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Microemulsion based vaginal gel of fluconazole: Formulation, *in vitro* and *in vivo* evaluation

Yogeshwar G. Bachhav, Vandana B. Patravale*

Department of Pharmaceutical Sciences and Technology, Institute Chemical Technology, Matunga, Mumbai 400019, India

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ABSTRACT

The objective of the present investigation was to develop and evaluate microemulsion based gel for the vaginal delivery of fluconazole (FLZ). The solubility of FLZ in oils and surfactants was evaluated to identify components of the microemulsion. The ternary diagram was plotted to identify the area of microemulsion existence. Various gelling agents were evaluated for their potential to gel the FLZ microemulsion without affecting its structure. The bioadhesive potential and anti-fungal activity of the FLZ microemulsion based gel (FLZ-MBG) was determined in comparison to the marketed clotrimazole gel (Candid V® gel) by *in vitro* methods. The vaginal irritation potential of the FLZ-MBG was evaluated in rabbits. The clinical efficacy of the FLZ-MBG and Candid V® gel was evaluated in females suffering from vaginal candidiasis. The FLZ microemulsion exhibited globule size of 24 nm and polydispersity index of 0.98. Carbopol® ETD 2020 could successfully gel the FLZ microemulsion without disturbing the structure. The FLZ-MBG showed significantly higher ($P < 0.05$) *in vitro* bioadhesion and anti-fungal activity as compared to that of Candid V® gel. The FLZ-MBG did not show any signs of vaginal irritation in the rabbits. The small-scale clinical studies indicated that the FLZ-MBG shows faster onset of action than Candid V® gel although no difference was observed in the clinical efficacy.

1. Introduction

Over the last two decades, there has been a dramatic increase in the rate of superficial and invasive fungal infections. Approximately three-quarters of all women experience at least one episode of vulvovaginal candidiasis during their lifetime and nearly half of them suffer from multiple episode. The manifestations of vulvovaginal candidiasis are often painful and uncomfortable and can include intense itching, irritation, vaginal discharge and dysuria (Horowitz et al., 1992; Sobel, 1993, 1997). The most commonly prescribed treatment for vulvovaginal candidiasis in recent years has been the imidazole antifungals. Imidazole antifungal agents are available in various dosage forms such as vaginal creams and pessaries and oral tablets. Fluconazole (FLZ) has emerged as the primary treatment option for virtually all forms of susceptible *Candida* infections in both immunocompetent and immunocompromised hosts (Meis et al., 2000). Moreover, treatment of vaginal candidiasis with FLZ is even more effective than for other sites of infection (Moosa et al., 2004). Currently, FLZ is available as oral tablets (Diflucan®, Pfizer Inc., NY) for the treatment of vulvovaginal candidiasis but there are no FLZ formulations available for the vaginal delivery. Recently, it has been demonstrated that Gynazole-1® (a vaginal cream containing imidazole antifungal agent) is more effective than oral fluconazole therapy with respect to fast relief from symptoms (Seidman and Skokos, 2005). This investigation highlights the utility of vaginal delivery of antifungal agents as compared to oral therapy. In view of this, it is desirable to develop a suitable delivery system for vaginal delivery of FLZ. The vaginal delivery of FLZ would provide high local tissue levels, more rapid drug delivery, and lower systemic exposure. This may be especially important for treating pregnant patients (Champman, 2007).

The hydrophobic nature of FLZ poses problems in a suitable topical dosage form for vaginal delivery. Hence, for solubilization of FLZ, 'formulation of microemulsion' appeared to be a viable approach. Microemulsions have gained a great attention for delivery of hydrophobic agents for systemic and local treatment (Lawrence and Rees, 2000; Date and Patravale, 2007). The solubilization of FLZ in microemulsions would improve its vaginal availability. However, it is also essential to have a dosage form which adheres to the vaginal mucosa and increases the residence time of FLZ in vagina. This functionality can be imparted by gelling of the FLZ microemulsion using bioadhesive agent. Thus, in the present investigation, the potential of microemulsion based bioadhesive gel of FLZ was explored for vaginal delivery. The developed microemulsion based bioadhesive gel of FLZ was evaluated for *in*

* Corresponding author. Tel.: +91 22 24145616; fax: +91 22 24145614.
E-mail address: vbpatravale@udct.org (V.B. Patravale).

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vitro antifungal activity, vaginal irritation in rabbits and its *in vivo* efficacy was evaluated by a small-scale clinical trial in women.

2. Materials and methods

2.1. Materials

Fluconazole was kindly gifted by Cipla Pharmaceuticals Ltd., Mumbai, India. Cremophore EL (BASF India Ltd., Mumbai, India), Carbopol ETD-2020 (Noveon India Ltd., Mumbai, India), Gattefosse excipients such as Capryol 90, Plurol oleique, Peceol, Labracfac CC, Labrafil 2125 CS and Hydroproyl methylcellulose (Colorcon Asia Ltd., Mumbai, India) and sodium alginate (Anshul Agencies Ltd., Mumbai, India) were received as gift samples. Methanol (HPLC grade), Tween 80, citric acid anhydrous, disodium hydrogen phosphate, chlorocresol and benzyl alcohol (All AR grade) were purchased from s.d. Fine Chemical Ltd., (Mumbai, India). Sabaraud dextrose agar was purchased from HiMedia Ltd, (Mumbai, India).

Candid-V® gel (Glenmark Pharmaceuticals Ltd., Mumbai, India), a marketed vaginal gel of clotrimazole was purchased from local market. Double distilled water was used whenever required.

2.2. Solubility studies

The solubility of FLZ in various oils and surfactants was determined using the shake flask method. Briefly, an excess amount of FLZ was added to each vial containing 5 ml of the selected vehicle, i.e. either oil or surfactant. After sealing, the mixture was vortexed using a cyclomixer for 10 min in order to facilitate proper mixing of FLZ with the vehicles. Mixtures were shaken for 72 h in an isothermal shaker (Remi, Mumbai, India) maintained at 37 ± 1 °C. Mixtures were centrifuged at 5000 rpm for 15 min, followed by filtration through membrane filter (0.45 μm, 13 mm, Pall Life sciences, Mumbai, India). The concentration of FLZ in the supernatant was determined by high-performance liquid chromatography (HPLC) method.

2.3. HPLC analysis of FLZ

The solubility of FLZ in various excipients was determined by a validated reverse-phase HPLC method developed in house. The HPLC apparatus consisted of Jasco PU-2080 Plus Intelligent HPLC pump (Jasco, Japan) equipped with a Jasco UV-2075 Intelligent UV/VIS detector (Jasco, Japan), a Rheodyne 7725 injector (Rheodyne, U.S.A.), a Jasco Borwin Chromatography Software (version 1.50) integrator software and a Hi-Q-Sil RP-18 (4.6 mm × 250 mm and 10-μm particle size) column. The mobile phase consisted of a mixture of methanol:monobasic ammonium phosphate (0.1 M) buffer (75:25 v/v) at a flow rate of 1 ml/min that led to a retention time of 5.1 min when detection was carried out at 214 nm. The assay was linear ($r^2 = 0.9996$) in the concentration range 1–5 μg/ml with the lowest detection limit of 438 ng/ml of FLZ. The method was validated with respect to accuracy and inter- and intra-day precision as per ICH guidelines and the relative standard deviation was less than 2% in both the cases.

2.4. Phase diagrams

An oil titration method was employed in present investigation to construct phase diagrams (Corswant et al., 1997). Briefly, mixtures of the double distilled water with Cremophore EL were prepared at ratios (%, w/w) of 9:1, 8:2, 7:3, 6:4, 5:5, 4:6, 3:7, 2:8, 1:9 into different vials. A small amount of Capryol 90 in 0.5% (w/w) increment was added into the vials. Following each addition, the mixtures in vials were vortexed for 2–3 min and were allowed to equilibrate at 25 °C for 30 min. After equilibration, the mixtures were examined visually for phase separation, transparency and flow properties. In addition, the mixtures were observed through crossed polarizers (fabricated in house by using polarizing lenses, Nikkon, Japan) for determining the optical isotropy of the systems. The point at which the mixture became turbid or showed signs of phase separation was considered as the end point of the titration. The area of microemulsion existence was determined and denoted as ME.

Table 1
Composition of FLZ microemulsion

Ingredient	Content
Fluconazole (%, w/w)	2
Capryol 90 (%, w/w)	14
Cremophor EL (%, w/w)	43.5
Benzyl alcohol (%, w/w)	2
Chlorocresol (%, w/w)	0.1
Water to make (g)	100

2.5. Formulation of microemulsion

From the phase diagrams, suitable composition was chosen for the further studies. The composition is shown in Table 1. Briefly, FLZ was dissolved in Capryol 90 with vortexing. To this solution, required amounts of Cremophore EL, benzyl alcohol and cholocresol were added and the mixture was cyclomixed to yield a homogenous solution. To this solution, required amount of water was added to yield microemulsion.

2.6. Globule size analysis of the microemulsion

The average globule size and polydispersity index of the FLZ microemulsion were determined in duplicate by the photon correlation spectroscopy (PCS; Beckman Coulter N4 plus, Wipro, India). Measurements were carried at an angle of 90° at 25 °C. Microemulsion was diluted with double distilled water to ensure that the light scattering intensity was within the instrument's sensitivity range. Double distilled water was filtered through 0.45 μm membrane filters (Pall Life sciences, Mumbai) prior to globule size determination.

2.7. Formulation of microemulsion based gel (MBG) of FLZ

Various gelling agents namely, sodium alginate, hydroxypropyl methylcellulose (Methocel K4M) and Carbopol® ETD 2020 were evaluated for their ability to gel FLZ microemulsion. Briefly, FLZ microemulsion was prepared as described in the Section 2.5. Gelling agent was dispersed slowly in 10 ml of the FLZ microemulsion with the help of overhead stirrer. In case of Carbopol ETD 2020, the dispersion was neutralized by using 50% (w/w) triethanolamine to obtain the gel. The suitable gelling agent was selected on the basis of compatibility with microemulsion structure, feel and ease of spreadability.

2.8. Characterization of the FLZ-MBG

2.8.1. Determination of drug content, pH and spreadability

For determination of drug content, about 1 g of the gel was weighed in a 100-ml volumetric flask and dissolved in methanol; it was diluted appropriately and analyzed by the HPLC method described earlier. The spreadability of the gel was determined using the following technique: 0.5 g gel was placed within a circle of 1 cm diameter premarked on a glass plate over which a second glass plate was placed. A weight of 500 g was allowed to rest on the upper glass plate for 5 min. The increase in the diameter due to spreading of the gels was noted. The pH of the 10% (w/w) gel was determined using

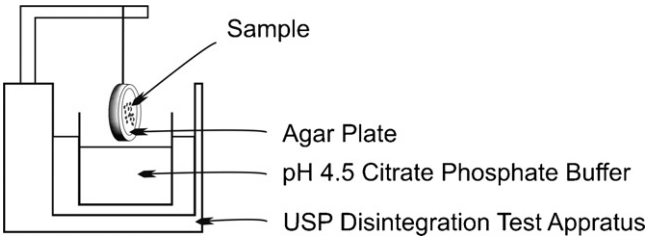

Fig. 1. Apparatus used for *in vitro* bioadhesion study.

Equip-tronic digital pH meter Model EQ 610, standardized using pH 4.0 and 7.0 standard buffers before use.

2.8.2. Rheological studies on the MBG

Brookefield Synchro-Lectric Viscometer (Model RVT) with helipath stand was used for rheological studies. The sample (30 g) was placed in a beaker and was allowed to equilibrate for 5 min before measuring the dial reading using a T-C spindle at 0.5, 1, 2.5, and 5 rpm. At each speed, the corresponding dial reading on the viscometer was noted. The spindle speed was successively lowered and the corresponding dial reading was noted. The measurements were carried in duplicate at ambient temperature. Direct multiplication of the dial readings with factors given in the Brookfield viscometer catalogue gave the viscosity in centipoises.

2.9. In vitro bioadhesion study

The bioadhesive potential of the FLZ MBG was evaluated in comparison with the marketed clotrimazole gel (Candid-V® gel) by an *in vitro* method reported by Nakamura et al. (1996). Candid-V® gel was used for the comparison, as there is no FLZ vaginal gel available in the market. Briefly, an agar plate (1%, w/w) was prepared in pH 4.5 citrate phosphate buffer. Test sample, 50 mg was placed at the center of plate. After 5 min, the agar plate was attached to a USP disintegration test apparatus (Fig. 1) and moved up and down in pH 4.5 citrate phosphate buffer at $37 \pm 1\,^{\circ}C$. The sample on the plate was immersed into the solution at the lowest point and was out of the solution at the highest point. The residence time of the test samples on the plate was noted visually.

2.10. In vitro antifungal activity

Antifungal activity of FLZ-MBG, Candid-V® gel, and FLZ standard (FLZ dissolved in DMSO) was evaluated against *Candida albicans* ATCC 10231 by using a cup plate method. The mean zone of inhibition was recorded for all the test samples.

2.11. Primary vaginal irritation studies

The vaginal irritation potential of FLZ-MBG and Candid-V® gel was evaluated in rabbits by using a method reported by Francois et al. (2003). Animal care and handling throughout the experimental procedure were performed in accordance to the CPCSEA guidelines. The experimental protocol was approved by the Animal Ethical Committee of University Institute of Chemical Technology. Female White New Zealand rabbits weighing 2.5–3 kg were obtained from Nicholas Piramal Research Centre, Mumbai, India and were acclimatized before the beginning of the study.

Animals were divided into three groups ($n = 6$) as follows:

Group 1: No application (Control).
Group 2: Marketed formulation (Candid-V® gel, Glenmark Pharma, India).
Group 3: FLZ-MBG.

All the formulations were administered using a 1-ml plastic syringe such that the material was administered at a dose of 0.25 ml/kg. After a single vaginal application, vaginal cavity was observed for 3 days for any signs of possible irritation of the vaginal mucosa (i.e., erythema and edema) and related mucosal reactions. The mean erythemal scores were recorded (ranging from 0 to 4) depending on the degree of erythema as follows: no erythema = 0, slight erythema (barely perceptible-light pink) = 1, moderate erythema (dark pink) = 2, moderate to severe erythema (light red) = 3, and severe erythema (extreme redness) = 4.

2.11.1. Evaluation of clinical efficacy in females

The small-scale clinical trial (pilot study) was conducted to evaluate the efficacy of developed FLZ-MBG in comparison to the Candid-V® gel which is currently used for the treatment of vaginal candidiasis. A double-blind and randomized clinical trial was conducted at Gadre Hospital, Parel, Mumbai, India. Protocol of the study was approved by the ethical committee of the Gadre Hospital. To be eligible for participation in the study, patients had to meet the following inclusion criteria: female, aged 18 years or older, not pregnant as determined by urine pregnancy test and suffering from vaginal candidiasis. Confirmation of current vulvovaginal candidiasis infection was made by use of KOH wet mount preparation, pelvic examination and patient's reporting of signs and symptoms. Written consent was obtained from all the patients before initiation of the study. Patients were divided into two groups A (FLZ-MBG group; $n = 6$) and B (Candid-V® gel; $n = 5$). Before the initiation of the study, for each patient, the severity of various symptoms of vaginal candidiasis such as itching, burning, irritation, erythemea, edema was recorded on a scale from 0 (no symptoms) to 5 (severe symptoms) by a gynecologist. After this, both the groups were treated with 5 g of the test formulation for 6 days. After the treatment, the scores for the symptoms of vaginal candidiasis were again recorded by a gynecologist. The data obtained for group A and B were evaluated by a two-tailed paired '*t*'-test (GraphPad InStat Demo Version). Differences were considered statistically significant at $P < 0.05$.

3. Results and discussion

3.1. Solubility studies

The results of the solubility studies are shown in Figs. 2 and 3. It is evident from Fig. 2 that all the oils exhibited similar solubilizing potential for FLZ (13.3–15.6 mg/ml). However, Capryol 90 was selected further due to its low molecular volume and better microemulsifying properties as compared to the other oils. All the surfactants used in the study showed similar solubilizing potential for the FLZ (Fig. 3). Hence, the selection of the surfactants was on the basis of compatibility with mucosal surfaces and/or

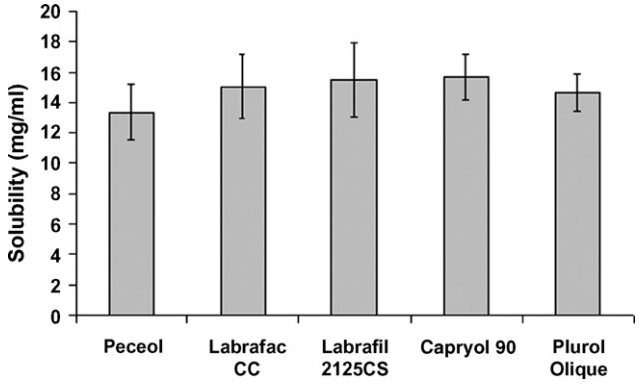

Fig. 2. Solubility of FLZ in various oils ($n = 3$).

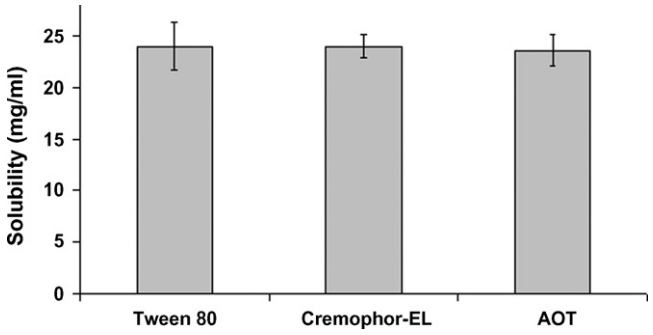

Fig. 3. Solubility of FLZ in various surfactants ($n = 3$).

microemulsification ability for Capryol 90. Anionic surfactants like AOT are known to cause irritation to the mucosal surfaces (Tenjarla, 1999; Lawrence and Rees, 2000; Date and Patravale, 2007). Hence, it was not selected further. Both Cremophore EL and Tween 80 have good mucosal acceptability. However, between Cremophore EL and Tween 80, Cremophore EL has better microemulsification ability for Capryol 90 as compared to Tween 80 (Date and Nagarsenker, 2007). Hence, Cremophore EL was selected for the further studies.

3.1.1. Phase diagrams and globule size analysis of the microemulsion

It is reported that nonionic surfactants alone can yield microemulsion without help of cosurfactant. The phase diagram of Cremophore EL–Capryol 90–water system is shown in Fig. 4. It is evident from the figure that Cremophore alone could give considerable microemulsification region (>30%). The microemulsion for the further studies was selected from the phase diagram. The selected microemulsion was composed of Capryol 90 (14%, w/w), Cremophore EL (43.5%, w/w) and water (42.5%, w/w). The selection of the microemulsion composition was on the basis of its ability to solubilize desired amount of FLZ. The selected microemulsion had globule size of 24 nm and polydispersity index of 0.98. The incorporation of FLZ did not have considerable influence on the globule size of the microemulsion.

3.2. Formulation and characterization of the MBG

Various gelling agents such as sodium alginate, hydroxypropyl methylcellulose and Carbopol® ETD 2020 were evaluated for the gelling of FLZ microemulsion. The FLZ microemulsion used for this purpose contained 2% (w/w) FLZ. The concentration of the FLZ was selected to enable comparative evaluation with the currently marketed 2% (w/w) clotrimazole formulations. It was observed that sodium alginate affected the structure of the microemulsion and resulted in separation of oily phase. This observation could be attributed to that fact that salts like sodium alginate can affect the structure of the microemulsion (Tenjarla, 1999). Hydroxypropyl methylcellulose was unable to yield gels of acceptable consistency. Only Carbopol® ETD 2020 could yield clear gel without disturbing the microstructure of the FLZ microemulsion. Furthermore, Carbopols are known to have mucoadhesive properties and have been used in the formulation vaginal delivery systems (Wang and Lee, 2002; Baloglu et al., 2003; Sharma et al., 2006). Hence, Carbopol® ETD 2020 was selected for the formulation of MBG.

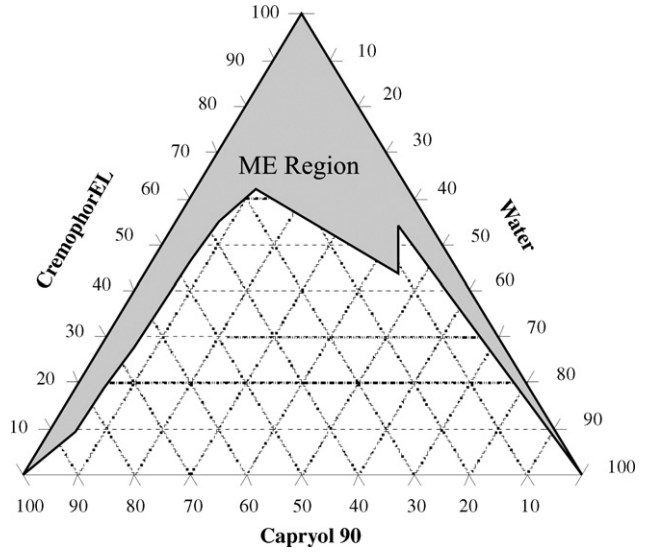

Fig. 4. Ternary phase diagram of Cremophore EL–Capryol 90–water system.

The FLZ content of the MBG was found to be 97.2 ± 4.2% of the theoretical value (2%, w/w). The pH of FLZ-MBG was 4.53 which is equivalent to the vaginal pH. Spreadability is an important property of topical formulation from patient compliance point of view. The diameter was found to be 7.2 cm which is indicative of good spreadability. The FLZ-MBG showed pseudo-plastic behavior and the viscosity of FLZ-MBG at 5 rpm was 9.8×10^6 mPa s.

3.3. In vitro bioadhesion study

The bioadhesive potential of FLZ-MBG and commercial formulation (Candid-V® gel) was evaluated by *in vitro* method. The retention times showed by FLZ-MBG and Candid-V® gel were 45 ± 3.0 and 24 ± 1.5 min, respectively ($n = 3$). The retention time shown by FLZ-MBG was significantly higher as compared to Candid-V® gel. This clearly indicates that the FLZ-MBG may have higher residence time in vagina as compared to Candid-V® gel. The increased bioadhesivity of FLZ-MBG can be attributed to the presence of Carbopol as its bioadhesive potential is highest at vaginal pH (Repka and Mcginity, 2001).

3.4. In vitro antifungal activity

The values of zone of inhibition produced by FLZ standard, FLZ-MBG and Candid-V® gel were 5.0 ± 0.18, 5.5 ± 0.1 and 3.0 ± 0.15 mm, respectively ($n = 3$). It is evident that FLZ-MBG showed higher anti-fungal activity as compared to the marketed Candid-V® gel ($P < 0.05$) and FLZ standard. The anti-fungal activity was significantly higher than Candid-V® gel ($P < 0.05$) whereas it was slightly higher than FLZ standard ($P > 0.05$). The enhanced *in vitro* antifungal activity of FLZ-MBG may be attributed to enhanced penetration of oil globules containing FLZ through fungal cell walls to inhibit ergosterol synthesis.

3.5. Primary vaginal irritation studies

The vaginal irritation studies were carried out to evaluate the tolerability of the FLZ MBG components after application. It was observed that FLZ-MBG was very well tolerated by the rabbits and no sings of erythema and/or edema were seen even after 3 days. Studies indicated that the marketed formulation (Candid-V® gel) was also well tolerated by the rabbits and it did not show any irritation.

3.6. Evaluation of clinical efficacy in females

All the patients completed the treatment. The results of the clinical studies are shown in Figs. 5 and 6. All the patients showed severe symptoms of vaginal candidiasis before starting the treatment. At the end of the study, both the groups showed considerable

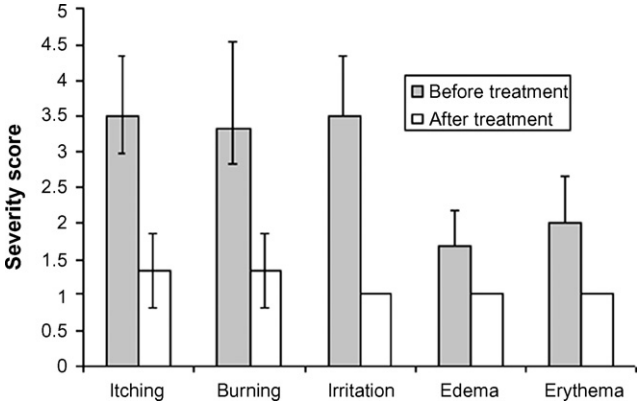

Fig. 5. Clinical efficacy of FLZ-MBG in reducing the clinical symptoms of vaginal candidiasis ($n = 6$).

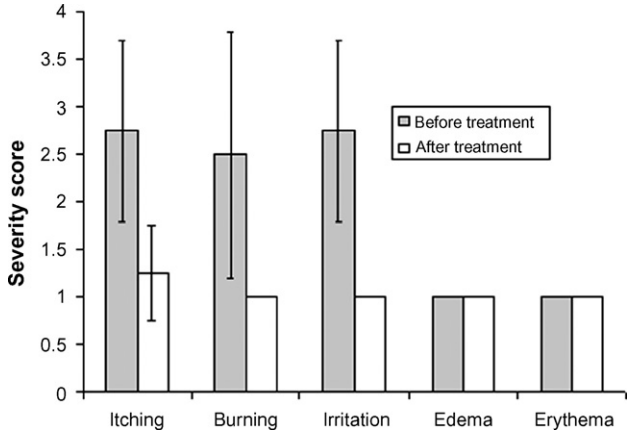

Fig. 6. Clinical efficacy of Candid-V® gel in reducing the clinical symptoms of vaginal candidiasis ($n = 5$).

reduction in the various symptoms of the vaginal candidiasis. In case of FLZ-MBG treated group, patients reported decline in the severity of the symptoms on the second day and the symptoms continued to decline further. After 6 days, most of the clinical symptoms of the vaginal candidiasis were minimal. In case of Candid-V® gel, the reduction in clinical symptoms was reported by the patients on third day. At the end of the study, this group also showed a considerable reduction in the clinical symptoms of the candidiasis. There was no significant difference ($P > 0.05$) in the clinical efficacy of the FLZ-MBG and Candid-V® gel at the end of the treatment. The future studies will be focused at evaluating potential of FLZ-MBG on larger scale and for longer duration. Nonetheless, FLZ-MBG has provided a good alternative to the current topical treatments available for the vaginal candidiasis. Moreover, it can also circumvent the disadvantages associated with the oral FLZ therapy. Our future studies will include comparison of topical FLZ-MBG with oral FLZ therapy.

4. Conclusion

The FLZ-MBG could be successfully formulated for the topical treatment of vaginal candidiasis. The developed FLZ MBG did not show any vaginal irritation in rabbits proving its tolerability. The clinical studies indicated that FLZ MBG could be a viable alternative to the current topical formulations available for the treatment of candidiasis.

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