrophilicity.[34] Therefore both acyclovir and TPGS are hydrophobic. The reason that niosomes with TPGS exhibited slower release compared to other niosomal formulations may be due to the increased interaction between acyclovir and TPGS and thus caused the slower drug release. This finding can be efficient in retarded release of drug in acyclovir drug delivery.

Cellular Toxicity

The toxicity of all formulations were studied by MTT method. The viability values were depicted in percent compared to non-treated cells. The data of Figure 4, proved that no considerable toxicity of different formulations, after 48 h of incubation up to 100 µM ($p = 0.007$). The lack of toxicity in uninfected cells was due to this fact that acyclovir is a pro-drug and need to be phosphorylated by viral thymidine kinase.[35]

Antiviral Activity of Acyclovir-Niosomes

The antiviral activity of ACV-N and ACV against HSV-1 were evaluated by $TCID_{50}$. Figure 5, compares the influence of different concentration of ACV-N and ACV on the HSV-1 replication at 24 h. The efficient concentration to inhibit 50 % of virus replication (IC_{50}) for ACV-N and the drug solution was 1 µM and 3 µM respectively. This result shows that the niosomes containing acyclovir have greater antiviral activity than free drug. Whereas niosomes without acyclovir show no antiviral activity (data not shown). The higher antiviral activity of ACV-N compared to ACV as a free drug may be attributed to the interactions of niosomes with cells, which have been reported to enter cells by fusion or endocytosis.[36] On the other hand, this higher antiviral activity might be attributed to the uptake of different colloidal carriers that is correlated to the membrane perturbation caused by the virus.[37]

CONCLUSION

In the current study, acyclovir niosomes were prepared and characterized regarding their size, encapsulation efficiency and in vitro drug release. The antiviral activity of prepared niosomes also investigated. ACV-N did not show cytotoxic effects on Hela cells. We found that niosomes containing acyclovir had 3 times greater antiviral activity than acyclovir. Therefore the niosomal formulation could be a promising drug delivery system

capable of increasing the antiviral activity of acyclovir.

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Table 1: Formulation of various niosomes containing acyclovir

Formulation	Lipid compositions	Molar ratio	%EE ± SD (n=3)	Mean vesicle diameter (nm)
F1	Span 20:Cho	70:30	15 ± 1.6	987± 0.7
F2	Span 60:Cho	70:30	17 ± 1.4	176.6 ± 0.3
F3	Span20:Cho:DCP	65:30:5	13.5 ± 1.8	302 ± 0.2
F4	Span60:Cho:DCP	65:30:5	17.1 ± 0.8	129.5 ± 0.1
F5	Span60:Cho:TPGS	55:30:15	24 ± 0.4	122.6 ± 0.2

Table 2: Effect of cholesterol on encapsulation efficiency

	Molar Ratio (µmol) Surfactant : cholesterol	EE %(mean ± SD, n = 3)
Span 60	80/20	17 ± 1.4
	70/30	17 ± 1.4
	50/50	15 ± 1.6
Span 20	80/20	14 ± 0.92
	70/30	15 ± 1.6
	50/50	16 ± 1.4

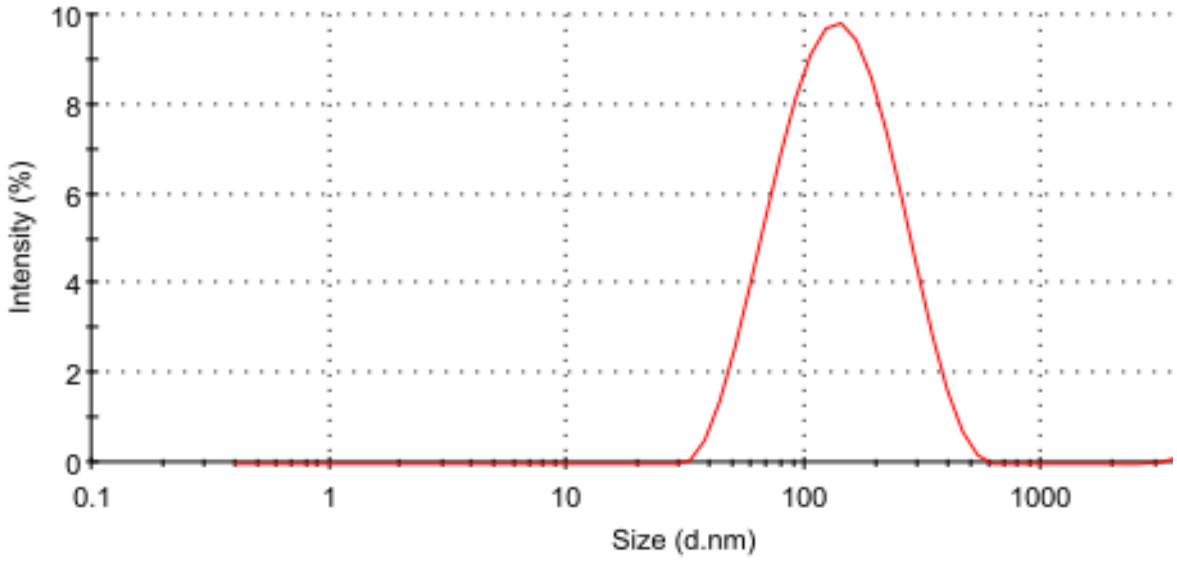

Figure 1 : Particle size distribution of niosomes (Span60:Cho:TPGS)

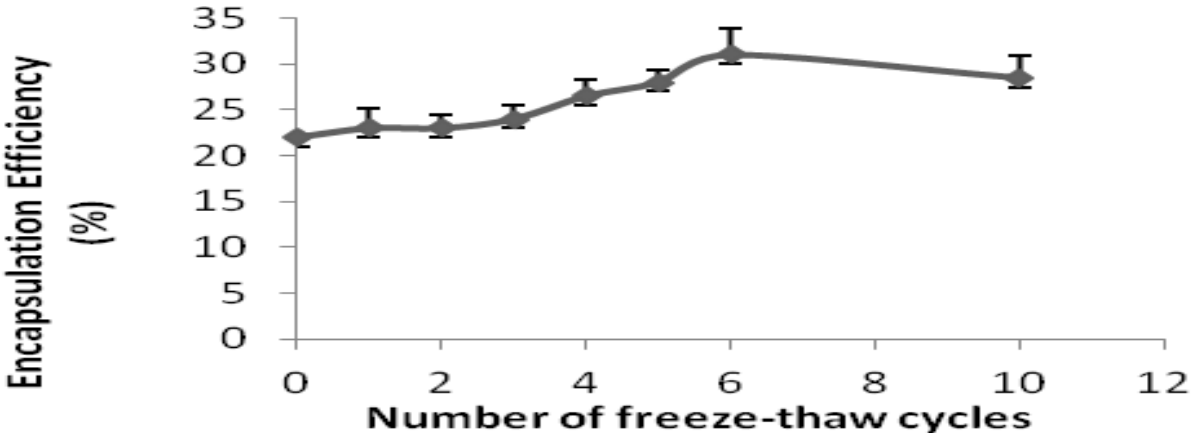

Figure 2 : Influence of the number of freeze-thaw cycles on the EE % of MLVs made of Span 60 : Cho (70 : 30 molar ratio) (n=3 ± S.D.)

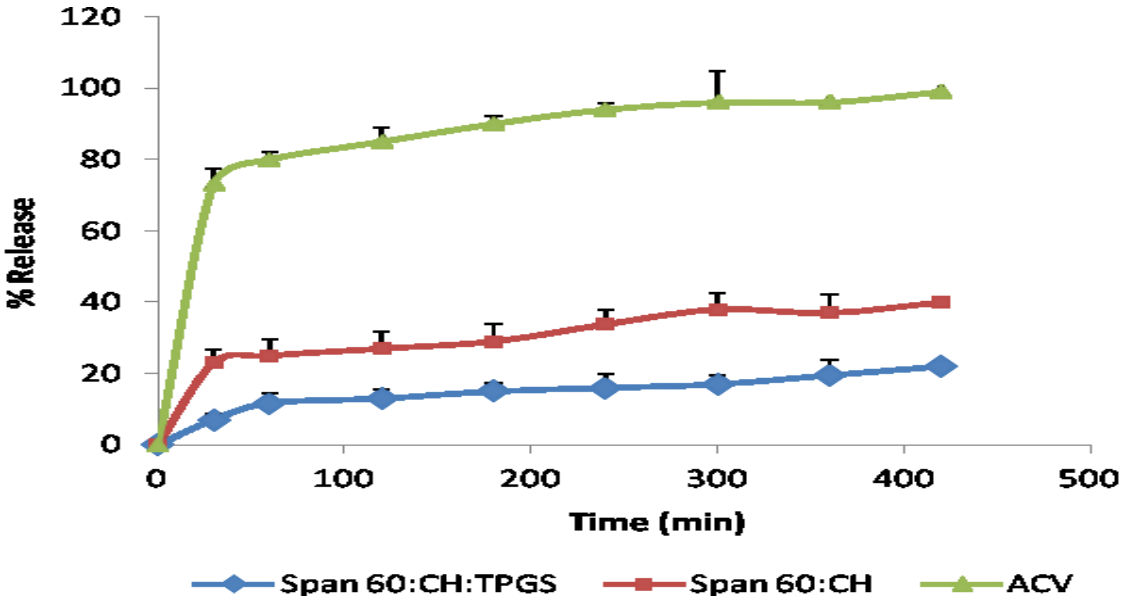

Figure 3 : The drug release of acyclovir from niosomal formulation and free drug.

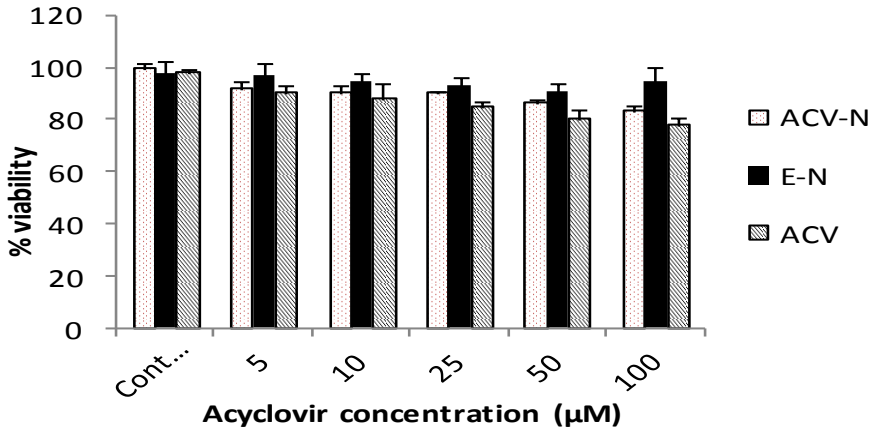

Figure 4 : Cytotoxicity of different acyclovir formulations against non-infected Hela cells for 48 hours. Each point represent the mean ± S.D. (n=3).

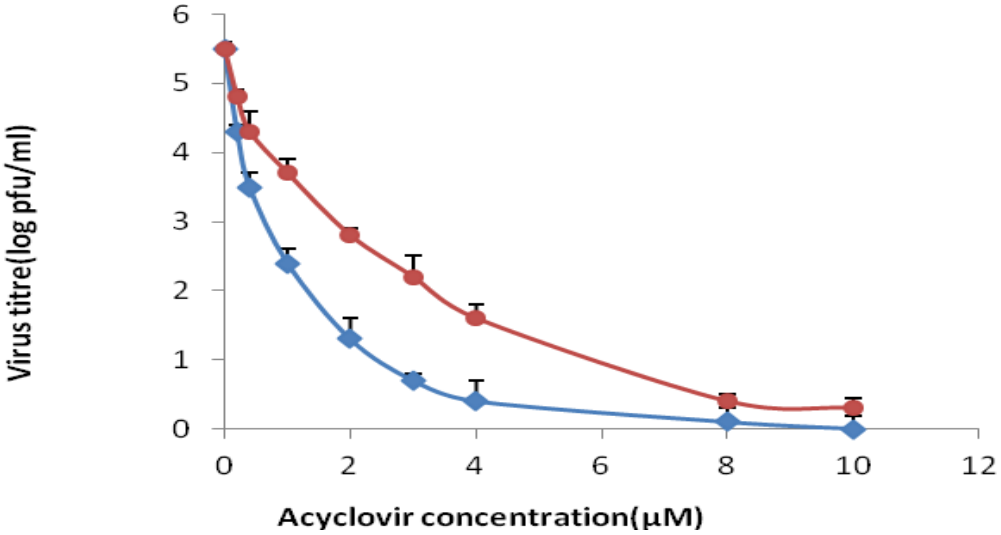

Figure 5 : Effect of different concentration of acyclovir- niosomes (F5) and acyclovir on HSV replication at 24 h

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