Chromoblastomycosis Murine Model and *In vitro* Test to Evaluate the Sensitivity of *Fonsecaea pedrosoi* to Ketoconazole, Itraconazole and Saperconazole

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An experimental model of murine chromoblastomycosis and in vitro tests with Fonsecaea pedrosoi *were used to test the sensitivity of this fungus to three different antimycotics. The experimental model was standardized in BALB/c mice inoculated intraperitoneally with a 10^6 CFU/ml suspension of a F.* pedrosoi *isolate. Clinical infection was evident after 5 days of inoculation. Three groups of 27 mice each were used in the experiment. One group was treated with ketoconazole (KTZ), another with itraconazole (ITZ) and the other with saperconazole (SPZ). Antimycotic therapy was continued for 21 days. The control group consisted of 40 mice which were inoculated, but not treated. Infection was documented by macroscopic and microscopic examination of affected tissue in addition to culture of tissue macerates. Minimal inhibitory concentrations (MIC) and minimal fungicidal concentrations (MFC) for the* F. pedrosoi *strain used were done. The in vitro results showed that SPZ was the most active with MIC 0.01* **m**g/ml *and MFC 0.1* **m**g/ml, *followed by ITZ. SPZ was also the most effective in vivo since 63% of the treated animals (p=0.01) showed a curative effect after the observation period. We concluded that SPZ had the best in vitro and in vivo activity against* F. pedrosoi.

Key words: chromoblastomycosis - murine model - *Fonsecaea pedrosoi* - minimal inhibitory concentration - minimal fungicidal concentration

Chromoblastomycosis is a chronic infectious disease produced by a dematiaceous fungi (dark pigmented) that affects the skin and the cellular subcutaneous tissue (Zaias 1978, Rippon 1988, Milam & Fenske 1989, Restrepo 1991). Four genera and 5 species of the dematiaceous fungi produce chromoblastomycosis: *Fonsecaea pedrosoi, Fonsecaea compactum, Phialophora verrucosum, Cladosporium carrionii* and *Rhinocladiella aquaspersa* (Londero & Ramos 1989, Wagner 1990). The most frequent one in Colombia is *F. pedrosoi* (Duque 1962, Rippon 1988).

These fungi live in vegetation, soil and water, and infect man by direct inoculation through trauma caused by contaminated material (Rocha & Gutiérrez 1972, Borelli & Reyes 1976).

Chromoblastomycosis is an incapacitating disease whose clinical evolution can vary from months to years. Duration, size of lesion and anatomic site can lead to functional limitations, concomitant bacterial infections, as well as physical deformities which alter the patient's well-being and, in some cases, cause his/her social isolation (Wackym et al. 1985, Rippon 1988, Milam & Fenske 1989).

Treatment for this mycotic infection has been difficult due to the fungus' resistance to existing antimycotics and to the chronicity of the lesions (Restrepo 1991). Several treatments have been tried, but response has not been adequate or uniform (Mittag & Kreysel 1982). Surgical resection is a good theraupeutic practice when lesions are circumscribed and small. Cryotherapy, local heat and carbon dioxide laser lights, some physical practices utilized, have not been totally effective (Conti-Diaz et al. 1964, Bopp 1974, Tagami et al. 1979, Tuffanelli & Milburn 1990).

Chemoteraphy is the treatment of choice; several agents have been tried individually or combined. Amphotericin B (parenteral or intralesional) has shown minimal utility and frequent and serious secondary effects (Tuffanelli & Milburn 1990). If used with 5-flourocytocin, better results are obtained, but notorious side effects persist (Borelli 1958, Bayles 1974, Vitto et al. 1979).

The prognosis of the disease has improved since imidazolic derivates and triazoles began to be used.

Thiabendazole, ketoconazole (KTZ), saperconazole (SPZ) and itraconazole (ITZ) have been tested and the latter seems to be the most adequate (Battistini & Sierra 1969, Bayles & Durban 1971, Drouhet et al. 1982, Silber et al. 1983, Espinel-Ingroff et al. 1984, Restrepo et al. 1988, Grant & Clissold 1989, Franco et al. 1992). Small lesions have responded to treatments with ITZ administered for periods of 18 to 24 months, in doses of 100 to 200 mg/day. Improvement in patients with larger lesions subjected to prolonged treatment, has also been considerable (Restrepo et al. 1988). SPZ, has been tested in patients with systemic and subcutaneous mycoses (Cutsem et al. 1989). Chromoblastomycosis patients, treated with SPZ at doses of 100 to 200 mg/day, presented complete resolution of their lesions after 12 months of therapy, with cultures becoming negative after 6 months of treatment (Franco et al. 1992), unfortunately this antimycotic is not available.

Due to the causative agents resistance to the different antimycotics, *in vitro* tests would be advisable before beginning the patient's therapy (Stevens 1984, Espinel-Ingroff & Shadomy 1988, Kobayashi & Spitzr 1989). This problem is specially complex because of the inherent variability of all *in vivo* systems and the fact that host factors (e.g. immune parameters, mechanical factors such as penetration of the drug to the specific site and undrained abscesses) can potentially have more influence on clinical outcome than intrinsic drug susceptibility. The outcomes of three general groups of fungal infections might be correlated with *in vitro* results: animal models of infection, clinical studies of cutaneous and mucosal infections and clinical studies of deep, invasive fungal infections. Studies with animals offer one solution to the problem of compensating for the effect of host factors. Ideally, infecting strains of fungi that differ only in their *in vitro* susceptibility to an antifungal agent are study in parallel (Ghannoum et al. 1996).

Several animal models have been developed to evaluate the evolution of the chromoblastomycosis and other therapeutic possibilities. Borelli used lactant mice and tried different inoculation sites and paths (Borelli 1972, Borelli & Reyes 1976). To evaluate the disease immunological process Balb/c mice strains nu/+, nu/nu and bg/bg have been used (Ahrens et al. 1989).

These studies permit clear demostration of relationships between the MIC and the minimal effective dose. A recent review of 17 of such studies found that 12 of the studies found coherent relationships between *in vitro* results and treatment efficacy for amphotericin B, flucytosine and a variety of azoles (Ghannoum et al. 1996).

MATERIALS AND METHODS

In vitro study

Cultures - *F. pedrosoi* strain no. 23790 isolated from a chromoblastomycosis patient was used (Laboratorio de Micología, Corporación para Investigaciones Biológicas, CIB, Medellín, Colombia). The cultures were plated both in sabouraud dextrose agar (SDA) (Becton Dickinson Microbiology Systems, Cockeysville, MD, USA), and mycosel agar (Becton Dickinson Microbiology Systems, Cockeysville, MD, USA) at room temperature for 14 days.

Inoculum - A suspension of the fungus in saline solution at 0.85% was prepared, both conidia and hyphae fragments were counted, and the inoculum was adjusted to the order of 10^4 colony forming units/ml (CFU/ml) in a Neubauer camera (Espinel-Ingroff & Shadomy 1988, Kobayashi & Spitzr 1989, Smith & Pfaller 1991). Inoculum viability was tested using an Evans Blue (E 2129 Sigma Chemical Company, St Louis, MO, USA) based vital stain with eosin yellow (E 4009 Sigma Chemical Company, St Louis, MO, USA) and serial dilutions were plated in SDA for 14 days at room temperature. At the end of that period, the CFU/ml grown was determined (Vermorel et al. 1988, Kobayashi & Spitzr 1989, Smith & Pfaller 1991).

Antimycotics evaluated - KTZ (R4 1400), ITZ (R51211), SPZ (R66905) (Janssen Pharmaceutical). These antimycotics were dissolved in polyethylene glycol 200 (P2263 Sigma Chemical Company, St Louis, MO, USA) with agitation and heating up to 75-80°C, according to the manufacturer's suggestion.

Minimal inhibitory concentration (MIC) - MICs were determined for each drug and the smallest non-fungal growth concentration was chosen (Espinel-Ingroff et al. 1984, Espinel-Ingroff & Shadomy 1988, Smith & Pfaller 1991).

Minimal fungicidal concentration (MFC) - MFC was determined from the MIC which, when plated in SDA and incubated at room temperature for 14 days, would yield fungal growth. CFU/ml was calculated and fungal viability demonstrated by its growth capacity after being subjected to the antimycotics tested (Espinel-Ingroff et al. 1984, Espinel-Ingroff & Shadomy 1988, Smith & Pfaller 1991).

In vivo study

Animal model - The standardization of the animal model was done on 202 male BALB/c mice distributed in groups according to the variables to be evaluated, i.e., age, weight, site and inoculation path (Brammer & Tarbil 1978, Kan & Bennet 1988,

Ahrens et al. 1989). For practical and standardization purposes (dose antimycotic administration) male Balb/c mice weighing 23 ± 2 g were placed in boxes containing 9 mice each, and supplied with food and acidified water *ad libitum* (Corporación para Investigaciones Biológicas, CIB, Bioterio). The inoculum was administered in brain heart infusion (BHI) (Becton Dickinson Microbiology Systems, Cockeysville, MD, USA) to contain 10^6 CFU/ml. The inoculum was administered intraperitoneally to mice in 5 different sites, (0.2 ml per site), the same day it was prepared. Inoculations in different sites (base of tail, auricular region, loins, rump, plantar pad, abdominal region), and at different depth levels (intradermic, subcutaneous, intramuscular, intraperitoneal) showed that the appropriate site for inoculation was the abdominal area, via intraperitoneum, since easily palpable gray-black nodular lesions involved the skin, were detected regularly in daily follow-ups. This standardization was confirmed by autopsies, biopsies and cultures which supported the diagnosis and contributed to the determination of the infection's natural evolution.

Study groups - Nine groups of 9 mice each were studied. Nine treatments were tested (3 doses of each antimycotic) (Table I). The control group consisted of 40 mice, infected but not treated, which received polyethilene glycol 200.

Antimycotics - The *F. pedrosoi*-infected mice were treated with the same agents used for the *in vitro* tests. The doses and the number of mice treated are recorded in Table I. These doses were chosen based on previous studies (Drouhet et al. 1982, Radetsky et al. 1986, Hughes et al. 1988, Kan & Bennett 1988, The RW Johnson Pharmaceutical Research Institute Zurich 1989).

Treatment duration - The antimycotics were given to the mice orally via cannula (Biomedical Animal Feeding, Popper & Sons, Inc, NY, USA),

TABLE I

Study groups

Group	Doses mg/kg/day	No. mice
Ketoconazole	10	9
	20	9
	40	9
Itraconazole	1.25	9
	2.50	9
	5.00	9
Saperconazole	1.25	9
	2.50	9
	5.00	9
Control-PEG 200	1ml/day	40

during 21 consecutive days, necessary for the stabilization of the antimycotic level in tissue (Drouhet et al. 1982, The RW Johnson Pharmaceutical Research Institute Zurich 1989, Grant & Clissold 1989).

Fungal load in tissues - On the 21st day, when the treatment was coming to an end, an autopsy was conducted on 27 mice (9 per treatment group) and 30 mice from the control group. Samples were taken from abdominal skin, peritoneum and from organs which showed macroscopical changes (nodules, gray or black pigmentation).

Thirty and 60 days after the treatment was finished, 27 mice (9 from each group) and 5 mice from the control group, were submitted to the same procedure, on each occasion. Macerates on tissue obtained in the autopsy were done, with macerator (Tekmar Tissumixer Cincinnati, Ohio, USA) in BHI. To determine fungi viability in the infected tissue, each sample was weighed in a pre-weigther container. From this macerate, serial dilutions were made and plated in SDA at room temperature; one month later a tissue CFU/g count was done.

Histopathological study - For histophatology, samples were taken systematically from skin and subcutaneous tissue. Samples from striated muscle, liver, spleen, gastrointestinal tract, pancreas, mesenteric ganglions were taken when there were macroscopically suspicious organs (gray-black nodules). The tissues were fixed in neutral formaldehyde at 10% for at least 12 hr, and embedded in paraffin. Serial sections (3μ), methenamine-silver and hematoxilin-eosine stains were performed.

Information processing - The information obtained was collected in a data-base program (dBase III plus) and transferred to a Statgraphics statistics program. The data were then analyzed by variance-analysis and the intervals for the medians were determined based on the Scheffe test with 95% reliability. The data were classified according to the type of drug versus a control group with no treatment.

RESULTS

In vitro tests

Minimal inhibitory concentration - KTZ MIC's was 1μg/ml, ITZ MIC's was 0.1μg/ml and 0.01μg/ml of SPZ was sufficient to inhibit *F. pedrosoi* strain 23790 CIB's growth.

Minimal fungicidal concentration - 1μg/ml of KTZ was the MFC for *F. pedrosoi* strain 23790 CIB, the ITZ MFC's was 0.5μg/ml and SPZ MFC's was 0.1μg/ml (Table II).

In vivo tests

Standardization of the animal model - After inoculation of 202 male BALB/c mice of various

TABLE II

Minimal inhibitory concentration (MIC) and minimal fungicidal concentration (MFC) of *Fonsecaea pedrosoi* strain 23790 CIB

Test agent	MIC (µg /ml)	MFC (µg/ml)
Ketoconazole	1.00	1.00
Itraconazole	0.10	0.50
Saperconazole	0.01	0.10

ages, it was determined that age was not an important factor in the evolution of the infection (p>0.05).

We found that BALB/c male mice developed clinical infection 4-5 days after being inoculated intraperitoneally, the average clinical infection lasting 25 days (Fig. 1). At this time the lesions changed to scars in 85% of the mice. This clinical change did not mean a cure for the infection, inasmuch as 95% of the mice presented deep tissue nodules confirmed by positive cultures and histopathology.

Eleven mice died during the experiment; 5 of them had a systemic infection that compromised skin, peritoneum, liver, spleen, mesenteric ganglions and abdominal muscle. The cause of death in 6 animals is unknown.

Histopathology and tissue cultures - The histopathology was interpreted according to its own results, positive or negative, positive is the presence of hyphae, sclerotic bodies and muriform cells (Figs 2,3); the tissue culture results were interpreted according to their own results (positive or negative fungal growth) and were classified according to the antimycotic so that histopathology and tissue culture results could be compared to the control group.

Therapeutic trial - SPZ was the only drug showing a positive effect in controlling the disease, compared to the control group p=0.01 (Table III).

Fig. 2: *Fonsecaea pedrosoi* hyphae in skin biopsie. Methenamine-silver stain. X100.

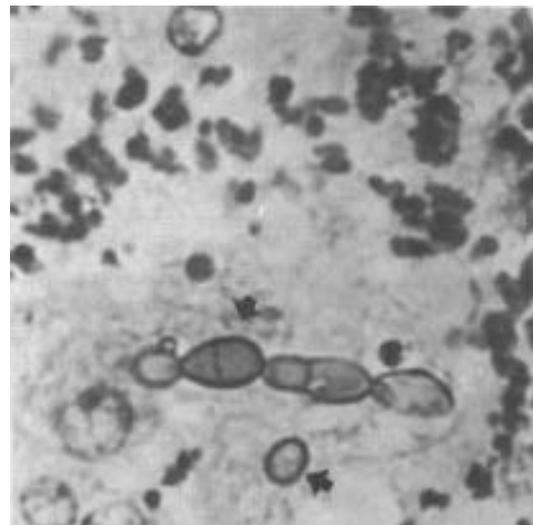Fig. 1: black nodular lesion of chromoblastomycosis in the inoculation site.

Fig. 3: *Fonsecaea pedrosoi* sclerotic bodies and muriform cells in subcutaneous tissue biopsie. Hematoxilin-Eosin stain. X400.

TABLE III

Percentage of results and statistical significance (ANOVA) of results from histopathology (HP) and tissue cultures (TC) according to the study group

Study group	Results HP and TC		p
	Negatives	Positives	
Saperconazole	63%	37%	0.01
Itraconazole	44%	56%	>0.05
Ketoconazole	37%	63%	>0.05

When the same test was carried out with the various treatment doses acting as classifying factors, SPZ at 1.25 mg/kg showed both lowest average of biopsies and positive cultures, and statistically significant differences (p=0.02) compared with the other doses. On the other hand, KTZ presented the highest average of biopsies and positive cultures.

After these analyses, each result was discriminated among positives and negatives for the accumulated values. According to this analysis, 17 of the 27 SPZ-treated mice were classified as negative for histopathology and cultures. According to this, SPZ could prevent the appearance of symptoms in up to 63% of the cases; ITZ appeared to have the same effect on 44% of the cases, 37% of the mice treated with KTZ were negative for cultures and histophatology.

DISCUSSION

The experiments showed that SPZ presented a greater *in vivo* and *in vitro* activity than ITZ and KTZ. SPZ inhibited fungal growth *in vitro* at a 0.01 µg/ml concentration and was fungicidal at a 0.1 ug/ml concentration; both concentrations were the lowest among the antimycotics tested. Other researchers have reported that *F. pedrosoi*'s response to SPZ *in vitro*, shows a 0.1 µg/ml MIC (Cutsem et al. 1989). The difference between our results and those mentioned may be due to the difference in isolates or to the laboratory techniques used.

ITZ was the second in efficacy. In the *in vitro* experiments a 0.1 µg/ml concentration was needed to inhibit fungal growth while a 0.5 µg/ml concentration was needed to produce a fungicidal effect. These concentrations were 10 and 5 times greater, respectively, than those required for SPZ to produce the same results. The MIC found was the same as that found by other researchers (Cutsem et al. 1986).

KTZ was the antimycotic which required the greatest concentration in the *in vitro* experiments, 1 µg/ml, to produce inhibitory and fungicidal effects. This concentration is 100 times greater than that required for SPZ and 10 times greater than that required for ITZ to produce inhibitory effects; 10 times greater than SPZ and 2 times greater than ITZ, to produce fungicidal effects.

The MIC found in our experiments, coincide with those found by other researchers (Cutsem et al. 1986).

In the *in vivo* experiments, the SPZ, ITZ and KTZ supplied to infected mice, resemble what Franco and colleagues found (Franco et al. 1992). They found that the action SPZ had on chromoblastomycosis patients was greater than that observed with either KTZ or ITZ. SPZ was the most effective drug, yielding 63% negative cultures and histopathology in mice, and a 100% cure-rate in humans. This could be explained because the drug's behavior and infection evolution are different in humans and in the animal model (Brammer & Tarbil 1978, Ahrens et al. 1989, Esterre et al. 1991).

The *in vivo* experiments with ITZ yielded a 44% cure-rate. In clinical studies with chromoblastomycosis patients, a cure rate of 80 to 90% was reported, which is a greater effective cure-rate than that found by us in the animal model (Restrepo et al. 1988).

A cure-rate of 37% was obtained with KTZ; this cure-rate data is closer to that found in clinical studies, which have rendered a 41 to 50% cure-rate (Drouhet et al. 1982, Hughes et al. 1988). The *in vitro* findings and the murine animal model can be compared to results obtained in other studies and to clinical experiments on humans, keeping in mind, though, that they deal with two different models which have different natural evolutions. The difference in antimycotic effectiveness between humans and the murine experimental model can be explained by the model's own nature, metabolism and immunity, among other differences.

Further studies of the *F. pedrosoi* infection on BALB/c mice could render this experimental model useful for other studies concerning phenomena, such as: mechanisms and efficiency of antimycotics and other chemotherapeutic agents, cellular and humoral immunological mechanisms during the disease evolution, host resistance and self-healing phenomena.

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