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Pathological Changes of the Blood Cells in Fluconazole Treated Toads

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ABSTRACT: Fluconazole is a triazole antifungal agent used for the treatment of certain superficial and systemic infections which predominantly affect immunocompromised individuals. Force–feeding toads with fluconazole at therapeutic dose level (0.26 mg/toad) for 20 weeks resulted in pronounced alterations of the blood cells of the peripheral blood. Most leucocytes showed irregularities in nuclear configuration, cytoplasmic vacuolation, pleomorphic granules and a paucity of organelles. These alterations are more or less similar to those reported in humans with leukaemia and were comparable to those observed after the administration of the carcinogenic chemical 7,12 dimethylbenz(a) anthracene (DMBA).

KEYWORDS: fluconazole, DMBA, toad, leukaemia.

INTRODUCTION

Microorganisms including fungi can cause great harm and damage. They infect people, animals and plants, producing diseases that range in seriousness from mild infections to death[1].

Fluconazole is a new triazole antifungal agent. It was introduced in early 1990 as prophylactic antifungal after bone marrow transplantation[2]. Fluconazole acts by selective inhibition of lanosterol 14-alpha-demethylase, a key enzyme for maintenance of the fungal cell wall[3]. It has a favourable pharmacokinetic profile that includes a long serum half-lifetime, which makes once-daily administration possible, more consistent absorption from the gastrointestinal tract than that of ketoconazole, excellent penetration into the cerebrospinal fluid, and elimination predominantly by a renal mechanism[4]. Prophylactic fluconazole prevents colonization and superficial infection by *Candida species* other than *Candida krusei* in patients undergoing chemotherapy for acute leukaemia. It is also used in the oral treatment of oropharyngeal, oesophageal, vaginal or systemic candidiasis and for fungal skin infection[5]. Havlir *et al*[6] pointed out that, administration of a 200 mg daily dosage of fluconazole is effective in reducing deep fungal infection in patients with AIDS.

The possible adverse effects of chronic, high-dose fluconazole therapy are detailed from analysis of a multicenter, dose – escalating study of the therapy of invasive mycoses[7]. Headache, hair loss, nausea, vomiting and anorexia were the most common symptoms experienced, and eosinophilia and aspartate aminotransferase increase were the most common laboratory findings. Leukopenia, thrombocytopenia and agranulocytosis were also reported in patients treated with fluconazole, but they recovered after withdrawal of antifungal theraphy[8]. The induction of leukaemia by the antifungal drugs, griseofulvin and nizoral has been also well documented. El-Mofty *et al*[9,10] found that toads force-fed with griseofulvin or nizoral had clear alterations of the blood cells similar to those of leukaemic cells.

El-Mofty *et al.*[11,12] proved that the Egyptian toads, *Bufo regularis* could be considered as an advantageous model for detecting the carcinogenicity and hazardous effects of chemicals and drugs. In contrast to mammalian experimental animals, toads are sensitive to smaller doses of chemicals and respond after shorter times. Hence the present work was undertaken to study the pathological effect of fluconazole on the blood cells of the Egyptian toad, *Bufo regularis*. In addition, we wished to determine whether fluconazole induces changes similar to those produced by 7,12-dimethylbenz(a) anthracene (DMBA), which was used in this study as a carcinogenic control.

MATERIALS AND METHODS

Experimental Animals : Sexually mature male and female toads, *Bufo regularis,* weighing 45 – 50g each were used. They were maintained in the laboratory at

22° C and fed earth worms twice a week. They were kept in large glass aquaria with some water that was changed twice daily.

The animals were divided into four groups, 50 of each. Toads of the first group (G1) were force- fed daily for 20 weeks with 0.26 mg fluconazole dissolved in 0.5 mL amphibian saline. This dose represents what is equal to the human therapeutic dose. Each toad in the second group (G2) was force-fed with 7,12-dimethylbenz(a) anthracene (DMBA) at a dose level of 0.5 mg, dissolved in 0.2 mL olive oil, twice a week for 20 weeks. The third group (G3) were force-fed with 0.2 mL of olive oil (as control for G2). Each animal of the fourth group (G4) was force-fed with 0.5 mL of amphibian saline (as control for G1).

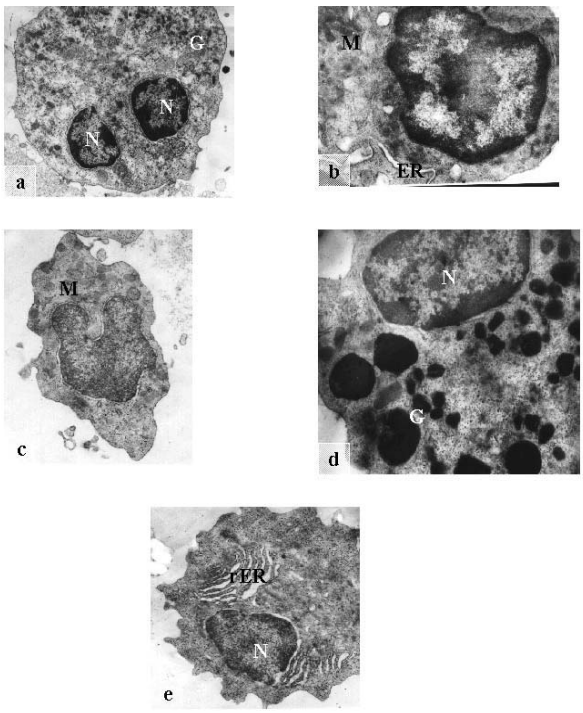

Fig 1. Transmission Electron Micrographs of Leucocytes in control toads, showing:
(a) Neutrophil with bilobed nucleus (N). G: Granules. (×7,500)
(b) Lymphocyte with relatively regular plasma membrane and nuclear envelope. ER: Endoplasmic reticulum, M: Mitochondria. (×15,000)
(c) Monocyte with a horseshoe shaped nucleus. Nuclear membrane and cell membrane are relatively regular in shape. Arrow points at endoplasmic reticulum enclosing the nucleus entirely. M: Mitochondria. (×10,000)
(d) Basophil with relatively rounded nucleus (N) and pleomorphic basophilic granules (G). (×10,000)
(e) Plasma cell with an eccentric nucleus (N) and abundant rough endoplasmic reticulum (rER). (×7,500)

Chemicals Used : Fluconazole was obtained from Pfizer, Egypt under authority of Pfizer Inc. , U.S.A. The carcinogenic chemical DMBA was obtained from Sigma Chemical Company (St. Louis, MO, USA).

Haematological Studies : Blood smears fixed in methyl alcohol and stained with Giemsa were used and prepared from blood samples obtained from the ventricle of the heart of the experimental animals. Blood cells were examined under light microscopy using 100X objective lens.

Blood Buffy Coat Preparation for Transmission Electron Microscope: Blood of toads from each experimental group (5 mL with 1 % heparin) was centrifuged for 20 min at 1,200 g. A thin, white buffy coat was formed between red blood cells below and the plasma above. After gently removing the plasma with a pipette 2.5 % glutaraldehyde in 0.1 M phosphate buffer was added dropwise. The buffy coat tube was allowed to stand for 18 h at 4 ° C and an approximately 1 mm^3 slice of the plug was cut and post-fixed in 1 % OsO4 for 1 h, then washed in buffer (pH 7.6), dehydrated in a graded series of increasing acetone concentrations followed by incubation in propylene oxide before embedding in araldite.

Thin sections were cut on an LKB ultramicrotome equipped with a glass knife. After double staining with uranylacetate and lead citrate, the sections were examined with a JEOL 100 CX electron microscope (JEOL Corp, Tokyo, Japan).

Results

Twenty weeks after the beginning of the experiment,

Table 1. Effect of force-feeding of fluconazole and 7.12 Dimethylben(a)anthracene (DMBA) on the peripheral blood of Egyptian toad, after 20-weeks of the experiment.

Chemical	No. of Exp. Toads	No. of Autopsied Toads	No. of Toads Bearing Blood Cell Changes	% of Toads Bearing Blood Cell Changes
DMBA	50	30	24	80%
Fluconazole	50	30	18	60%

examination of light microscopic preparations revealed changes in the structure of leucocytes of 80% of the toads force-fed with DMBA and 60% of the toads force-fed with fluconazole (Table 1) . However, no changes in leucocytes were detected in the peripheral blood of any toad of the control groups which were therefore not included in the table.

Electron microscopic examination showed that ultrastructural changes did occur in leucocytes of toads following administration of fluconazole or its carcinogenic control DMBA (Figs. 2 and 3), unlike the untreated toads which had no change (Fig 1).

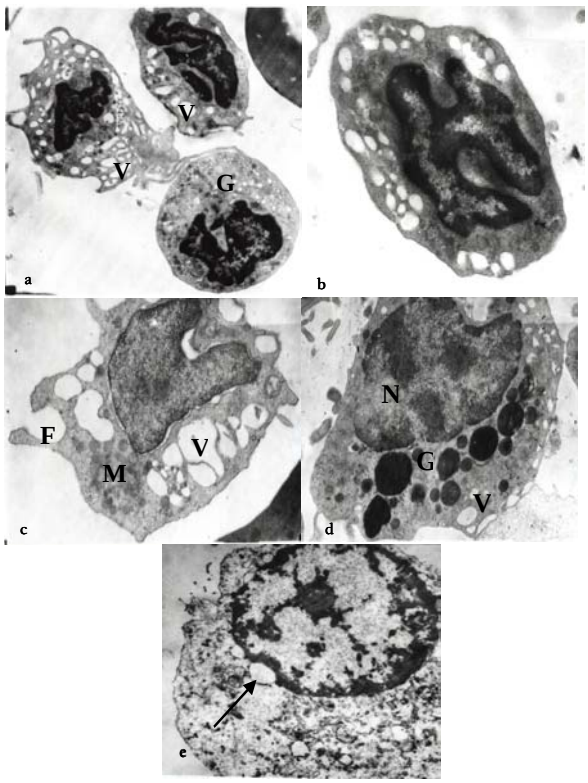

Fig 2. Transmission Electron Micrographs of Leucocytes in DMBA-treated toads, showing:
(a) Extensive segmentation, lobulation and hyperchromatism of nuclei in neutrophils . G:Granules, V:Vacuoles. (× 5,000)
(b) Lymphocyte with vacuolated cytoplasm and abnormal dividing nucleus. (×10,000)
(c) Vacuolated monocyte with dilated nuclear envelope, abundant euchromatin and irregular plasma membrane. F: Filopodia, M: Mitochondria, V: Vacuoles. (× 10,000)
(d) Basophil with irregular nucleus (N), vacuolated cytoplasm and numerous pleomorphic dense granules (G). V: Vacuoles (× 10,000)
(e) Plasma cell with evidences of necrosis. Note the margination of chromatin of the eccentric nucleus and lack of most cytoplasmic organellae. Arrow points at dilation of outer nuclear membrane.(× 7,500)

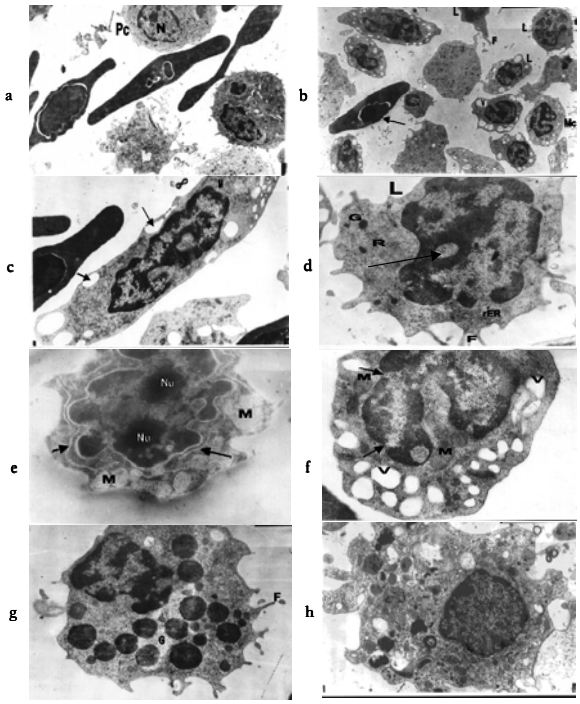

Fig 3. Transmission Electron Micrographs of blood film in fluconazole treated toads, showing:
(a) Altered erythrocytes with fragmented nucleus and long cytoplasmic projections. Note an altered segmented neutrophil (lower) and a plasma cell (Pc). N: Nucleus. (× 4,800).
(b) Altered leucocytes with altered heterochromatin content and highly vacuolized cytoplasm. Arrow points at an erythrocyte with dilated nuclear envelope. F: Filopodia, L: Lymphocyte, Mc: Monocyte, V: Vacuole (× 4,800).
(c) Band neutrophil with predominant heterochromatin and extensive vacuolated cytoplasm. Note that the nucleus is deeply intricated and invaginated with cytoplasmic material. A large number of small mitochondria (M) are present. (arrows point at endocytic vesicles) (× 7,500).
(d) Lymphocyte (L) with irregular nuclear envelope, dilated nuclear pores and intranuclear inclusion (arrow). The cytoplasm contains many ribosomes (R), rough endoplasmic reticulum (rER) and some granules (G). F: Filopodia . (× 10,000).
(e) Lymphocyte with 2 nucleoli (Nu) and an irregular hyperchromatic nucleus with dilated nuclear membrane. Arrows point at endoplasmic reticulum. M : Mitochondria (× 15,000).
(f) Monocyte with U-shaped nucleus, dilated nuclear pores (arrows) and predominant heterochromatin. The cytoplasm is highly vacuolated. M: Mitochondria, V: Vacuoles (× 13,000).
(g) Basophil with an eccentric heterochromatic irregular shaped nucleus and numerous large pleomorphric dense granules (G). F: Filopodia (× 7,500).
(h) Plasma cell with an eccentric nucleus, pleomorphic granules and folded plasma membrane. (× 7,500).

The administration of fluconazole resulted in erythrocytic anaemia detected by the presence of altered erythrocytes with fragmented or degenerated nuclei, long cytoplasmic projections and vacuolated cytoplasm. (Fig 3a and b).

Ultrastructural studies of leucocytes of fluconazole-treated toads revealed severe ultrastructual changes, including nuclear abnormalities, hyperchromatism, pleomorphic granules, cytoplasmic vacuolations and ruffled cell surfaces (Fig 3b). Neutrophils had large eccentric irregular shaped nucleus with peripherally condensed heterochromatin and vacuolated cytoplasm (Fig 3b and c). The plasma membrane of these cells form short projections, the fusion of which forms endocytic vesicles. Lymphocytes were observed with irregular shaped nuclei, dilated nuclear pores, vacuolated cytoplasm and irregular plasma membrane (Fig 3b and d). Some lymphocytes with irregular hyperchromatic nuclei and mitochondria with light matrices were also depicted (Fig 3e). Monocytes are characterized as having U- or W-shaped nuclei, dilated nuclear pores, pleomorphic mitochondria, ruffed cell surfaces, numerous filopodia, large endocytic vesicles and vacuolated cytoplasm (Fig 3b and f).

Basophils contain numerous pleomorphic basophilic granules, eccentric irregular shaped nuclei and many filopodia (Fig 3g).

Plasma cells were ultrastructurally different from their normal appearance. They showed eccentric nuclei with predominantly euchromatin and dilated nuclear envelope (Fig 3 h). The cytoplasm of these cells contains distorted rough endoplasmic reticulum in the form of vesicles and mitochondria with varying degrees of pleomorphism.

Discussion

In the present study, administration of fluconazole, or its carcinogenic control DMBA was found to cause significant changes in the cellular elements (erythrocytes and leucocytes) of the peripheral blood of toads, *Bufo regularis*. Erythrocytes exhibited anisocytosis and poikilocytosis. The majority of erythrocytes were observed with irregular cell membranes, elongated cytoplasmic projections and deformed nuclei. Similar changes were reported in toads treated with the antifungal drug griseofulvin[9] and antibiotics chloramphenicol[10] and are considered to be diagnostic features of haemolytic anaemia[13].

Electron micrographs of leucocytes showed ultrastructural features that are similar to those of leukaemic cells. Most cells showed irregularities in nuclear configuration with hyperchromatism, cytoplasmic vacuolation, pleomorphic granules, numerous ribosomes, ruffled cell surfaces and a paucity of organelles. Monocytes revealed idented, U- or W-shaped nuclei with dilated nuclear pores, vacuolated cytoplasm and plasma membrane with many protrusions and endocytic vesicles. Ghadially[14] described similar alterations of leucocytes of monocytic leukaemic patients. Lymphocytes were found with irregular hyperchromatic nuclei, mitochondria with light matrices and plasma membranes with numerous filopodia. Similar findings have been described by Komiyama *et al*[15] in humans suffering from leukaemia. Eosinophils and basophils have altered plasma membranes, eccentric nuclei and pleomorphic granules. Eosinophilia was the most common laboratory findings in patients treated with fluconazole for the therapy of invasive mycoses[7]. Altered plasma cells were also observed. Their presence in the peripheral blood indicates a change in the immune system of the body.

From the above discussed results, it could be concluded that, fluconazole has serious detrimental impacts on the blood cells of toads and these hazardous effects are comparable to those induced by the chemical carcinogen DMBA. Still the validation of this conclusion for human beings would require considerable further experimentation.

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