The Use of Swiss Albino Mice and Egyptian Toads (*Bufo regularis*) as Reliable Biological Test Animals for Screening Chemicals and Drugs Which Induce Leukaemia in Man. I: The Effect of Nizoral (Ketoconazole) on Leucocytes of Toads and Mice

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Abstract: Nizoral (Ketoconazole) is an antifungal drug used for the treatment of systemic mycosis and mucocutaneous candidiasis. Administration of Nizoral into toads and mice induced pronounced alterations in leucocytes. Electron microscopical examination revealed that these alterations are leukaemic alterations and they are more or less similar to the criteria reported in human Leukaemia. The changes were all comparable to those observed after the administration of the carcinogenic chemical 7,12 dimethylbenz (a) anthracene.

Key words: Nizoral (Ketoconazole), *Bufo regularis*, Swiss albino mice, biological test anirriel, Leukaemia

Introduction

Nizoral (Ketoconazole), a synthetic imidazole piperazine compound, is a broad-spectrum antimycotic drug for the treatment of superficial and systemic fungal infections (Jones *et al*., 1981). Ketoconazole (Nizoral) inhibits steroid biosynthesis in patients as it does in fungi by inhibition of cytochrome P₄₅₀ dependent enzymes (Loose *et al*., 1983). Thus nizoral impairs the biosynthesis of ergosterol for the cytoplasmic membrane and lead to the accumulation of 14-α methyl sterols (Vanden Bossche *et al*., 1986). These methyl sterols may disrupt the close packing of acyl chains of phospholipids, impairing the function of certain membrane-bound enzyme systems and inhibiting growth of fungi (Bennett, 1991). The primary metabolic pathways of ketoconazole in humans are oxidation of the imidazole ring, degradation of the oxidized imidazole oxidative 0-dealkylation, and degradation of the piperazine (Heel *et al*., 1982). In the rat, it has been shown that imidazol-piperazin compounds affect the microsomal production of hepatic enzymes (Razzouk *et al*., 1978). Nizoral was introduced in the united kingdom in 1981, by 1984 the committee on Safety of Medicines had received 82 reports of possible hepatotoxicity associated with the drug, including five deaths. The results of serum liver function tests suggested hepatocellular injury in 63 percent of patients (Lake-Bakaar *et al*., 1987).

Six of nine male patients, who received nizoral for more than 4 months showed sperm counts below normal and 2 of these patients showed azoospermia (Stevens *et al*., 1982). At high doses of nizoral, a variable number of males experience gynecomastia and decreased libido and potency (DeFelice *et al*., 1981). While doses of nizoral as low as 400 mg can cause a transient drop of plasma concentration of free testosterone and estradiol C-17 B (De Coster *et al*., 1995). The aim of the Present work is to study the possible induction of Leukaemia in the toads (*Bufo regularis*) and Swiss albino mice and to compare these with the leukaemic cells induced in the previous works of El-Mofty *et al*., (1995, 1997), in toads by the effect of griseofulvin, adriamycin and the chemical carcinogen 7,12-dimethylbenz(a)anthracene. Also these leukaemic cells will be compared with leukaemic cells of man described by Ghadially (1985).

Materials and Methods

Experimental animals

Toads: Sexually mature male and female toads (*Bufo regularis*) weighing approximately 35 grams were used. The toads were force fed with 0.2 mg of Nizoral suspended in ½ ml of amphibian saline solution. Food and tap water were provided ad libitum. Dead animals were removed quickly and the cages were cleaned daily.

Drug Used

Nizoral (Ketoconazole): Ketoconazole (Janssen, Pharamceutica) is in the form of white tablets, of 200 mg each. The milled tablets of the drug were administered orally, in the form of a suspension in each of the amphibian and mammalian saline solution.

Feeding Technique

Toads

Experimental animals (50 toads): Each Experimental toad was force fed with 0.2 mg of Nizoral suspended in ½ ml of amphibian saline daily for 3 months. The drug suspension was introduced into the stomach of each toad using an eye dropper (Fig.1). This is called the force feeding technique (El-Mofty *et al*., 1981).

Fig. 1: Force feeding technique of toads using an eye dropper (Natural size)
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![Fig. 2: An electron micrograph of peripheral blood of ketoconazole treated toad, showing a granular altered leucocytes with highly vacuolized cytoplasm. Arrows point irregular nuclear envelope and indentation of nucleus. Arrow head points dilation of the nuclear envelope. L: lymphocyte, MC: Monocyte, P: plasma cell. (X 10,000)](image1)

![Fig. 3: An electron micrograph of peripheral blood of ketoconazole treated toad, showing monocyte (Mc) with elongated U-shaped nucleus. L: lymphocyte, S: Sezary like lymphocyte. (X 10,000)](image2)

![Fig. 4: An electron micrograph of peripheral blood of ketoconazole treated toad, showing a monocyte (MC) with W-shaped nucleus (N) and large filopodia (F) of plasma membrane (X 20,000)](image3)

![Fig. 5: An electron micrograph of peripheral blood of Ketoconazole treated toad, showing a neutrophil with bilobed nucleus (N), rough endoplasmic reticulum (RER), mitochondria (M) and nuclear pore (NP) (X 15000)](image4)

**Control’s (50 animals):** Each control toad was fed ½ ml of amphibian saline daily for 3 months.

**Mice**

**Experimental animals (50 mice):** Each experimental mouse was fed daily with 0.2 mg of Nizoral suspended in ½ ml of mammalian saline for 12 months. The drug suspension was fed by gavage using a stomach tube.

**Control mice (50 animals):** Each control mouse was fed daily with ½ ml of mammalian saline for 12 months.

**Haematological study:** Blood samples were obtained from the ventricle of the heart and blood smears were fixed in methyl alcohol and stained with buffered Giemsa stain. Blood cells of all groups were examined for pathological changes.

**Blood buffy coat preparation for electron microscope:** Blood of toads and mice from each experimental group (5 ml, with 1% heparin) was centrifuged for 20 minutes, at 2,500 r.p.m. A thin white buffy coat was formed between the lower red blood cells and the upper plasma. After gently removing the plasma with a pipette 2.5 percent glutaraldehyde buffered with 0.1 M phosphate buffer was added. The buffy coat tube was allowed to stand for 18 hours at 4°C. 1 mm slice of the plug was cut into smaller pieces and post fixed in 1 percent OsO₄ for 1 hour, then washed in buffer (pH, 7.6), dehydrated in a graded series of acetone followed by propylene oxide before embedding in araldite. Thin
sections were cut on LKB-Ultramicrotome equipped with a glass Knife. After double staining with urinal acetate and lead citrate sections were examined in a Jeol X 100 electron microscope.

Results
Electron micrographs of Peripheral blood of ketoconazole treated toads or mice revealed morphologically altered forms of leucocytes.

Agranulocyte of toads: Elongated agranulocyte (lymphocytes and monocytes) of toads had elongated nuclei (Fig. 2). All the nuclei showed irregularities and indentation of the nuclear envelope, with a dilated space between the 2 membranes of the nuclear envelope (Fig. 2 arrow heads). Some lymphocytes that appeared partially cleared and with less margination heterochromatic eccentric nuclei showed irregularity of the plasma membrane (Fig. 3, lower left), while others had central nucleus with predominating heterochromatin and slightly central euchromatin. Vacuoles were also observed in some lymphocytes (Fig. 2, upper left). Asezary like lymphocytes with a cerebriform appearance nucleus were recorded changes (Fig 3,5, lower right).

Fig. 6: An electron micrograph of peripheral blood of ketoconazole treated toad, showing an eosinophil with pyknotic nucleus (N) and pleomorphic granules (G) (X 26,000)

Fig. 7: An electron micrograph of peripheral blood of ketoconazole treated toad, showing abnormal plasma cell. M: mitochondria, N: Nucleus, RER: rough endoplasmic reticulum (X 26,000)

Monocytes showed clear alterations, they were characterized by having different sized indented U- or W- shaped nuclei (Fig. 2-4), and abnormal chromatin distribution and with basophilic vacuolated cytoplasm (Fig. 2, 4). The cell membrane of some monocytes is thrown out into filopodia (Fig. 4), the fusion of which result in the formation of endocytic vesicles (Fig. 4).

Granulocyte of toads: Ultrastructurally, most granulocyte examined conform the structure of neutrophils (Fig. 5), they have large similar eccentric nuclear lobes. These lobes showed heterochromatin condensed on the periphery with few dilated nuclear pores (Fig. 5). Few disorganized mitochondria, few short profiles or rough endoplasmic reticulum scattered in the cytoplasm and moderated number of different sized specific pleomorphic granules were presented in the cytoplasm (Fig. 5).

Eosinophils of treated toads showed some abnormalities such as relatively less numerous granules that were pleomorphic and obvious pyknosis of the nucleus (Fig. 6). Also they had irregular plasma membrane.

Plasma cells were ultrastructurally different from their normal counter parts. They showed slightly irregular nuclei that were eccentric in position with predominantly euchromatin and dilated nuclear pores. The cytoplasm contains abundant clear rough
endoplasmic reticulum with dilated longitudinal cisternae bearing ribosomes on their outer surfaces. The mitochondria were numerous, oval in shape with orthodox configuration. Cristae are clear in all of them. The plasma membrane is irregular with short microvilli (Fig. 7).

Agranulocyte of mice: Lymphocytes of ketoconazole treated mice had large nuclei with marginated heterochromatin (Fig. 8). The nuclear membrane is irregular. Some lymphocytes had their cytoplasm filled with mylinoid bodies (Fig. 8).

Monocytes of ketoconazole treated mice had highly irregular U- or W-shaped nuclei. The heterochromatin content was subjacent to the nuclear envelope. The cytoplasm had few rounded mitochondria (Fig. 9).

Granulocyte of mice: Eosinophils contained numerous large pleomorphic eosinophilic granules. The nucleus had eccentric position and had an elongated shape (Fig. 10). Basophilic of treated mice showed pleomorphic basophilic granules and eccentric nuclei. The plasma cells of ketoconazole treated mice showed eccentric nucleus. The cytoplasm had poorly developed rough endoplasmic reticulum and numerous pleomorphic granules.

Discussion

Force feeding toads (Bufo regularis) or Swiss albino mice with nizoral (Ketoconazole) induced clear ultrastructural changes in the leucocytes of peripheral blood of the amphibian and mammalian experimental animals used in this work.

1. Lymphocytes have abundant marginated heterochromatin and some lymphocytes have sezary nuclei (cerebriform nuclei).
2. Monocytes have either irregular U-shaped or W-shaped nuclei.
3. Plasma cells have very clear rough endoplasmic reticulum with dilated cisternae. Some of them have active secretory Golgi apparatus. They also have clear pleomorphic mitochondria and eccentric nuclei.
4. Eosinophils have eccentric nuclei and large pleomorphic granules.
5. Basophils have pleomorphic basophilic granules and eccentric nuclei.

Ghadially (1985) suggested that these changes can be used as tentative markers for leukaemias and lymphomas in man. These changes are very similar to those induced in toads (Bufo regularis) in 2 previous works by El-Mofty et al. (1995, 1997), in which some toads were force fed with the antifungal drug griseofulvin, other toads were injected with the anticancer drug adriamycin and some other toads were force fed with the chemical carcinogen 7,12-dimethylbenz(a) anthracene. These ultrastructural changes are similar to the changes that takes place in leucocytes of men suffering from Leukaemia as described by Ghadially (1985) and Dickersin (1988). Also Komiyama et al. (1976) described similar alterations in human acute Leukaemia.

Our finding assures the previous 2 works of El-Mofty et al. (1995, 1997) which mentioned that toads (Bufo regularis) can be used as reliable biological test animals for screening Leukaemia in mammalian animals and man. Further studies are going on, however, to test other drugs that may cause Leukaemia in man by the use of toads or mice.

References


