ESTABLISHING THE EFFICACY OF NOVEL TOPICAL FORMULATIONS IN THE TREATMENT OF EXPERIMENTAL DERMATOPHYTOSIS IN GUINEA PIGS

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Summary. Dermatophytosis remains one of the most frequent infectious diseases in veterinary dermatology and clinical investigations are still required to better understand the epidemiology of the disease and provide new treatment options. Since dermatophytosis is highly contagious and zoonotic, its treatment must be effective, safe, comfortable to administer and inexpensive. Topical drug delivery formulations become more widespread in veterinary medicine. Topical therapy is often preferred to oral drug administration in the treatment of cutaneous fungal infections in pets. The aim of this study was to evaluate the efficacy of novel topical formulations in the treatment of dermatophytosis in guinea pigs. The clinical efficacy and safety of once daily topical administration of E-1 cream and T-1 cream was assessed in experimental tinea corporis in guinea pigs and compared with licensed antifungal topical preparations Imaverol, as well as the vehicle of the creams. The clinical features improvement after 1% terbinafine hydrochloride cream application varied from 41.25% (day 12) to 100% (day 36), after 1% econazole nitrate cream resorting - from 22.5% (day 12) to 100% (day 44). Clinical effectiveness of Imaverol solution varied from 16.25% (day 12) to 100% (day 48). When animals were treated with vehicle of the creams, mean percentage improvement of clinical features varied from 20% (day 12) to 100% (day 48). The experimentally infected untreated guinea pigs in control group showed spontaneous resolution of lesions within 56 days.

Keywords: antifungal, cream, dermatophytosis, guinea pig.

NAUJŲ ĮSORIŠKAI NAUDOJAMŲ PREPARATŲ EFEKTYVUMO ĮVERTINIMAS JŪRŲ KIAULYTĖMS NUO EKSPERIMENTIŠKAI SUKELTOS DERMATOFITIJOS GYDYTI

received far more attention than in animals (Moriello, 2003). Foil (1998) stated that dermatophytosis makes up 2% of all pet skin diseases. More than twenty dermatophytes species are the cause of clinical diseases in dogs and cats (Scott et al., 2001). Pets are frequently blamed for the transmission of dermatophytes between animals and humans. The most commonly isolated pathogens are Microsporum canis, Microsporum gypseum, Trichophyton mentagrophytes (Patel et al., 2005; Seebacher et al., 2008). M. canis, in dog hair, is found in 70% of all cases, Trichophyton - in 20% and M. gypseum – in 10%. M. canis is the cause of 98% of the cases of dermatophytosis in cats (Moriello, 2004).

M. canis, a zoophilic dermatophyte, is the most common agent in Europe to cause tinea capitis. 3-7 years children irrespective of gender, remain the ones most commonly affected by tinea capitis, however, recently there was an increase in infections in adults and the elderly (Seebacher et al., 2008). Iväikne and others (2009) have found that in the Kaunas city, Lithuania dogs and cats are asymptomatic carriers of pathogenic and saprophytic fungi in their coat. The most common pathogenic species isolated were M. canis (16%), M. gypseum (12%), T. mentagrophytes (1%) and saprophytic – Cladosporium spp. (66%), Aspergillus spp. (55%), Penicillium spp. (49%).

Although, dermatophyte infections generally are self-limiting, treatment helps to speed the resolution of the disease and minimise the risk of spread of infected spores to the environment (Scott et al., 2001). Topical therapy is indicated for animals with dermatophytosis and may be the sole therapy for local, nondiffuse lesions.

Range of antifungal drugs is not so wide that of antibacterial. Research although mycoses are widespread, there was a limited choice of effective and nontoxic antifungal agents for a long time (Vanden Bossche et al., 2003). Polyene antibiotics and pyrimidine derivatives were available for the treatment of fungal infections, however their limited antifungal spectrum ant toxicity to mammalian cell diminished their usage (Maertens, 2004). The continued search for new and less toxic antifungals led to the discovery of the azoles – imidazoles (ketoconazole, econazole), the modification of which led to the development of more potent azoles – triazoles (itraconazole, fluconazole) and bistriazoles (voriconazole, posaconazole, ravuconazole). In last decades, a new group of antifungal drugs allylamines has been synthesized. The antifungal action of allylamines is mediated by inhibition of main structural component of fungal cell membrane – ergosterol biosynthesis at a site much earlier in the pathway than theazole antifungal drugs (Matusevicius et al., 2008 a, b). Allylamines are more effective and less toxic to mammalian cell than azoles, because they are highly selective for the fungal enzyme and have a minimal effect on mammalian cholesterol synthesis.

The use of azoles and allylamines raised the development of number of antifungal formulations, which are licensed for use in human medicine. Recently, econazole and terbinafine are widely used in antifungal preparations for human mycoses, however these agents are not licensed for use in animals. In veterinary available topical formulations, such as Biopirox® (piroctonolamine), Surolan® (miconazole), Otomax® (clotrimazole), Imaverol® (enilconazole), Panalog® (nystatin), contain antifungals of first generation imidazole and polyene groups, that are fungistatic, have narrow spectrum of activity and the development of resistance. Formulations for systemic use, such as Norofulvlin® (griseofulvine) is licensed only for use in horses, Ketonfungol® (ketoconazole) is licensed for dogs only in France, Fulcin® (griseofulvine) is licensed for use in dogs, cats, cattle and horses (Rochette et al., 2003). A systemic use of these drugs often has a negative impact on the animal’s body, as ketoconazole and griseofulvine exert hepatotoxic activity. The use of an effective and safe antifungal therapy that shortens the time of treatment and shortens the exposure of the owners to the disease is important in veterinary medicine.

The Laboratory of Experimental and Clinical Pharmacology in Veterinary Academy of Lithuanian University of Health Science. The laboratory is an independent research unit that conducts research on the use of laboratory animals (permission Nr. 0185; 2009). Twenty Dunkin Hartley guinea pigs of both sexes, two months of age, weighing 500g, were used. Animals were anaesthetised subcutaneously by anaesthetic solution (ketamine 40 mg/kg and xylazine 5 mg/kg). The area of 2 cm x 2 cm on the back of each animal was clipped and...
gently scraped with single-use manual razor. Such gentle skin traumatization makes the animal more susceptible to infection (Saunte et al., 2008). That the inoculum size does not influence the severity of an infection (Saunte et al., 2008), therefore animals were inoculated with M. canis suspension adjusted to 0.5 McFarland turbidity standard (1-5x10<sup>6</sup> CFU/ml (colony-forming units/ml)). Suspension was prepared from M. canis colonies, initially cultivated on Potato dextrose agar (Liofilchem, Italy) for 14 days at 27 °C. The colonies were covered with sterile phosphate buffered saline (PBS, pH 7.4) and gently scraped with the tip of a Pasteur pipette.

The experimental animals were divided into five groups. The first group received the formulation T-1 containing 1% of terbinafine hydrochloride, the second – the formulation E-1 containing 1% of econazole nitrate, the third – the Imaverol deep as a comparator every 3 days till the end of clinical changes, the fourth – the vehicle-placebo (the formulation without antifungal agent and salicylic acid) and the fifth group was an untreated control group. Creams (E-1 and T-1) were applied on the day when clinical features of infection were most evident, in a volume of ~25 mg per application to the infected area, once a day and the treatment was continued till the resolution. Changes in lesion scaling, erythema, ulceration or alopecia were examined and recorded daily. To evaluate the clinical and mycological efficacy the methodology described by Ghammou et al. (2009) with little modifications was used.

To evaluate the clinical efficacy of different treatments, the infected area was divided into four equal quadrants. Each quadrant was scored on a scale from 0 to 5 as follows: 0 – no signs of infection, hair is re-grown; 1 – skin is calm, hair is half length long, no scaling; 2 – hair re-grows on full lesion surface, little scaling; 3 – no redness, little scaling, hair starts to re-grow, few bald patches; 4 – slightly erythematous skin, loss of hair, scaling; 5 – extensive damage to the skin, redness, crust formation, ulceration, loss of hair. These scores were summed for the four sites on each animal and were used to compare the efficacy of different treatments. Treatment efficacy in percent was calculated using the following equation:

\[
\text{Efficacy}=100 - \left( \frac{T}{C} \right) \times 100
\]

T - the total score of treated or control lesion in each animal
C - the score of 20 for the unhealed lesion

The total score for any group denotes the average clinical score from different animals in the same group.

Standardized mycological examination was performed on the day before the treatment and after that once a week. Ten hairs were removed from each lesion, or, if the lesion was bald, hairs were removed at the edges of the lesion and placed on the Potato Dextrose Agar in Petri dishes. Following incubation at 27 °C for seven days, the hairs, which showed fungal growth, were counted. Mycological evaluation was based on the number of culture positive hairs obtained from each lesion. Percent efficacy of different treatments and the control group was calculated with the same formula used to determine clinical efficacy; C score of 10 was assigned for the unhealed lesion.

Statistical analysis of data was performed by the use of SPSS statistical package (SPSS for Windows, SPSS Inc., 1989–1995, Chicago, IL, USA). Mean total (±SD) (SD – standard deviation) lesion severity scores and total count of infected hairs was calculated for each treatment group over the trial period. The Mann-Whitney Test was applied to compare two treatment groups. P value <0.05 was considered significant.

**Results.** All experimental animals were successfully infected. Figure 1 shows the appearance of guinea pig skin photographs taken on the days of inoculation, of severe clinical signs and every 8th day of treatment. During the present experiment the first signs of infection were observed on the 10.2±2.7 day after the inoculation and manifested themselves in erythema, swelling, scaling and bristle hair. These alterations became more evident on the 18.3±8.0 day and the treatment procedure was started. Lesions formed where the skin was abraded and didn’t extend to other sites of the body. Treatment with the T-1 cream removed infected material (crusts, large skin flakes and hairs) on the 8.5±1.9 day of treatment, treatment with E-1 cream – on the 13.0±4.1 day, treatment with Imaverol solution – on 14.8±3.8 day, and treatment with vehicle – on 13.3±6.7 day. Untreated lesions were free of infected material on 19.5±5.5 day since well-defined signs of infection developed. It was observed, that experimental creams and their vehicle alone took around 10 to 20 minutes to get absorbed by the skin. After these treatments skin looked clearer, moisturized, soft and elastic. Moistened crusts and skin flakes detached easier from the surface of the lesion and fell off. After “cleansing” the lesion the new hair started to grow back at a different time period, depending on treatment. When treating animals with T-1, it took 6.3±3.2 days for hairs to start shooting; when treating animals with E-1, it took 7.8±1.7 days, with Imaverol – 6.5±2.4, with vehicle – 8.3±2.9. In untreated control animals first hairs showed up in 8.25±0.9 days.

Table 1 and Figure 2 show comparative clinical efficacy of each tested preparations. The clinical efficacy of T-1 cream resulted in 18.75% (day 8), 53.75% (day 16), 72.5% (day 24), 95% (day 32) and 100% (day 36). The efficacy of E-1 cream varied from 13.75% (day 8), 31.25% (day 16), 45% (day 24), 68.75% (day 32), 90% (day 40) and 100% (day 44). Clinical effectiveness of Imaverol solution varied from 7.5% (day 8), 26.25% (day 16), 47.5% (day 24), 75% (day 32), 90% (day 40) and 100% (day 48). Treatments with Imaverol solution, repeated every 3 days, demonstrated clinical efficacy after 13.5±1.0 applications. When animals were treated with vehicle of the creams, mean percentage improvement of clinical features varied from 10% (day 8), 31.25% (day 16), 55% (day 24), 70% (day 32), 92.5% (day 40) and 100% (day 48). During our experiment, untreated guinea pigs recovered spontaneously. Clinical effectiveness varied from 3.75% (day 8), 12.5% (day 16), 25% (day 24), 37.5% (day 32), 57.5% (day 40), 87.5% (day 48) and 100% (day 56).
Comparing the clinical effectiveness between the test preparations and spontaneous recovery, statistically significant (p<0.05) differences of effectiveness determined between the: T-1 and E-1 creams on the 32 day of treatment; between the T-1 cream and spontaneous recovery on the 28, 32 and 36 days; between E-1 cream and self-

Figure 1. Guinea pig skin photographs taken on the day of inoculation, on the day of severe clinical signs appear and every fourth day of treatment

DI – day of inoculation
DS – day of severe clinical signs; treatment begins
recovery on the 28, 32, 36 and 40 days; between the vehicle of the creams and self-recovery on the 36 and 40 days. All experimental treatments produced a 100% mycological efficacy response, however it was time dependent (Figure 3). The 100% mycological efficacy in the group treated with T-1 cream, was achieved on the second week of treatment, in the group treated with E-1 cream – on the third week of treatment, treatments with Imaverol showed negative mycological results on the fourth week of the treatment, treatments with the vehicle – on the fifth week of treatment. Untreated control animals were culture negative on the eighth week after the appearance of well defined clinical signs of infection. Mycological efficacy of T-1 cream was superior compared with Imaverol, vehicle and control group (p<0.05). Mycological efficacy of E-1 cream was superior compared with untreated control group (p<0.05). Statistically significant differences between mycological efficacy of E-1 cream and Imaverol determined after first and second weeks of treatment, between E-1 cream and vehicle – after second week of treatment.

Table 1. Mean clinical scores ±SD and percentage expression of each tested formulation and control

<table>
<thead>
<tr>
<th>Group</th>
<th>Mean±SD Before the treatment</th>
<th>Treatment days</th>
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<td>8</td>
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<tr>
<td>T-1 cream</td>
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<td>I</td>
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<td>SD</td>
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<td>%</td>
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<td>10</td>
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<tr>
<td>E-1 cream</td>
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<tr>
<td>II</td>
<td>X 20,0</td>
<td>18,25</td>
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<tr>
<td>SD</td>
<td>0</td>
<td>1,26</td>
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<td>%</td>
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<td>8,75</td>
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<td>Imaverol</td>
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<td>III</td>
<td>X 20,0</td>
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<td>IV</td>
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<td>SD</td>
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<td>0</td>
<td>5</td>
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<tr>
<td>Control</td>
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<td>V</td>
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It was also noticed, that, however, treatment with E-1 cream induced skin irritation with such effects as redness and itching. These effects persisted in each treated animal of this group for few minutes after spreading the formulation and only at that period, when lesions had acute inflammation. Later, when the skin of a lesion regenerated, side effects were not present.

Summarizing the results we can say, that cream E-1 resolved clinical features of dermatophytosis in 44 days and eliminated pathogen in three weeks, cream T-1 eliminated the pathogen in two weeks and clinical symptoms disappeared in 36 days.

Discussion and conclusions. Summarizing the results we can say that the present work on dermatophytosis in guinea pigs represents a relatively simple model to perform the study of the pathogenesis of dermatophytic infection and evaluate the efficacy of antifungal therapeutic agents. Over the past decade, the effectiveness and tolerability of terbinafine actively investigated in the treatment of animal dermatomycoses. After a number of studies on animals, scientists have shown that orally administered terbinafine is effective in the treatment of cats suffering from experimentally induced or naturally occurring dermatophytosis (Castanon-Olivares et al., 2001; Kotnik, 2002; Kotnik, Cerne, 2006, Foust et al., 2007). Recently topically applied terbinafine showed superior effectiveness in the experimental Trichophyton mentagrophytes infection in guinea pigs (Ghannoum et al., 2009; 2010). Therefore our research is timely and relevant.

Evaluation of the efficacy of various preparations applied once daily demonstrated that T-1 cream, E-1 cream, their vehicle and Imaverol solution possess clinical and mycological efficacy against M. canis infection. Among the formulations tested, the T-1 cream caused more rapid and persistent clearing of tinea corporis signs and cured all experimentally infected animals in a shorter period of time, than E-1 cream and licensed product Imaverol.

The Fig.1 and Fig.2 show that the clinical efficacy of T-1 cream is beginning to grow rapidly on the 8 day of treatment compared with other groups, but statistically significant efficacy determined on the 28, 32 and 36 days of treatment comparing to the control group and on the 32
day - comparing to the effectiveness of E-1 cream. Clinical effectiveness of E-1 cream began to increase rapidly on the 16 day of treatment and statistically significant efficacy determined on the 28, 32, 36 and 40 days comparing to the control group.

Figure 2. Clinical effectiveness of the formulations

Figure 3. Mycological effectiveness of the formulations
On the 28th day of treatment the skin treated with T-1 cream was evaluated by 3.75±5.19 points (81.25%), as it looked slightly pink, was elastic and moisturized, had no scales, hair was re-grown up to half of full-length (6 mm). On the same day skin treated with E-1 cream was evaluated by 8.75±0.96 points (56.25%), as it looked dry, scaly, slightly reddened, the hair were 3 mm re-grown.

The effectiveness of T-1 cream was superior to E-1 cream on the 32nd day of treatment, as on this day skin treated with T-1 cream was almost fully healed, it looked elastic, slightly pink, moisturized, had no signs of scaling, hairs were almost re-grown and therefore such skin features were evaluated by 14.0±2.31 (30%).

The treatment with vehicle was effective in inhibiting fungal growth compared to the infected untreated control group. It is perhaps due to everyday moisturising effect on skin, which speeds up the cleansing of the lesion and helps to recover skin barrier properties. It was noticed that after the crust is gone and the hair had fallen off, both of which are the food source of dermatophytes, it is easier for the pharmacological formulation to reach the stratum corneum and the microscopic fungi present within. Therefore, after such “cleansing” the lesion heals and the hairs start to grow back.

According to the literature dermatophytosis is a common infectious skin disease in small animals. Since dermatophytosis is highly contagious and zoonotic, its treatment must be effective, safe, comfortable to administer and inexpensive. Topical drug delivery formulations become more widespread in veterinary medicine. Topical therapy is often preferred to oral drug administration in the treatment of cutaneous fungal infections in pets. The therapeutic efficacy of a topical formulation depends on the nature of the vehicle and the physicochemical properties of the active substance. To attain the same local drug concentration, a higher oral dose generally needs to be administered, hence increasing the risk of adverse effects (Ozcan et al., 2009). Preferred formulations for topical administration of an active substance are creams and lotions that are typically homogeneous oil-in-water emulsions, or "vanishing creams", with a continuous aqueous phase containing oily globules. Evaporation of the aqueous phase gives a cooling effect after application (Williams, 2003). Oil-in-water emulsions intensively hydrate the skin by donating the water. Increased skin hydration opens the structure of the superficial layers of the skin leading to an increase in penetration of active agents (Benson, 2005).

It is well known that emollients, moisturisers and keratolytic agents are essential in the topical treatment of skin diseases. They are adjuvant to classic treatments and help to normalize barrier function of stratum corneum, they exert anti-inflammatory effects and make the epidermis more resistant to external stressors. Salicylic acid is commonly used externally in ointments and solutions for its antiseptic, keratolytic and antipruritic properties; it increases hydration and softens the stratum corneum by decreasing its pH. Topical salicylates enhance the absorption and efficacy of other topical medications (Yosipovitch et al., 2001). In addition, the pH of creams was achieved to 6.2, because reduction in skin pH inhibits the proliferation of pathogenic microflora (Matussek et al., 2003).

Our results are in agreement with previous studies that demonstrated high effectiveness of topical terbinafine formulations in the treatment of experimental dermatophytosis in guinea pigs (Ghannoum et al., 2004; 2009; 2010). Terbinafine is a lipophilic drug, it tends to accumulate in stratum corneum and hair follicles and persists there at concentrations above the MIC for several weeks after a short-term therapy (Foust et al., 2007). Their absorption into the bloodstream after topical application is very low (Schäfer-Korting et al., 2008).

By this experiment we also confirmed the statement that dermatophytosis is a self-limiting disease (Colombo et al., 2001; Scott et al., 2001). The clinical symptoms in our control guinea pigs resolved spontaneously in 56 days. In the similar experiment with guinea pigs Cavalcanti and others (2002) observed spontaneous cure of lesions about 40 days after inoculation.

In conclusion, experimental creams E-1 and T-1 are effective in the treatment of experimentally induced dermatophytosis in guinea pigs.

1. Cream T-1 is more effective than cream E-1. Treatment with T-1 cream eliminated the pathogen in two weeks and the clinical symptoms disappeared in 36 days. Treatment with E-1 cream eliminated the pathogen in 3 weeks and a complete resolution of clinical signs of infection was achieved on 44 day.

2. Comparing to the effectiveness of creams, treatment with Imaverol solution resolved clinical features of disease in 48 days and eliminated the pathogen in four weeks, treatment with vehicle resolved clinical features in 48 days and eliminated the pathogen in five weeks.

3. Untreated guinea pigs were culture negative on the eighth week and cured spontaneously in 56 days after well defined signs of infection revealed.

4. The reservoir effect and the fungicidal activity of terbinafine, combined with the keratolytic effect of salicylic acid and the hydration effect of newly designed formulation, allow to reach fast results in treating dermatophytosis in pets.

References


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