

Effect of topical 0.5% povidone-iodine compared to 5% natamycin in fungal keratitis caused by *Fusarium solani* in a rabbit model: a pilot study

Efeito tóxico de iodo-povidona 0,5% comparado com natamicina 5% em ceratites fúngicas causadas por Fusarium solani em um modelo animal de coelhos: estudo piloto

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ABSTRACT

Purpose: To evaluate the efficacy of topical administration of 0.5% povidone-iodine in experimental *Fusarium solani* keratitis in rabbits. **Methods:** Fungal keratitis caused by *Fusarium solani* was induced in the right eye of 24 New Zealand rabbits. The rabbits were randomly divided into 3 different treatment groups: Group I (povidone-iodine) - treated with topical 0.5% povidone-iodine; Group II (natamycin) - treated with topical 5% natamycin; and Group III (control) - treated with topical saline solution. In all groups the rabbits were treated for three days and then sacrificed. The corneas were excised, macerated and immersed in 10 mL BHI. Culture samples were plated daily on Sabouraud's agar for 7 days, and the number of colony-forming units (CFU) was counted. The rabbits were clinically evaluated during the treatment period. **Results:** The povidone-iodine and natamycin groups demonstrated better efficacy than the control group based on the number of rabbits with no colonies growing. However, there were no statistically significant differences between the three groups when the number of CFU was analyzed ($p>0.05$). **Conclusion:** Our study demonstrates important methodological considerations in the use of *in vivo* animal models for the testing of antifungal agents. Using this sample size and methodology of counting CFU, topical 0.5% povidone-iodine demonstrated no benefit in the treatment of experimental *Fusarium solani* when compared with topical 5% natamycin.

Keywords: Keratitis/microbiology; Povidone-iodine/administration & dosage; Povidone-iodine/therapeutic use; *Fusarium*; Natamycin/administration & dosage; Natamycin/therapeutic use; Eye infections, fungal/drug therapy; Antifungal agents/administration & dosage; Antifungal agents/therapeutic use

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INTRODUCTION

Among suppurative corneal ulcerations, fungal keratitis is responsible for significant ocular morbidity and blindness, primarily in the developing world. Its incidence is variable, but it is clearly higher in regions with a warm, humid climate and/or in an agricultural economy⁽¹⁾. Studies from different continents identified fungi in 6% to 53% of cases of suppurative keratitis⁽²⁻³⁾. Filamentous fungi are responsible for the majority of these cases⁽⁴⁾. Höfling-Lima et al.⁽⁵⁾ cited filamentous fungi as responsible for 79% of fungal keratitis and showed that the genus *Fusarium* is responsible for 59% of the fungal keratitis in patients of the Federal University of São Paulo.

The key elements in successful treatment of this condition in a manner which spares vision are early diagnosis, timely identification of the pathogen, and application of effective antifungal drugs. While early diagnosis and identification of pathogens is generally straightforward, the therapy for fungal keratitis is restricted by a limited choice of effective antifungal drugs for topical administration.

The polyene, natamycin (pimaricin), has been recommended at a concentration of 5% as the topical drug of choice for first line treatment of filamentous fungal keratitis⁽⁶⁻⁷⁾. In recent years, azole compounds have demonstrated promise in the treatment of fungal keratitis. Prajna et al.⁽⁸⁾ reported that a 2% preparation of econazole for topical application was as effective as 5% natamycin in the management of filamentous fungal keratitis. Kalavathy et al.⁽⁹⁾ compared topical 1% itraconazole with 5% natamycin and reported that natamycin should continue to be the treatment of choice for filamentous fungal keratitis. However, in many developing nations, natamycin and azole compounds are not readily available or affordable.

Povidone-iodine has been used for many years as an effective broad-spectrum disinfectant in ophthalmic surgeries⁽¹⁰⁾. The solution is inexpensive and widely available. Povidone-iodine is a complex of polyvinylpyrrolidone and iodine. Its chemistry is only partially understood and has been subject of several reviews. Several studies have demonstrated its effectiveness against bacteria⁽¹¹⁾. It is also effective against viruses and fungi⁽²⁾.

We sought to evaluate the potential clinical efficacy of topical 0.5% povidone-iodine eyedrop preparation by comparing it with a standard therapeutic regimen with topical 5% natamycin for *Fusarium solani* fungal keratitis in a rabbit model.

METHODS

We performed an experimental, randomized, controlled study in a rabbit model. Permission for the experimental study was obtained from the Ethics Committee of the Federal University of São Paulo, and we strictly adhered to the experimental rules set forth in the ARVO resolution on animal experimentation.

A strain of *Fusarium solani* (IMI 314228, International Mycological Institute, Surrey, UK) was grown on potato-dextrose agar slants at 35°C for the first 48 hours and at 25°C until day 7. The formed colonies were covered with 2 mL sterile 0.9% saline, and suspended by gently probing them with the tip of a Pasteur pipette. The resulting mixture of conidia, hyphal fragments and agar particles was transferred to a sterile test tube. Heavy particles were allowed to settle for 5 minutes, and the upper homogeneous suspension was collected with a micropipette, transferred to another tube and mixed in a vortex for 15 seconds. The density of the conidial suspension was read in a spectrophotometer and adjusted to an optical density range of 0.15 to 0.17, which corresponds to 0.4×10^6 to 0.5×10^6 CFU/mL. Inoculum size was also checked by plating 10 μ L

of a 1:100 dilution of the suspension on Sabouraud agar plates and by counting the conidia in a Neubauer chamber.

We induced *Fusarium solani* keratitis in the right eye of 24 adult male New Zealand rabbits (1.4-2.6 kg in weight). Prior to the inoculation, the eyes received 5 daily subconjunctival injections of 0.2 mL 0.4% dexamethasone sodium phosphate, under topical anesthesia (proparacaine). Inoculation was performed on the last day of this preparation.

Animals were anesthetized with intramuscular ketamine hydrochloride (35-50 mg/kg), xylazine hydrochloride (5-7.5 mg/kg), and with topical 0.5% proparacaine hydrochloride drops. Under the operating microscope, the eye was gently proptosed and fixated. A temporal vertical incision, 2 mm in width and 250 μ m in depth, was made 2 mm from the center of the cornea with a diamond knife. The blunt needle of a microsyringe (model 80300, Hamilton Company, Reno, NV, USA) was advanced through the previous incision and 10 μ L of the inoculum were injected into the corneal stroma. Five days after inoculation, we verified the presence of a corneal infiltrate (Figure 1) (infiltrate ≥ 2 mm), and we divided the animals randomly into 3 treatment groups: *Gr.I*: treated with topical 0.5% povidone-iodine hourly (7am - 11pm) for three days; *Gr.II*: treated with topical 5% natamycin hourly (7am - 11pm) for three days; and *Gr.III*: treated with topical normal saline hourly (7am - 11pm) for three days (control). All animals underwent corneal scraping for debridement of the epithelium and necrotic tissue overlying the infiltrate to allow for better penetration of the topical antimicrobial drops before initiating the treatment. The three different drugs were prepared under sterile conditions by the same pharmaceutical company (Ophthalmos Fórmulas Oficinas Ltda., São Paulo, Brasil) using identical diluents. There were no preservatives in any of the three preparations. Prophylactic moxifloxacin (Vigamox®, Alcon, Fort Worth, TX, USA) drops were used every 6 hours on the right eye of all animals. During the treatment, we evaluated the rabbits daily with the slit lamp, and clinical features were scored. We considered the size and depth of the infiltrate, the

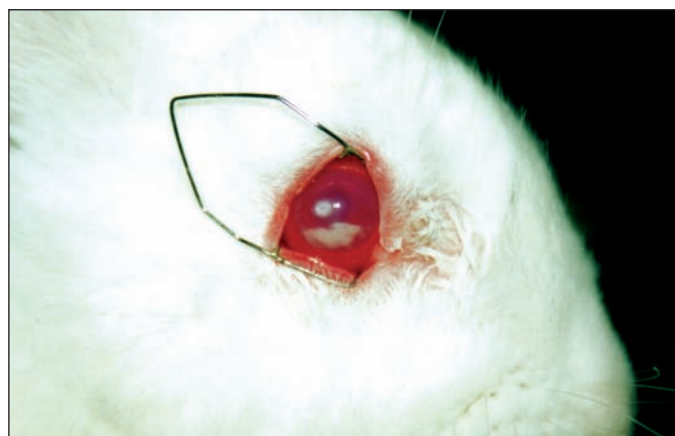


Figure 1 - Infected rabbit. 2 mm x 2.5 mm central corneal infiltrate with hypopyon in the anterior chamber.

growth of new vessels into the cornea, conjunctival injection, hypopyon, and discharge.

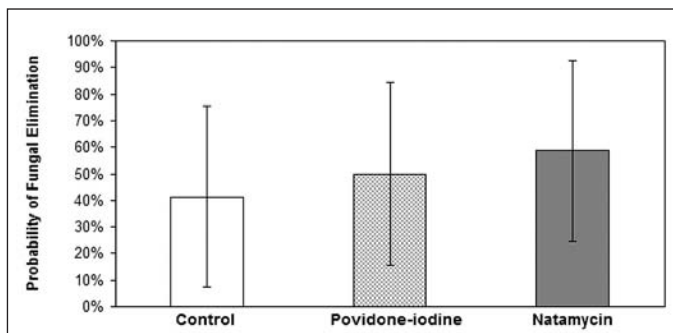
The day after conclusion of the treatment period, all rabbits were anesthetized with intramuscular ketamine hydrochloride (35-50 mg/kg) and xylazine hydrochloride (5-7.5 mg/kg), and were sacrificed with intravenous sodium pentobarbital (150 mg/kg). The right cornea of each animal was trephined, thoroughly washed with sterile balanced salt solution, macerated, and immersed in 10 mL BHI with gentamicin (50 mg/L) and chloramphenicol (40 mg/L). All surgical instruments were utilized on a single use base to avoid cross contamination.

Immediately after incubation, the test tubes contents were homogenized for 15 seconds and allowed to settle for 15 minutes. These solutions were diluted to 1/10 and to 1/100. A 10 µL aliquot of the upper homogeneous suspension of each solution and its dilutions were plated on Sabouraud's agar and kept at 25°C. This procedure was repeated every 24 hours for 7 days. The number of CFU was counted for 5 days after each seeding (total of seven seeding days). We established 100 CFU as the maximum growth. We considered days 1, 4, and 7 of seeding for analyzing the CFU at the standard solution and 1/10 diluted solution. For the 1/100 diluted solution, we considered days 1 and 7 of seeding. The comparisons of CFU counts for the three study groups were performed using the Kruskal-Wallis test.

RESULTS

We found no statistically significant difference in clinical appearance between the three groups after treatment. All groups had clinically indistinguishable disease during the treatment period and on the day of sacrifice.

Groups I and II, 0.5% povidone-iodine and 5% natamycin respectively, demonstrated better efficacy than Gr. III (control) when comparing the number of rabbits in which there were no colonies growing. Four rabbits in Gr.I, 5 rabbits in Gr.II, and 3 rabbits in Gr.III demonstrated no colony growth. However, the confidence intervals between the three groups overlapped, suggesting that there was no statistically significant difference between them (Graph 1).



Graph 1 - Confidence intervals for the three treatment groups of fungal keratitis due to *Fusarium solani* (intervals at 95% confidence level)

On days 1, 4, and 7 after incubation in BHI, samples were plated on Sabouraud agar and were counted on days 1 through 5. The standard solution (Table 1) and the 1/10 diluted solution (data not shown) demonstrated no statistical difference in the number of CFU between the 3 groups. Likewise, the 1/100 diluted solution demonstrated no statistical difference on days 1 and 7 after incubation in BHI, considering counting days 1 through 5 (Table 1).

DISCUSSION

Povidone-iodine has a broad spectrum of action, is cost effective, and has been used for preoperative antibiotic prophylaxis⁽¹⁰⁾. Its efficacy against *Fusarium solani* has been demonstrated *in vitro* by Martin et al.⁽²⁾, as well as *in vivo*⁽¹²⁾. White et al.⁽¹²⁾ reported that 1% povidone-iodine led to a significant reduction in the duration of experimentally induced disease with *Aspergillus niger*, in rabbits, without notable irritation. Because of its effectiveness against filamentous fungi *in vitro* as well as *in vivo*, we evaluated its benefits in a rabbit model of *Fusarium solani* keratitis.

Our study demonstrated no clinical difference between the three groups during the treatment period. It is possible that subconjunctival injection of corticosteroids may have partially suppressed the ocular inflammation and the rabbits got infected in such a similar situation, that no difference in clinical response could be determined during the three treatment days. Fiscella et al.⁽¹³⁾ reported the same lack of difference in clinical features for 10 days after corticosteroid administration in an experimental *Fusarium* keratomycosis. O'Day et al.⁽¹⁴⁾ noted that the use of glucocorticoids, usually given subconjunctivally, alters the course of disease and facilitates the establishment of infection. When corticoids are given, the fungal burden remains stable longer and the inflammatory response in the cornea is worsened⁽¹⁵⁾. It is also noted that the usual effective duration of this rabbit model of filamentous keratitis is about 5-10 days^(14,16). Moreover, three days of clinical evaluation is a very short period of time to discern significant clinical differences between the groups when dealing with fungal infection. On the other hand, this finding suggests that the methodology used to infect the animals worked similarly in all groups. Evaluation of clinical scores was, therefore, not considered a reliable measure of active infection in this study.

There was no statistical significance when we compared the number of rabbits in which there were no colonies growing. The confidence intervals between the three groups overlapped. If we assume repeatability of the point estimates, a calculation would give an appropriate sample size for future experiments. However, we did not have a normal distribution in our study, and consequently, it is difficult to determine the adequate sample size for future experiments. It is impressive that in 3 rabbits in the control group the infection resolved with no treatment. This reflects the strong inflammatory res-

Table 1. Number of colony-forming units according to the study group at a dilution of 1/100 and the standard solution

	Incubation time in BHI	Group	Days in Petri dish									
			1 Day		2 Days		3 Days		4 Days		5 Days	
			CFU Median (min-max)	P	CFU Median (min-max)	P	CFU Median (min-max)	P	CFU Median (min-max)	P	CFU Median (min-max)	P
SS	1 Day	Control	0 (0-0)	1.00	0 (0-0)	1.00	0 (0-0)	1.00	0 (0-0)	1.00	0 (0-0)	1.00
		Povidone	0 (0-0)		0 (0-0)		0 (0-0)		0 (0-0)		0 (0-0)	
		Natamycin	0 (0-0)		0 (0-0)		0 (0-0)		0 (0-0)		0 (0-0)	
SS	4 Days	Control	10 (0-38)	0.38	64.5 (0->100)	0.62	69.5 (0->100)	0.73	72.5 (0->100)	0.65	72.5 (0->100)	0.65
		Povidone	0 (0-49)		0 (0->100)		0 (0->100)		0 (0->100)		0 (0->100)	
		Natamycin	0 (0-86)		0 (0->100)		0 (0->100)		0 (0->100)		0 (0->100)	
SS	7 Days	Control	3 (0-18)	0.53	>100 (0->100)	0.70	>100 (0->100)	0.70	>100 (0->100)	0.79	>100 (0->100)	0.79
		Povidone	0 (0-13)		50 (0->100)		50 (0->100)		50 (0->100)		50 (0->100)	
		Natamycin	0 (0-12)		0 (0->100)		0 (0->100)		1 (0->100)		1 (0->100)	
1/100	1 Day	Control	0 (0-0)	1.00	0 (0-0)	1.00	0 (0-0)	1.00	0 (0-0)	1.00	0 (0-0)	1.00
		Povidone	0 (0-0)		0 (0-0)		0 (0-0)		0 (0-0)		0 (0-0)	
		Natamycin	0 (0-0)		0 (0-0)		0 (0-0)		0 (0-0)		0 (0-0)	
1/100	7 Days	Control	0 (0-0)	0.89	9 (0-19)	0.77	11 (0-24)	0.75	11.5 (0-24)	0.75	11.5 (0-24)	0.77
		Povidone	0 (0-0)		0.5 (0-15)		0.5 (0-20)		0.5 (0-20)		0.5 (0-20)	
		Natamycin	0 (0-5)		0 (0->100)		0 (0->100)		0 (0->100)		0 (0->100)	

BHI= brain heart infusion; CFU= colony-forming unit; min= minimum; max= maximum; SS= standard solution; 1/100= 1/100 dilution

ponse presented by rabbits to infectious agents. In some cases demonstrating healing in a period of time up to 8 days after inoculation, even with subconjunctival corticosteroid administration previous to inoculation⁽¹⁷⁾.

Another limitation in our study was fixing the maximum number of CFU at 100. We used that number since in our previous pilot study, the colonies were confluent and became somewhat indistinct one from another. Preestablishing that number fixed the limit for statistical analysis for all groups; in some cases the CFU would clearly be higher than that, if it had been possible to count the colonies further. For example, when we observe the control group on the 7th day of incubation in BHI, and on the 2nd day of colony counting, the number of colonies was higher than 100 (Table 1). It is evident that this number would be higher than that on the 5th day of colony counting. Establishing 100 CFU as the maximum limit for all groups rendered the statistical analysis limited. We recognized the problem with counting more than 100 colonies per plate in previous pilot studies. An alternative solution was to dilute the standard solution to 1/10 and 1/100. However, analyzing diluted solutions did not demonstrate statistical significance between the groups in any of the evaluated incubation and counting days. We plated the standard solutions and dilutions on different plating days (days 1, 4, and 7 for standard solution and the 1/10 diluted solution; and days 1 and 7 for the 1/100 diluted solution). None demonstrated statistical significance. However, it is clear that the CFU are higher with longer incubation in BHI. For future studies we recommend fixing the number of days in BHI, fixing the dilution level, and the number of counting days in the Petri dish. It seems reasonable to choose the 7th day in BHI and to observe the response on the 5th day in the Petri dish in order to allow full

opportunity for colony growth. The methodology of counting colony forming units or any quantitative isolate recovery technique should be taken into consideration when measuring responses to therapy in keratomycosis. More sensitive and specific measures are clearly needed.

Toxicity was not evaluated in our study. According to York et al., concentrations of 0.5% topical PVP-I or less (6 to 8 times daily) are practically non-irritating and might delay epithelial healing in one day⁽¹⁸⁾.

With the above mentioned limitations, our results do not suggest that topical 0.5% povidone-iodine is efficacious for the treatment of *Fusarium solani* keratitis. However, one cannot conclude from this study that the treatment modalities are equivalent. According to recently published data in the literature, topical natamycin continues to be considered the therapy of choice for treatment of filamentous keratitis⁽⁹⁾. Alfonso⁽¹⁹⁾ reviewed cases of *Fusarium* keratitis at the Bascom Palmer Eye Institute, University of Miami, in a recent contact lens related outbreak of this disease in the United States. Topical 5% natamycin was used in 91% of the patients. Newer antifungal agents such as voriconazole were promising in both *in vitro* susceptibility testing against ocular fungal isolates and in clinical settings⁽²⁰⁾. However, comparative clinical trials are necessary to prove superior efficacy and safety in order to recommend voriconazole over natamycin as topical agent for *Fusarium* keratitis.

Using this methodology of counting colony-forming units and with this sample size, topical 0.5% povidone-iodine demonstrated no significant benefit in the treatment of experimental *Fusarium solani* fungal keratitis when compared with topical 5% natamycin. It will be valuable to enlarge the sample size and to adjust the methodology in order to allow for a better evaluation of these drugs. The present study demon-

trates important methodological considerations in the use of *in vivo* animal models to test the effectiveness of antifungal agents. Larger numbers and alterations in the quantitative microbiology may yet demonstrate the antifungal efficacy of these agents.

RESUMO

Objetivo: Avaliar a eficácia do uso tópico de iodo-povidona 0,5% em ceratite experimental por *Fusarium solani* em coelhos. **Métodos:** Ceratite fúngica por *Fusarium solani* foi induzida no olho direito de 24 coelhos da raça New Zealand. Os coelhos foram divididos aleatoriamente em 3 diferentes grupos de tratamento: Grupo I (iodo-povidona) - tratados com iodo-povidona 0,5%; Grupo II (natamicina) - tratados com natamicina 5%; Grupo III (controle) - tratados com solução salina. Os coelhos dos 3 grupos foram tratados por 3 dias e sacrificados em seguida. As córneas foram removidas cirurgicamente, maceradas e incubadas em meio BHI. Semeou-se culturas em placas de ágar Sabouraud, diariamente, durante 7 dias, e contou-se o número de unidades formadoras de colônias (UFC). Os coelhos foram avaliados clinicamente durante o período de tratamento. **Resultados:** Os grupos iodo-povidona e natamicina demonstraram melhor eficácia do que o grupo controle considerando-se o número de coelhos nos quais não houve crescimento de colônias. Entretanto, não houve diferença estatística significativa entre os 3 grupos quando se analisou o número de UFC ($p > 0,05$). **Conclusão:** Este estudo demonstrou considerações metodológicas importantes na utilização de modelos animais para o teste de agentes antifúngicos. Usando a metodologia de contar UFC e com este tamanho amostral, administração tópica de iodo-povidona 0,5% não demonstrou benefício do tratamento de ceratite fúngica experimental causada por *Fusarium solani* quando comparado com a administração tópica de natamicina 5%.

Descritores: Ceratite/microbiologia; Povidona-Iodo/administração & dosagem; Povidona-Iodo/uso terapêutico; *Fusarium*; Natamicina/administração & dosagem; Natamicina/uso terapêutico; Infecções oculares fúngicas/quimioterapia; Antimicóticos/administração & dosagem; Antimicóticos/uso terapêutico.

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