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Benznidazole-induced ultrastructural and biochemical alterations in rat esophagus

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Abstract

Benznidazole (Bz) is a drug used in the chemotherapy of the acute and intermediate phases of Chagas' disease (American Trypanosomiasis), an endemic parasitic disease afflicting more than 16 million people in Latin America. Serious toxic side effects of Bz have been reported in treated human beings and in experimental animals. Bz toxicity would be linked to its nitroreductive bioactivation to reactive intermediates and to the corresponding amine known to occur in vivo and mediated by different enzymatic systems. In the present study the presence of Bz nitroreductases in rat esophagus and the occurrence of Bz induced esophageal cell injury are described. Already 1 and 3 h after an intragastric Bz administration to Sprague–Dawley male rats (240–260 g body weight) at a dose of 100 mg/kg esophageal levels of the drug were 66.4 ± 4.0 and 149.2 ± 14.3 nmol per g tissue, respectively. The esophageal mucosa homogenates exhibited Bz nitroreductase activity attributable to the participation of cytochrome P450 reductase and xanthine oxidoreductase (XOR). The ultrastructural observation of esophageal tissue from Bz treated animals 24 h after its administration evidenced: detachment and conglomeration of polyribosomes, reduction in the presence of desmosomes and of the amount of bacteria on its surface. The potential significance of these alterations is not fully clear at present. However, these deleterious effects might be additive or synergistic with those induced by the evolution of the disease.

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Keywords: Benznidazole; Nitroreductases; Drug therapy; Esophageal injury

1. Introduction

Chagas' disease (American trypanosomiasis) is an endemic parasitic disease of major concern in Central and South American countries. It is estimated that 16–18 million people are currently infected with *Trypanosoma cruzi*, the causative agent of the disease, and that 100 million people (i.e. 25% of the population of Latin America) are at risk of acquiring the infection (WHO, 2000). Around 100 000 infected individuals are living in USA, most of them immigrated from Mexico and Central America (Pinto Dias, 1992). Two drugs

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are currently in use against Chagas' disease, nifurtimox (Nfx) and benznidazole (Bz) (Rodriques Coura and de Castro, 2002). Both are unsatisfactory because of their serious side effects, which frequently force physicians to stop treatment (Docampo and Moreno, 1988; Castro and de Toranzo, 1988; Castro, 2000). These Bz toxic side effects were attributable to Bz nitroreductive processes mediated mostly by cytochrome P450 reductase but also by cytochrome P450; xanthine oxidoreductase (XOR) and aldehyde oxidase (AO) (Masana et al., 1984; Walton and Workman, 1987; Docampo and Moreno, 1985; Castro and de Toranzo, 1988; Castro, 2000). The anaerobic reduction of Bz to reactive intermediates able to covalently bind (CB) to proteins and lipids (de Toranzo et al., 1984; Masana et al., 1985) and to the corresponding Bz amine (Walton and Workman, 1987) was evidenced to occur in situ under in vivo conditions.

Further, the intracellular intensity of a given Bz nitroreductive pathway in a given tissue (e.g. testes, ovaries, adrenals, colon) correlated with the intracellular location of the ultrastructurally detectable cell injury (Bernacchi et al., 1986; de Castro et al., 1989, 1992; Díaz et al., 2000).

More recently, the use of Bz was extended to the period after the acute phase of the disease and prior to the chronic one (Sgambati de Andrade, 1997; Sosa Estani et al., 1997; Viotti et al., 1994). The usefulness of the use of Bz in that intermediate phase of the disease is still controversial (Docampo, 2001). The chronic stage includes life threatening effects on the heart and CNS and intense inflammatory processes in both, the colon and the esophagus (Pinto Dias, 1984). Further, there are reports in literature giving evidence of significant increased incidence of colon and esophageal cancer in patients suffering Chagas' disease (Camarca-Lopes, 1961; de Lustig et al., 1980). Those reports, however, did not analyze whether those patients were treated or not with the nitrocompound. This is important because Bz is known to have carcinogenic effects in laboratory animals (Teixeira et al., 1994). Those effects would require Bz nitroreductive metabolism to reactive metabolites able to react with DNA (Gorla et al., 1986) and of mutagenic nature (Gorla and Castro, 1985; Gorla et al., 1988; Nagel and Nepomnaschy, 1983; Ferreira and Ferreira, 1986).

In this study, we verified whether esophageal mucosa was able to nitroreduce Bz and whether Bz administration produced ultrastructurally detectable cell injury. These studies might help to have a more clear evaluation of risks of Bz therapy in the late or intermediate stages of the disease until new effective and safer chemotherapeutic agents under research may become available (Docampo, 2001; Fairlamb, 1999; Urbina, 2002).

2. Materials and methods

2.1. Chemicals

Benznidazole, *N*-benzyl-2-nitro-1-imidazole acetamide (Bz) was a gift from F. Hoffmann La Roche and Company, Ltd; diphenylene iodonium chloride (DPI), hypoxanthine and allopurinol were purchased from Sigma Chemical Co. (St. Louis, MO). All other chemicals employed were of analytical grade.

2.2. Animals and treatments

Sprague–Dawley male rats of 240–260 g body weight were bred in our laboratory. Rats were maintained in a controlled room on a 12-h light:12-h dark cycle (light phase 7–19 h). Temperature in the animal room was (23 ± 2) °C and the relative humidity was between 35 and 65%. Animals were fasted 12–14 h with free access to water. They were sacrificed by decapitation and bled and their esophagi were dissected out in its entire length.

In the in vivo studies Bz was given intragastrically at a dose of 100 mg/kg suspended in carboxymethylcellulose (CMC) 1%. Animals were sacrificed at different times after Bz administration and the esophageal samples were rapidly excised and processed.

2.3. Determination of Bz content in esophageal tissue

Sample preparation for the determination of Bz content in esophageal tissue was as follows. After a single dose of Bz, esophageal samples were homogenized with saline solution (12 volumes per g) in a high speed cutting, dispersing and emulsifying Ultra Turrax T_{25} tissue homogenizer (IKA Werk Germany). Control rats were run simultaneously. The extraction of 1 ml of the homogenate with 6 ml of dichloromethane was carried out with an Extrelut® column. The solvent was evaporated to dryness under nitrogen and the residue dissolved in 0.5 ml of mobile phase and analyzed by HPLC.

2.4. Bz nitroreductase activity determinations

Esophagi were homogenized in an Ultra Turrax T_{25} with 4 volumes of 20 mM potassium phosphate buffer pH 7.4. The homogenates were centrifuged at $600 \times g$ for 10 min. All procedures were performed at 0–4 °C.

All incubations were run in 20 ml aluminum-sealed-neoprene septum stoppered glass vials with agitation at 150 oscillations/min in a covered Dubnoff shaker at 37 °C and were gassed for 5 min with oxygen-free N_2. The incubation mixtures contained the following: $600 \times g$ supernatant (final concentration 3.0–4.6 mg protein/ml); 20 mM potassium phosphate buffer pH 7.4; 28.8 μM Bz (in *N*,*N*-dimethylformamide, 2% v/v final concentration). To determine the NADPH dependent nitroreductase activity, 0.5 mM NADPH-generating system was included in the incubation mixture described above (Díaz et al., 2000). When checking for participation of flavoenzymes in this process, 10 μM DPI was also added. To establish XOR participation in Bz nitroreduction, 0.25 mM hypoxanthine was included in the mixture in place of NADPH. To confirm XOR participation, the inhibitory effect of 0.15 mM allopurinol was tested.

After incubation for 4 h at 37 °C, the reaction was interrupted placing the flasks on ice and adding 15% (w/v) $ZnSO_4$, 1 ml. A fraction of the incubation mixtures was poured over 1 g of NaCl and extracted with 4.0 ml of ethyl acetate. An aliquot of the organic layer was evaporated under N_2. The residue was dissolved in 0.5 ml of mobile phase and analyzed by HPLC. In these studies Bz nitroreductase activity was followed by substrate decrease. This was calculated by the difference in Bz concentration at the beginning and at the end of the reaction.

2.5. Bz concentration measurement by HPLC

The resuspended sample was filtered through nylon filter membranes (pore size 0.45 μm) prior to HPLC analysis. Samples (10 μl) were analyzed at 40 °C using a Hewlett Packard model 1090 Serie II liquid chromatograph with a Hewlett Packard ODS Hypersil column (20 cm × 2.1 mm ID, 5 μm particle size) and diode array detector. The mobile phase, consisting of 60% methanol–water, was delivered at a constant flow-rate of 0.2 ml/min. The column effluent was monitored at 320 nm. Quantification was by peak-area ratio with reference to standards treated identically.

For the determination of Bz content in esophageal tissue, the efficiency of recovery of Bz added to a esophageal homogenate was $> 70\%$. Calibration curves were prepared by analyzing 1 ml of homogenized sample spiked with known amounts of the compounds (ranging from 1.0 to 4.0 μg Bz/ml). Plots of peak areas against concentration were linear over the concentration ranges studied (correlation factor 0.9703). The detection limit was 0.5 μg Bz/ml using 1 ml of homogenate and allowing a signal-to-noise ratio of 2.

2.6. Protein concentration determination

Protein concentrations were determined using the method of Lowry et al. (1951), using bovine serum albumin as standard.

2.7. Histochemical procedure

Portions of esophageal tissue were frozen at −70 °C in hexane in a mixture of solid carbon dioxide and absolute alcohol. Tissue blocks were stored at −80 °C until further use. Sections 8 μm thick were cut on an International Cryostat IEC at −24 °C. The sections were picked up onto clean

glass slides and incubated immediately for xanthine oxidase activity using the cerium capture method in the presence of polyvinyl alcohol described by Frederiks et al. (1994). Briefly, the incubation mixture contained 100 mM Tris–maleate buffer, pH 8; 10 mM cerium chloride; 100 mM sodium azide; 0.5 mM hypoxanthine and 10% polyvinyl alcohol. Incubations lasted 60 min at 37 °C. After that, sections were washed in hot distilled water (60 °C). Visualization was performed by incubating sections for 30 min at room temperature in 50 mM acetate buffer, pH 5.3; 42 mM cobalt chloride; 100 mM sodium azide; 1.4 mM diamino benzidine and 0.6 mM H_2O_2. After rinsing the sections were embedded in glycerol jelly.

2.8. Transmission electron microscopy

Five male rats per group (control and Bz-treated animals) were anesthetized by diethyl ether 24 h after CMC or Bz administration. The entire esophagi were rapidly removed and immediately placed in chilled 2% formaldehyde–2% glutaraldehyde in 100 mM cacodylic buffer containing 0.02% Cl_2Ca, pH 7.4, and promptly cut longitudinally under the fixative to improve exposure of the luminal surface to it. After adequate fixation, ten cubes (3 mm^3) per each rat esophagus were washed with barbital buffer and postfixed with 1% osmium tetroxide. Then, they were stained as a whole with uranyl acetate, dehydrated with graded ethanol and vertically embedded in epoxy resin to ensure proper orientation for sectioning perpendicular to the plane of the luminal surface.

Sections 1 μm thick were stained with toluidine blue and examined with a light microscope in order to select epithelial areas for thin sectioning. Thin sections were cut with diamond knives and were mounted on a copper grid (300 mesh) stained with uranyl acetate and lead citrate and examined in a Philips EM 300 transmission electron microscope (de Castro et al., 1989, 1992; Bernacchi et al., 1986).

2.9. Statistics

The significance of the difference between two mean values was assessed using the Student's t-test as described by Gad and Weil (1982).

3. Results

3.1. Bz content in esophageal tissue of rats

Bz given intragastrically to rats reached the esophagus. The tissue level of Bz was (66.4±4.0) and (149.2±14.3) nmol/g, 1 and 3 h after the drug administration, respectively.

3.2. Bz nitroreductase activity in rat esophagus

An NADPH dependent Bz nitroreductase activity was observed in the 600 × g supernatants from rat esophagi under a nitrogen atmosphere. In the absence of NADPH no nitroreductive activity was observed. That NADPH-dependent Bz nitroreductase activity was significatively inhibited by DPI (Table 1).

A minor nitroreductase activity attributable to XOR was observed using hipoxantine as cosub-

Table 1
Bz nitroreductase activity in 600 × g rat esophageal supernatants

Experimental conditions	Bz nitroreductase activity (nmol/min per mg protein)
Control	0
NADPH	13.96±1.73
DPI	0.64±0.37
NADPH+DPI	2.50±0.67[a]
Hypoxanthine	2.91±0.26
Hypoxanthine+allopurinol	1.28±0.12[b]

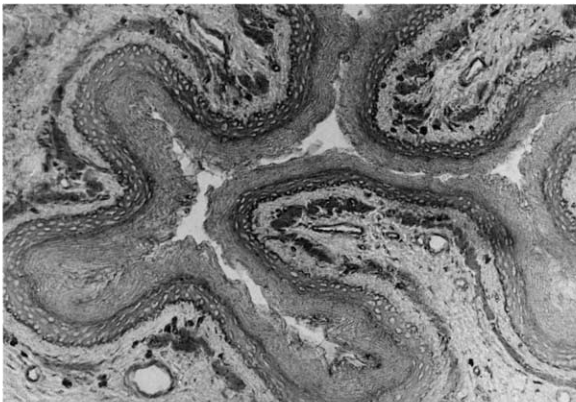
All the samples contained the 600 × g and 28.8 μM Bz. Some vials additionally contained either NADPH generating system or 10 μM DPI or NADPH+DPI or 0.25 mM hypoxanthine or hypoxanthine+0.15 mM allopurinol. Flasks were incubated 4 h at 37 °C under anaerobic conditions as discussed in Section 2. Values are the mean±S.D. of three determinations.

[a] $P < 0.001$ (vs. NADPH).

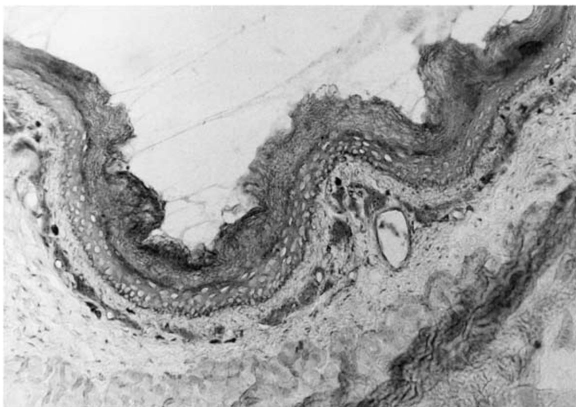
[b] $P < 0.005$ (vs. hypoxanthine).

strate and confirmed by allopurinol inhibition (Table 1).

Histochemical studies have shown that XOR was present in the esophageal mucosa (Fig. 1). The XOR activity was particularly intense in the muscularis mucosa cells of the esophagus. A slightly less intense but more widely and homogeneously distributed activity was found in the stratified squamous epithelium particularly in the polyhedral and columnar cells (Fig. 1a). A further detail of the XOR distribution pattern can be seen in Fig. 1b.



(a)



(b)

Fig. 1. Esophageal section from a control rat. (a) Distribution of xanthine oxidase activity in esophageal mucosa, $\times 150$. (b) Further detail of the same section, $\times 240$.

3.3. Ultrastructural observations

The esophagus is a muscular tube connecting the pharynx with stomach. The lamina mucosae is lined by a thick stratified squamous epithelium keratinized in the rat. The observation of the esophageal mucosa from control rats showed numerous polyhedral cells from the intermediate layers with round or oval nuclei, mitochondria and Golgi complex. These cells also exhibited endoplasmic reticulum membranes studded with ribosomes, group of polyribosomes possessing four or five ribosomes and bundles of tonofilaments (Fig. 2a). The Bz treated polyhedral epithelial cells contained important accumulation of polyribosomes as conglomerates. They also showed polyribosomes with various configurations as helical or rosette-like formations, exhibiting a larger number of ribosomes per polysome than those of the control group (e.g. from 8 to 12) (Fig. 2b).

Squamous epithelium of control rat showed numerous desmosomes. The desmosomes were distinct plaques at the cell surface, button-like structures. The size of desmosomes was variable and identified by the presence of a dense plaque and filaments on their cytoplasmic faces (Fig. 3a). In the Bz treated rats, the points of firm intercellular adhesion (desmosomes) were depressed. Many cells showed nuclei with irregular nuclear shape (Fig. 3b).

The esophageal surface in all rats was covered with bacteria. Control animals showed a great number of bacteria (Fig. 4a). After treatment with Bz the amount of bacteria present in all esophageal samples was markedly reduced (Fig. 4b).

4. Discussion

As shown in Section 3, already 1 and 3 h after direct intragastric administration of Bz the drug was present in the esophagus. The rat esophageal tissue probed to be able to anaerobically metabolize the nitro group of Bz in a NADPH-dependent reaction. It was previously reported that Bz can be metabolized in liver and other tissues such as testes, ovaries, adrenals and colon via nitroreductive mechanisms. Most of the activity was attribu-

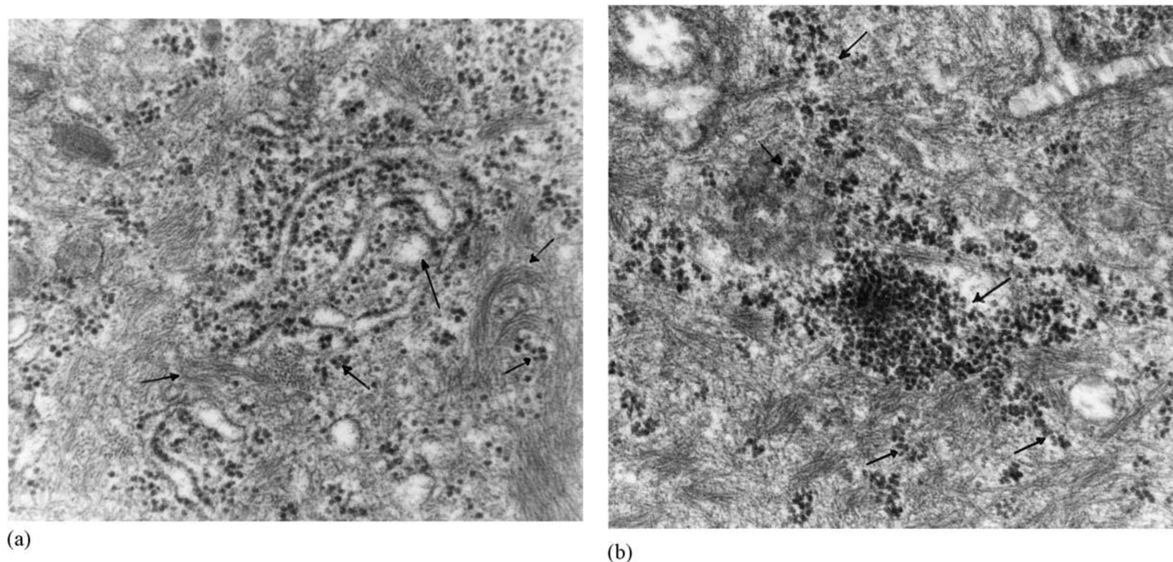
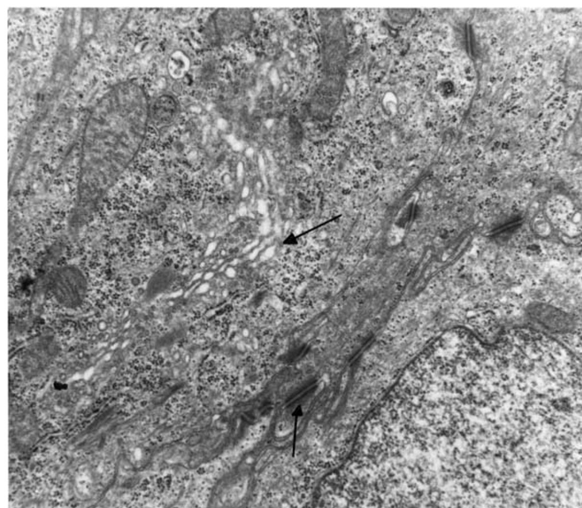


Fig. 2. (a) Electron micrograph of esophageal mucosa of a control rat following CMC treatment. A group of cisternae of rough endoplasmic reticulum shows ribosomes at irregular intervals along the cytoplasmic surface of the membrane. The cisternae of the rough endoplasmic reticulum are moderately distended with proteinaceus secretory product (arrow). Groups of polyribosomes possessing four or five ribosomes are lying free in the cytoplasm (arrows) and numerous bundles of tonofilaments (arrows) are also found, × 23 600. (b) The cytoplasmic matrix of the squamous epithelia cells of Bz treated rat contains important accumulation of polyribosomes as conglomerates (arrow). Polyribosomes with various configurations as helical (arrows) or rosette-like formations (arrows), exhibiting a larger number of ribosomes per polysome than control group (e.g. from 8 to 12) (Fig. 2b), × 23 600.

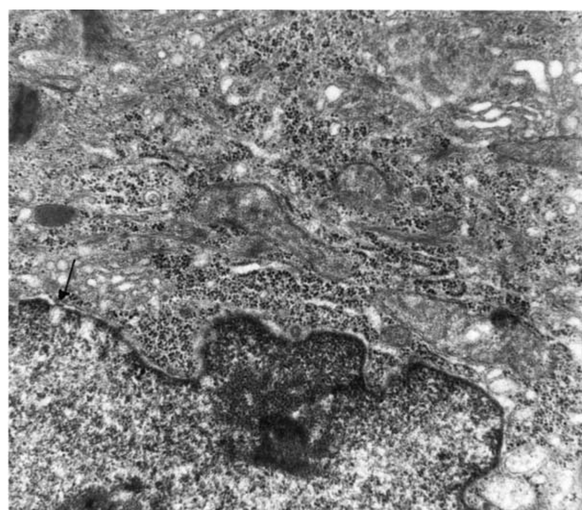
table to cytochrome P450 reductase and to a minor extent to cytochrome P450 and to other enzymes such as XOR or AO (Masana et al., 1984; Bernacchi et al., 1986; de Castro et al., 1989, 1992; Díaz et al., 2000; Walton and Workman, 1987).

These Bz nitroreductive processes producing reactive intermediates covalently binding to proteins or lipids or giving the corresponding Bz-amine occurred significantly in vivo (de Toranzo et al., 1984; Masana et al., 1985; Walton and Workman, 1987). That is, the Bz nitroreductive process despite being more intense under anaerobic conditions evidenced to also occur in normoxic tissues. The potential acetylation of Bz-amine or of the Bz-hydroxylamine produced in the gut bacteria or elsewhere via e.g. *N*-acetyltransferases (Levy and Weber, 2002) did not appear to play a significant role in Bz biotransformation. In effect no *N*-acetylated derivatives of Bz-amine were detected in urine of ^{14}C-Bz treated rats, dogs, monkeys or humans (Schwartz and Hofheinz, 1982) or in liver, kidney or blood from Bz-treated rats (Walton and Workman, 1987). This apparently contradictory behavior of Bz is not unique, other 2-nitroimidazole derivative, the well-known radiosensitizer misonidazole behave similarly (Cobb et al., 1990). In those misonidazole studies, the authors reported covalent binding of misonidazole nitroreductive metabolites in vivo to different tissues. The highest levels of covalent binding observed occurred in the esophageal epithelium when compared with those obtained for many other tissues.

In the case of esophageal tissue again, P450 reductase appeared to be responsible for most of the Bz nitroreductase activity as suggested by the potent inhibitory effect of DPI (Table 1). In effect, it is well known that DPI is a specific inhibitor of flavoenzymes and of P450 reductase as well (McGuire et al., 1998). The latter contains both, FAD and FMN prostetic groups in their molecules (Opsian and Coon, 1982) and that might be the reason for its susceptibility to DPI.

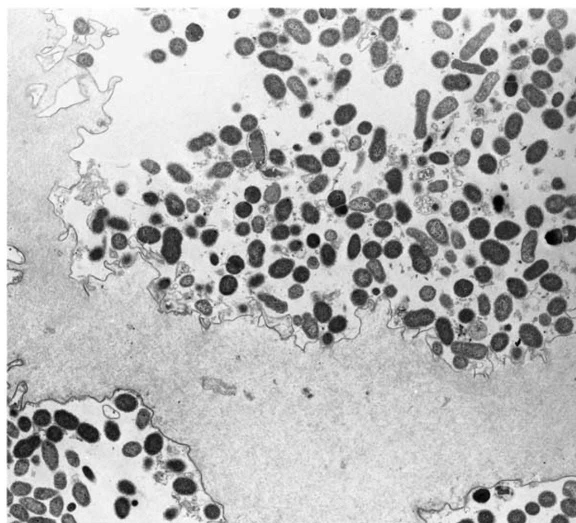


(a)

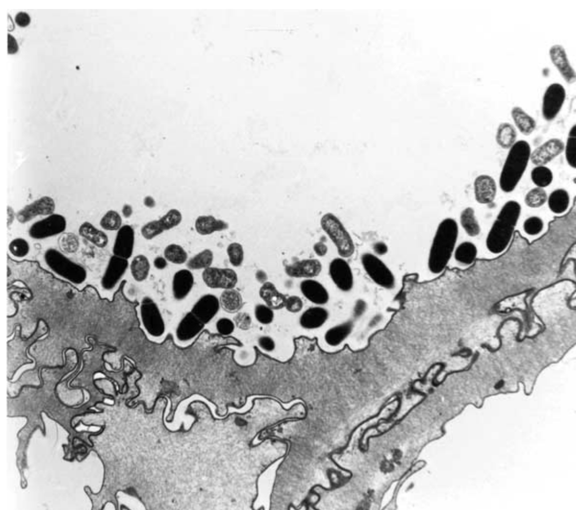


(b)

Fig. 3. (a) Esophageal mucosa of a control rat following CMC treatment. The nucleus has the nuclear envelope studded with many ribosomes. A prominent Golgi (arrow) and few mitocondria are seen. Desmosomes binding cells together show dense plaques with converging filaments on their cytoplasmic surfaces (arrow), × 15 000. (b) Few desmosomes are seen in the cytoplasm of the epithelial cell of Bz treated rat. Folded and invaginated nuclei showing profiles of pores with and without diaphragms (arrow) and numerous polyribosomes are also seen, × 17 500.



(a)



(b)

Fig. 4. (a) Esophageal surface covered with bacteria in control specimen, × 10 900. (b) Fewer amounts of bacteria are seen in esophageal lumen of Bz specimen, × 10 900.

A quantitatively less important proportion of the nitroreductive ability of the esophagus can be attributable to XOR as evidenced by the need of hypoxanthine as cosubstrate for the nitroreduction of Bz and by its susceptibility to allopurinol inhibitory effects (Table 1) (Frederiks et al., 1994). On the other hand, our histochemical studies have shown that XOR is present in the esophageal epithelial cells (Fig. 1).

The nitroreductive biotransformation of Bz is known to lead to reactive metabolites that bind

covalently to tissue constituents such as DNA, proteins and lipids. Those interactions would be the basis of its ability to cause cell injury and toxicity (Bernacchi et al., 1986; de Castro et al., 1989, 1992; Castro and de Toranzo, 1988; Castro, 2000; Díaz et al., 2000).

Our present ultrastructural studies showed alterations that involved different cellular components. In the Bz treated animals the epithelial esophageal cells have much more polyribosomes lying free in the cytoplasm than in controls. It is not clear to us whether this represents a detachment of polyribosomes previously present in the RER membranes or the result of a cell response to Bz insult. On one hand, previous studies from our laboratory evidenced that during Bz bioactivation in the endoplasmic reticulum reactive metabolites are formed which bind covalently to the lipids and proteins of their membranes (Masana et al., 1984). That could result in polysomal detachment from membranes. However, additional effects in polyribosomes were observed after Bz treatment. Not only the number of ribosomes per polyribosome was markedly increased but also in several instances they tended to exhibit a helical configuration. Further, conglomerates of many of them were also observed (Fig. 2). Alterations in polyribosomal structure of this type were previously reported in other pathologies (Ghadially, 1982).

The increased proportion of polyribosomes in the cytoplasm may reflect detachment of them from the RER as considered above but it could also indicate that the cell switched to increased synthesis of proteins for endogenous cellular needs (Ghadially, 1982). If that was the case the endogenous protein under synthesis should be some of larger molecular weight than usually synthesized in controls. The presence of abundant polyribosomes in the cytoplasm may additionally indicate active protein synthesis for cell growth and division (Ghadially, 1982).

The esophageal cells of Bz treated animals also exhibited alterations in their nuclei. In agreement with the possibility for increased cellular activity in the Bz treated group would be the observation of nuclear alterations in the epithelial esophageal cells. In effect, the nuclei from esophageal cells of the Bz treated animals exhibited irregular forms (Fig. 3). Previous studies from our laboratory evidenced that at least for the case of liver nuclei, Bz reactive metabolites are able to bind covalently to nuclear macromolecules and lipids (Gorla et al., 1986). At present, we do not know whether equivalent interactions occur in nuclei from esophageal cells and if they occurred, whether they would be related to observable ultrastructural effects.

Other interesting effect occurring in the esophageal epithelial cells from Bz-treated rats, was a significant reduction in the number of the desmosomes (Fig. 3). In general terms, it is known that desmosomes are particularly numerous and well developed in epithelia subjected to strong mechanical work (Ghadially, 1982) like esophageal epithelia. The observed decrease in their number in Bz treated animals might result from the deleterious effects of harmful Bz reactive metabolites produced in situ during Bz nitroreduction. This decrease in the number of desmosomes may imply a decreased mechanical resistance of the esophagus.

The observed bactericide effects of Bz on the microbiological flora close to the esophagus should not surprise (Fig. 4). Bacteria have nitroreductases able to nitroreduce and bioactivate Bz (de Toranzo et al., 1983). As it was previously observed for the case of radiation, the disappearance of bacteria from the esophagus neighborhood would be transitory and they would reappear after the Bz treatment is discontinued (Albertsson et al., 1987).

In summary, esophageal epithelial cells were found to bioactivate Bz to deleterious reactive metabolites via XOR and P450 reductase. As result of these processes covalent binding of Bz metabolites to cellular constituents would be produced and cause cell injury. Whether these observed deleterious effects of Bz on the esophagus might add or potentiate the cell injury already in course in the esophagus of people being in the intermediate stage of Chagas' disease remains to be an open question that deserves attention.

Acknowledgements

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