PREPARATION AND EVALUATION OF NOVEL TOPICAL GEL PREPARATIONS FOR WOUND HEALING IN DIABETICS

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ABSTRACT

Diabetes mellitus is one of the metabolic disorders that impedes normal steps of wound healing process. Recently, statins have been shown to have anticoagulant, immunosuppressive and antiproliferative effects that could conceivably affect wound healing or the risk of wound complications after surgery or injury. The objective of the present study was to develop formulations of Atorvastatin in different types of gels including hydroalcoholic , Hydrogel, microemulsion, Anhydrous, and Alcoholic gel bases. The prepared gels were evaluated for physical appearance, rheological behavior, drug release through a standard cellophane membrane and wound-healing power in streptozotocin-induced diabetic male albino rats. Results showed that all gel formulations showed good and acceptable physical properties. The data obtained from release studies revealed that the total amount of drug released was affected by the nature of the base with maximum drug release was observed with the alcoholic gel base. The rank order of the various gel formulations based on their maximum drug release was alcoholic > hydrogel > hydroalcoholic > anhydrous > microemulsion gel base. Statistical analysis showed that the higher release rate of the first three gel bases was significant (P<0.1) when compared to the anhydrous and the microemulsion gel bases. However, the differences between these three bases were statistically insignificant, (P>0.05). The release kinetics of ATR from the prepared gels, according to various kinetic models gave linear relationships. The best linearity was found in Higuchi´s equation and first order equation. Results of In-vivo wound healing studies showed a time dependant increase in percent wound contractions which is higher than that produced by the control groups. These contractions were statistically significant (P<0.001), during the first 10 days of the study in case of hydroalcoholic, Hydrogel, and Anhydrous. The anhydrous base showed the highest percent wound contraction with complete wound closure and epithelization was observed on 7th day of wound induction. All the animals tolerated the applied gels and no signs of irritations were noticed during the whole period of study

Keywords: Atorvastatin – Gels – Release – Wound healing.

INTRODUCTION

The healing of cutaneous wounds is a dynamic, complex, and well-organized process and requires the orchestration of many different cell types and cellular processes[1]. The classic model of wound healing is divided into three sequential phases: inflammation, proliferation, and maturation[2]. Each phase is characterized by the sequential elaboration of distinctive cytokines by specific cells.

Adult skin consists of two major tissue layers: a keratinized stratified epidermis and an underlying thick layer of collagen-rich dermal connective tissue providing support and nourishment. Appendages such as hairs and glands are derived from, and linked to, the epidermis but project deep into the dermal layer. Because the skin serves as a protective barrier against the outside world, any break in it must be rapidly and efficiently mended. A temporary repair is achieved in the form of a clot that plugs the defect, and over subsequent days steps to regenerate the missing parts are initiated. Inflammatory cells and then fibroblasts and capillaries invade the clot to form a contractile granulation tissue that draws the wound margins together; meanwhile, the cut epidermal edges migrate forward to cover the denuded wound surface[3]. However, various physiologic and mechanical factors may impair the healing response, resulting in a chronic wound that fails to proceed through the usual stepwise progression.

Diabetes mellitus is one such metabolic disorder that impedes normal steps of wound healing process. Many histopathological studies show prolonged inflammatory phase in diabetic wounds, which causes delay in the formation of mature granulation tissue and a parallel reduction in wound tensile strength[4]. Type 2 DM, in particular, is more prevalent in older patients[5], whom age-related skin changes already negatively impact the healing process. Complications resulting of DM include ischemia and neuropathy which may lead to foot ulceration[6]. Diabetic foot ulcers frequently become infected and are a major cause of hospital admissions. They also account for more than half of non-traumatic lower limb amputations in this patient population[7,8]. For these reasons, it is important to manage diabetic wounds effectively.

More recently, statins have been shown to have a number of beneficial effects that are not related to lipid lowering. Statins have anticoagulant, immunosuppressive, and antiproliferative effects that could conceivably affect wound healing or the risk of wound complications after surgery or injury. Studies also showed that local atorvastatin therapy may be useful for healing the wounds in diabetic rats. More encouraging, the safety profile of statins is excellent and the major side effects are rare and mostly reversible[9,10].

The objective of the present study was to develop formulations of Atorvastatin in different types of gels. The prepared gels were evaluated for physical appearance, rheological behavior, drug release through a standard cellophane membrane, and wound-healing power in streptozotocin-induced diabetic male albino rats.

MATERIAL AND METHODS

Materials

Atorvastatin (Gift sample, Medical union of pharmaceutical co., Egypt), Carbopol-941 (Sigma-Aldrich, USA), Triethanolamine (Oxford laboratory reagents, Mumbai), HPMC (winlab, UK), Tween 80 (Columbus chemicals industries, USA). All other chemicals are of analytical grade.

Preparation of Hydroalcoholic gel

Gels were prepared by dissolving the specified amount of drug in methanol. (Formula F1, table 1). Carbopol 941 was soaked in a mixture of water: methanol: PG (4:4:2 w/w). The mixture was kept under magnetic stirrer for 10 hrs then the dispersion was neutralized (pH 7.2) and its viscosity was improved by adding triethanolamine (TEA).

Preparation of Hydrogel

The specified amount of Carbopol was soaked in a mixture of water and PG (8: 2 w/w). The drug was added to the mixture and kept under magnetic stirrer for 10 hrs. The viscosity and pH are finally adjusted with TEA until the desired values were approximately reached (Formula 2, table 1).

Preparation of microemulsion gel

The appropriate amount of Tween 80, PG, and oleic acid were weighed accurately and placed in a stoppered flask. ATR was added as a solution in 1 ml alcohol into the flask. The mixture was stirred using a magnetic stirrer for 10 minutes, then the microemulsion gel was formed by adding water with continuous stirring for another 10 minutes. The gel was stored at room temperature for 48 hrs for equilibration (Formula 3, table 1). The method was previously described by Nagia et al.[11]

Preparation of Anhydrous gel

The drug was first dissolved in alcohol before the incorporation of the specified amount of glycerin (Formula F4, table 1). Carbomer 941 was added with stirring for 24 hrs using magnetic stirrer. The prepared gel was stored at room temperature for 48 hrs for equilibration. Anhydrous glycerin-based Carbopol gels can be prepared without the need for neutralization and they are suitable for oxygen/water-sensitive drugs[12].

Preparation of Alcoholic gel

The method was carried out as previously described by valencia et al.[13] with modifications. The formulation was prepared by soaking HPMC in distilled water at 30 °C for 12 hrs until the polymer was fully hydrated, then alcohol (6 ml) was added with stirring. Carbomer 941 was added and stirring continued for 10 hrs until the mixture was homogeneous. ATR was added as a solution in 1 ml alcohol with stirring. Finally the specified amount of glycerin was added as a gelling agent to HPMC-carbomer 941 combination. TEA may be added to adjust the viscosity and obtain a more accepted appearance of the gel. (Formula F5, table 1).

Table 1: Compositions of the prepared gels

	F1	F2	F3	F4	F5	F6
Atorvastatin (% w/w)	1.0	1.0	1.0	1.0	1.0	1.0 *
Carbomer 941 (% w/w)	1.0	1.0	-	1.5	1.0	1.0
PG (g)	2	2	0.5	-		2
Oleic acid (g)	-	-	2	-		-
Methanol (ml)	4	-	1	1	7	4
Tween 80 (g)	-	-	2.5	-		-
HPMC (mg)	-	-	-	-	75	-
Glycerin (g)	-	-	-	9	2	-
TEA	q.s.	q.s.	q.s.	-	q.s.	q.s.
Distilled Water (g) to	10	10	10	-	10	10

* ATR is introduced into the formula (F6) as an inclusion complex with HP-B-CD

Physical Examination

The prepared gels were inspected visually for their color and homogeneity. The spreadability of the gel formulations was determined by measuring the spreading diameter of 1 g of gel between two horizontal plates (20 cm × 20 cm) after one min. The standardized weight tied on the upper plate was 125 g [14]. The results obtained were average of three determinations. The pH was measured, at room temperature, in each gel sample using digital pH meter which was calibrated before each use with standard buffer solutions. The pH of the gel formulations was performed at 1, 10, 45 and 60 days after preparation to detect any pH changes with time.

Drug Content Studies

A specific quantity (100 mg) of each gel was taken and dissolved in 100ml of methanol. The stoppered volumetric flask containing the gel was stirred for 2 hrs on magnetic stirrer, at 250 rpm, to get complete solubility of the drug. The solution was filtered to remove the undissolved residues and estimated spectrophotometrically for the drug content. An unloaded gel was also subjected to a similar determination to observe the effect of excipients on the absorbance.

Viscosity Measurement

Viscometer (Brookfield digital viscometer DV-III, USA) was used to measure the viscosities (in cPs) of the gels. The spindle was rotated at 0.3 rpm. Samples of the gels were allowed to settle over 30 min at the assay temperature (25 ±1ºC) before the measurements were taken.

In vitro drug release studies

A sample of two grams gel was accurately weighed and placed on a semipermeable standard cellophane membrane (previously immersed in phosphate buffer, pH 7.4, for 24 hrs). The loaded membrane was stretched over the lower open end of a glass tube of 3 cm diameter and sealed with a rubber band. The glass cylinder w as then immersed in a 250 ml beaker containing 200 ml of the phosphate buffer (pH=7.4) in such a manner that the membrane was located just below the surface of the sink solution. The whole dialysis unit was placed in a thermostatically controlled shaker water-bath adjusted at 37± 0.1 °C with a constant stirring at 50 rpm to avoid any development of a concentration gradient. At appropriate sampling intervals an aliquot, 2ml, was collected and replaced by equal volume of the phosphate buffer at the same temperature to keep the volume of the sink solution constant during the experiment. Samples were then assayed spectrophotometrically at λ_{max} 241 nm. The concentration of ATR in each sample was determined from the standard curve previously constructed. Blank gel samples were carried out simultaneously to check for any interference.

Kinetic studies

To analyze the mechanism of drug release from the prepared gels, the following plots were made: cumulative % drug release vs. time (zero order kinetic model); log cumulative of % drug remaining vs. time (first order kinetic model); cumulative % drug release per surface area of membrane vs. square root of time (Higuchi model), and cube root of drug % remaining in matrix vs. time (Hixson-Crowell cube root law). The correlation was used as an indicator of goodness-of-fit.

In addition, Korsmeyer et al[15] derived a simple relationship which described drug release from a polymeric system. To find out the mechanism of drug release, log cumulative % drug release was plotted against log time according to equation (1):

$$M_t / M_\infty = K_{KP} t^n \dots (1)$$

Where M_t / M_∞ is fraction of drug released at time t, k is the rate constant and n is the release exponent. The n value is used to characterize different release mechanisms as the following

(n)	Diffusion mechanism
0.45	Fickian diffusion
0.45 < n < 0.89	Anomalous diffusion
0.89	Case-II transport
n > 0.89	Super case-II transport

In vivo Evaluation of wound healing activity

The study was conducted on male albino rats of 200-250 g and maintained under standard conditions (room temperature 22 – 27 °C and humidity 50-60 %) with 12 hr light and dark cycle. The in vivo experimental protocol was approved by the ethical committee of faculty of pharmacy, El-Minia university.

The overnight fasted rats were made diabetic by a single intraperitoneal injection of streptozotocin in a dose of 150 mg/kg [16]. Seven days later, rats with blood glucose levels between 300-400 mg/dL were selected for the in vivo evaluation. The selected animals were divided into six groups with six animals in each group. The first was the control group that is treated with hydroalcoholic gel base, without the drug. The others were the test groups and received the prepared gels. The rats were anesthetized by administering urethane (0.5 ml/kg i.p.). The back of the mouse was shaved and then sterilized using an alcohol swab. Wounds of approximately 2 cm² in surface area and 2 mm in depth were made with sterile scalpel on the shaved area of the back skin below the shoulder blades. A wound placed in this area cannot be reached by the animal and therefore prevent self-licking. The wounding day was considered as day zero. The wounds were treated with topical application of the prepared gels (100 mg once daily) till the wounds were completely healed.

The wounds were monitored and the area of wound was measured daily. The % of wound contraction is calculated as the following[17,18,19]:

% of wound contraction =

$$\frac{\text{Wound area on day (zero) - Wound area in day (n)}}{\text{Wound area on day (zero)}} \times 100$$

Where, (n) = number of days

Skin irritation studies

Rats (200-250 g) of either sex were used for testing of skin irritation. The animals were maintained on standard animal feed and had free access to water. The animals were kept under standard conditions. Hair was shaved from back and area of 4 cm² was marked on both the sides, one side served as control while the other side was test. Gel was applied (500 mg/animal) twice a day for 7 days and the site

was observed for any sensitivity and the reaction if any, was graded as 0, 1, 2, 3 for no reaction, slight patchy erythema, slight but cofluent or moderate but patchy erythema and severe erythema with or without edema, respectively [20].

Statistical analysis

All values were expressed as Mean ± SEM. The statistical analysis was performed using one way analysis of variance (ANOVA). The value of p less than 5% ($p < 0.05$) was considered statistically significant.

RESULTS AND DISCUSSION

Physical Examination

The physical properties of the gel formulations are shown in Table 2. All gel formulations showed good homogeneity and spreadability. The physical appearance of most formulations was colorless and transparent. The pH of the gel formulations was in the range of 6.9 ± 0.04 to 7.2 ± 0.28, which lies in the normal pH range of the skin and would not produce any skin irritation. It is important to say that there was no significant change in the pH values as a function of time for all formulations. The drug content of the gel formulations was in the range of 97.4 ± 0.83% to 99.3 ± 1.82 %, indicating the uniformity of drug contents. The viscosity values of all the prepared formulations was considered acceptable with the formulations F3 and F4 were less viscous than the others.

In vitro drug release studies

The data obtained from release and kinetic studies were shown in figure 1 and table 3. It was observed that the total amount of drug released was affected by the nature, rather than the viscosity, of the base. Maximum drug release was observed with the formulation F5 (Alcoholic gel). This higher release rate was statistically significant ($P < 0.01$) when compared to the microemulsion and the anhydrous gel, formulations F3 and F4 respectively.

Table 2: Physical properties of the prepared gels

	Physical Appearance	Homogeneity	Spreading diameter after 1 min (mm)	pH	Viscosity (cp)	Drug Content
F1	Colorless, Transparent	Homogeneous	54 ± 0.82	7.1 ± 0.06	172942 ± 1653	99.2 ± 1.37
F2	White, Opaque	Homogeneous	58 ± 0.63	6.9 ± 0.13	161650 ± 2157	98.2 ± 0.92
F3	Colorless, Transparent	Homogeneous	73 ± 0.66	7.1 ± 0.61	98984 ± 2328	99.1 ± 2.11
F4	Colorless, Transparent	Homogeneous	79 ± 0.72	----------	85699 ± 1572	99.3 ±1.82
F5	Colorless, Transparent	Homogeneous	63 ± 0.84	7.2 ± 0.04	106820 ± 4283	98.82 ± 1.02
F6	Colorless, Transparent	Homogeneous	52 ± 0.27	7.2 ± 0.28	185365 ± 2743	97.4 ± 0.83

* Values are expressed as mean ± SD, n=3

The rank order of the various gel formulations based on their maximum drug release is F5>F2>F1>F4>F3. The cumulative amounts released at 120 minutes were 3.9, 3.6, and 2.7 mg/cm² for the alcoholic gel, hydrogel, and hydroalcoholic gel bases respectively. However, the differences between these three gel bases were statistically insignificant, ($P > 0.05$). The enhanced drug release from the formulation F5 could be ascribed to the effect of alcohol that may facilitate the partitioning of drug into the receptor solution and decreasing the viscosity of the gel. These effects were previously suggested by Chi and Jun[21].

Atorvastatin is introduced into formulat F6 as an inclusion complex with HP-β-CD, as the former is known to be sensitive to heat, humidity, and low pH and the later is thought to protect the drug against such environmental conditions[22,23,24]. Results showed that presence of ATR as an inclusion complex with HP-β-CD did not affect the total amount released or the mechanism of release. Statistical studies confirmed this result, as the P value between F1 and F6 was higher than 0.05, considered a nonsignificant. The decreased release of ATR from microemusion gel base (F3), as observed from the figure, is probably, due to the solubility of ATR in oleic acid, 2.4 mg/ml [11] that decreased partitioning of the drug into the receptor solution, and hence, decreased the release rate. The low release rate of ATR from the anhydrous gel (F4) could be attributed to the poor

water content of the solvent mixtures and the increase in glycerin content. This could yield a more viscous fluid behavior of the gel structure. The type of solvent can markedly affect the viscoelasticity of neutralized carbopol polymers by influencing the degree of entanglement between different polymer chains; the entanglement is greater when the polymer chains are more extended. In solvents with higher water content, polymer- solvent interactions are favored over the polymer chain-chain interactions, thus polymer chains are well expanded. In a poor solvent composition, such that used in formula F4, the intermolecular interactions between the polymer segments are greater than the segment-solvent affinity, and the molecular chain would tend to be more contracted. This explanation was supported by the findings of Chu et al[25], and in agreement with the studies conducted by Rathapon et al,[26] who found also that glycerin through its plasticizing properties, can increase the flexibility of polymer chains, and therefore decreases the gel elastic behavior.

The release kinetics of ATR from the prepared gels was shown in table 3. The plots drawn, according to various kinetic models were giving linear relationship. The best linearity was found in Higuchi´s equation for formulations F1, F4 and F6 and in first order equation for formulations F2, F3, and F5. Mechanism of release as indicated by n values of Korsmeyer equation was also illustrated in the table 3.
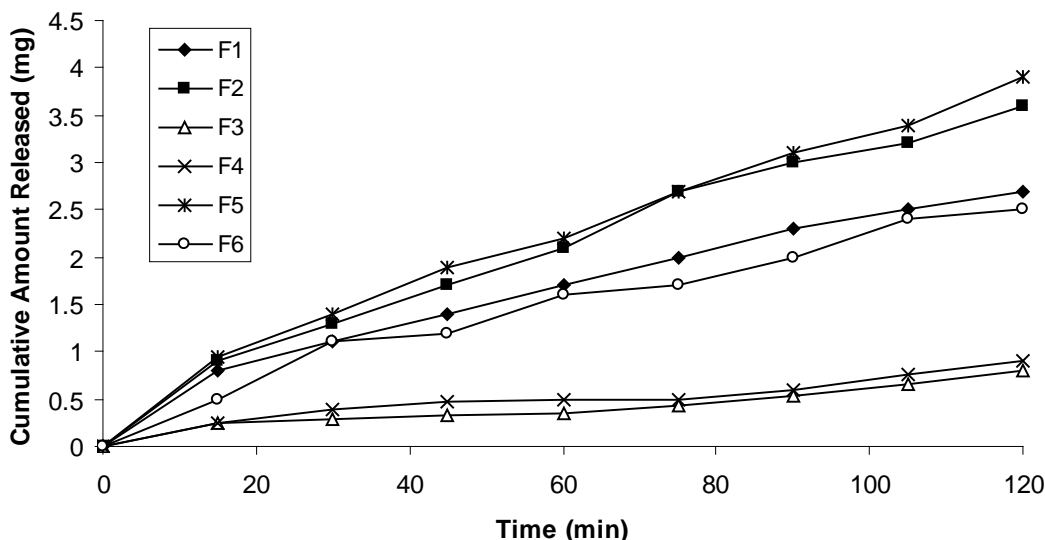
Fig. 1: Release profile of ATR from different prepared topical gel bases

Table 3: Kinetic data of the release studies:

Formula	Zero Order		First Order		Diffusion Model (Higuchi)		Hixon-Crowell		Korsmeyer-Peppas	
	r	K $\mu g/min$	r	K Min^{-1}	r	K $\mu g. t^{-0.5}$	r	K $h^{-1/3}$	n	K h^{-n}
F1	.982	0.105	.986	0.0011	.990	0.165	.984	0.0017	.604	0.735
F2	.988	0.142	.992	0.0015	.978	0.213	.990	0.0023	.687	0.659
F3	.966	0.028	.986	0.0002	.934	0.042	.966	0.0004	.541	0.235
F4	.961	0.031	.962	0.0003	.972	0.049	.962	0.0005	.557	0.267
F5	.990	0.150	.994	0.0016	.978	0.225	.993	0.0025	.679	0.721
F6	.983	0.100	.986	0.0002	.988	0.149	.985	0.0016	.737	0.375

In-vivo wound healing effect

Results of In-vivo wound healing studies were represented in figure 2. From the figure, it could be noticed that all treated groups showed a time dependant increase in % wound contractions higher than that produced by the control group. These contractions were statistically significant (P<0.001), during the first 10 days of the study in case of formulations F1, F2, and F4.

The anhydrous base (F4) showed the highest % wound contraction from 12.3 ± 2.1 to 95.4 ± 1.2 from 2nd day to 6th day, while complete wound closure and epithelization was observed on 7th day of wound induction. This fast and higher wound contraction rate may be ascribed to the dual effect of the drug and the vehicle, glycerol. Glycerol is thought to have many properties that would be beneficial for wounds. The compound can help wound healing more quickly in some cases. Xiangjian and Wendy[27] explained that glycerin, when applied to the skin, signals the cells to mature in normal fashion. Okan and Rondon[28] noticed that a hydrogel dressing of high glycerin content is better and more suitable to manage post-laser wound healing. In addition, glycerin is known to have humectants, demulcent and preservative effects, thus promote drug penetration, relive inflammation and protect wounds from contaminations that may delay healing.
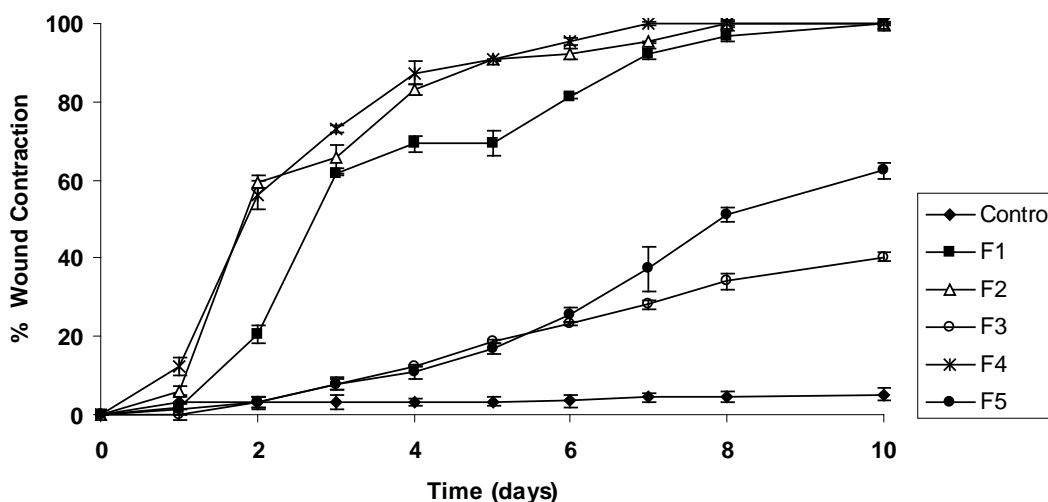

Fig. 2: Wound healing effects of the prepared gel formulations containing 1% ATR

Wound treated with hydrogel bases (F2) contracted progressively and also showed a time dependant increase in % wound contraction from 6.1 ± 1.3 to 95.3 ±1.2 from 2nd day to 7th day, while complete wound closure and epithelization was observed on 8th day of wound induction. Similar observations were noticed with formula (F1), hydroalcoholic gel base, but complete wound closure and epithelization was observed on 10th day of wound induction. No significant differences were detected (P>0.05) between animal groups that treated with formulations F4, F2, and F1. It is noticed that after complete wounds closure in the three groups, hair began to grow and the wounds area were completely covered with hair within one week.

Animals treated with formulations (F3) and (F5) showed a smaller wound area than the control groups, however, the reduction of wound area are statistically insignificant till the 10th day, the period of study.

Skin irritation studies

All the animals tolerated the applied gels and no signs of irritations were noticed during the whole period of study.

CONCLUSIONS

Atorvastatin could be formulated topically in various pharmaceutical gel bases. The anhydrous gel base showed the highest wound healing power with complete wound closure and epithelization within 7 days. No signs of irritations were noticed with all the prepared bases, during the whole period of study.

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