The ultrastructural effects and immunolocalisation of fumonisin B_1 on cultured oesophageal cancer cells (SNO)

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Numerous investigations have shown that fumonisin B_1 (FB_1) is the causal agent in a range of animal toxicities, including leucoencephalomalacia, pulmonary oedema and renal and hepatic cancer in rats and mice. Fumonisin B_1 has also been implicated in the aetiology of oesophageal cancer in South Africa. Human data are lacking, however, and the International Agency for Research on Cancer has accordingly classified this mycotoxin as a Type 2B carcinogen. This study investigated the ultrastructural effects of FB_1 cytotoxicity on a human oesophageal carcinoma cell line (SNO). The pathological changes induced by FB_1 were determined using transmission and scanning electron microscopy. Immunocytochemistry was used to immunolocalise FB_1 (monoclonal anti-FB_1) within the cells. The results showed marked pathological changes that included enlargement or microsegregation of the nucleus, microsegregation of the nucleolus, and swelling and elongation of mitochondria, as well as signs of membrane damage. These cytotoxic effects were associated with the action of FB_1, since the toxin was internalised in nuclei, mitochondria and the cytoplasm of affected cells. This study shows that FB_1 may exert its biological effects in SNO cells through binding to cellular macromolecules or membrane components within the affected organelles.

Key words: oesophageal cancer, fumonisin B1, cell culture, immunocytochemistry

Introduction

Cancer of the oesophagus (OC) follows the increasing incidence of cancer worldwide. There is a high incidence in the black population in certain parts of Transkei, South Africa and in parts of China;[1] both have increased in recent times.[1,2]

Maize is the staple food of the population of Transkei.[3] Fumonisins are mycotoxins produced by *Fusarium verticillioides* and other *Fusarium* fungi, found worldwide on maize and maize-based foods.[4,5] Maize from an area of high OC incidence in the Transkei contained higher levels of fumonisin B_1 (FB_1) (44 ppm) than did commercial maize meal (<10 ppm).[6]

Fumonisin B_1, a strongly polar compound,[5] is the most prevalent of the fumonisin mycotoxins.[7–10] The polarity of the toxin determines its level of carcinogenicity[11] i.e. the more polar the molecule, the greater the cytotoxic response. In addition to polarity, other determinants, such as the presence of a free amino group, carboxyl groups and the location of the hydroxyl group, could also affect the biological activity of these compounds. Thus, both the amino group and the intact molecule play an important role in the toxic and cancer-promoting activity of fumonisins.[8,12] This would be compatible with the association of FB_1 with both soluble and insoluble portions of the cell.[13]

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One of the established characteristics of fumonisin toxicity is its species specificity. A causal role of FB_1 in equine leucoencephalomalacia,[14] porcine pulmonary oedema,[15] and liver and kidney carcinoma in rats[16] has been reported. An initial study by Marasas *et al.*[17] showed that BD IX rats chronically exposed to *F. verticillioides* developed oesophageal hyperplasia, forestomach papillomas and carcinomas, hepatocellular carcinomas and cholangiocarcinomas. Several subsequent studies with laboratory animals in which cultures of *F. verticillioides* or fumonisins were fed found no signs of cancerous or precancerous lesions of the oesophagus,[16,18,19] however. Thus, there is no consistent animal model to support the theory that FB_1 may be related to human OC.

In human health, the role of fumonisins is still unclear,[12] but the consumption of *Fusarium*-contaminated maize has been correlated with human OC in areas of South Africa, China and other countries.[20,21] The high incidence of OC in the Transkei has been demographically associated with a prevalence of FB_1-contaminated corn.[22,23] Although other factors, such as smoking, alcohol consumption and certain dietary and environmental components could be involved in the aetiology of the disease, several recent studies have implicated fumonisins as a possible contributing factor. Nitrosamines or other carcinogenic agents may be responsible for the increased incidence of OC in humans, with fumonisins contributing to the problem through their potent tumour-promoting activity.[24] In a previous study,[25] we showed that FB_1 (2–34 μM), a type 2B carcinogen, was cytotoxic to cultured SNO oesophageal cancer cells. We speculate that FB_1 would alter organelle ultrastucture in cultured SNO human oesophageal cells.

In this study, the SNO epithelial cell line (cells that retain most of the functions associated with primary cells), derived from a well-differentiated squamous cell carcinoma explanted from a 62-year old indigenous black male,[26] was used to determine the effects of FB_1 on cellular ultrastructure. The pathological changes induced by FB_1 were determined using transmission electron microscopy. The cells were then immunocytochemically probed for the presence of FB_1.

Materials and methods

Reagents

Fumonisin B_1 (98%) was purchased from Sigma (Johannesburg, South Africa). A monoclonal FB_1 antibody was purchased from Neogen (Michigan, U.S.A.). All other immunochemicals were purchased from Sigma (Johannesburg, South Africa). Cell culture media and disposables were purchased from Adcock Scientific (Durban, South Africa). All HPLC and other chemicals were purchased from Merck (Johannesburg, South Africa).

Maintenance of the SNO cell line

The SNO epithelial cells[26] (a well-differentiated squamous carcinoma line) were grown and maintained in Eagle's minimum essential medium (EMEM) containing 0.25 mM Hepes

buffer supplemented with 5–10% foetal calf serum (FCS), 1% L-glutamine and 1% penstrep fungizone (complete culture media). The cells were maintained in a 37°C incubator and grown to confluency (25 cm^2 flasks) before use.

Transmission electron microscopy

Confluent 25 cm^2 flasks of SNO cells were treated with FB_1 at concentrations of 1 μM, 2 μM, 4 μM, 8 μM, 16 μM or 32 μM for 24 h; these concentrations were in keeping with a previous *in vitro* study.[25] Flasks containing untreated cells served as controls. After a 24-h incubation period, the treatment medium was removed and stored for subsequent HPLC analysis. The cells were washed twice with Hanks' balanced salt solution (HBSS) (3 ml), and the cells were then fixed with 1% glutaraldehyde in HBSS for transmission electron microscopy (TEM) and immunocytochemistry (ICC). After a 30-min fixation period, the epithelial cells in flasks were washed twice with HBSS (5 ml), and then processed for microscopy. Briefly, cells were post-fixed in 1% osmium tetroxide in distilled water (4°C) (omitted for flasks used for immunocytochemistry) and then dehydrated with increasing strengths of alcohol (70%, 90%, 100%) for 15 min each.

Flasks containing SNO cells were infiltrated with Spurr's resin (2 × 60 min) and then left to polymerise at 60°C for 24 h. The blocks, which comprised cells sandwiched between the resin and plastic flasks, were trimmed and vertically sectioned on a Reichert Ultracut microtome. Sections (0.5 μm) were picked up on 200 mesh copper grids, stained with uranyl acetate in alcohol:water (1:1), counterstained with Reynold's lead citrate, and viewed and photographed using a JEOL-JEM 100S transmission electron microscope. Sections (0.5 μm) were also picked up on 200 mesh nickel grids and probed immunochemically for FB_1.

Immunocytochemistry

Non-osmicated sections on 200 mesh nickel grids were etched to block endogenous peroxidase activity by placing in 5% H_2O_2 (20 μl, 5 min) and then washed in distilled water (10 ml), and finally drained on fibre-free paper. The sections were incubated in normal goat serum diluted 1:20 in 50 mM Tris (pH 7.2) for 30 min, in order to block non-specific binding sites, before placing them in the primary antibody (monoclonal mouse anti-FB_1 diluted 1:100 in 50 mM Tris with 0.2% bovine serum albumin (BSA), pH 7.2) for 3 h. Grids were washed in 50 mM Tris (pH 7.2), 50 mM Tris containing 0.2% BSA (pH 7.2), and finally in 50 mM Tris containing 1% BSA (pH 7.2). The secondary antibody used was goat anti-mouse IgG conjugated to a 10 nm gold probe (1:15 in 50 mM Tris with 1% BSA, pH 7.2) to localise FB_1. The grids were thoroughly washed in 50 mM Tris 0.2% BSA (pH 7.2), 50 mM Tris (pH 7.2) and finally in distilled water. Untreated cultured cells served as the negative controls. The grids were stained with 1% uranyl acetate, counterstained using Reynold's lead citrate and then viewed using the Joel JEM 100 TEM.

Method controls (in which the primary antibody was omitted and replaced with PBS, pH 7.4) were used to determine the method specificity. This results in the exclusion of staining caused by mechanisms other than the immunological interactions between the primary antibody and antigens(s).

Scanning electron microscopy

The SNO cells were grown on 20 mm^2 coverslips in six-well plates until confluency. The cells were then treated with FB_1 (at the same concentrations as those used for TEM), fixed, post-fixed and dehydrated as described for TEM. Coverslips were then critical point-dried, coated to 10 nm thickness with gold particles, and viewed using a Hitachi S520 scanning electron microscope.

Results and discussion

SNO epithelial cells in culture have to be firmly attached to the substrate on which they are grown. Such anchorage-dependence was evident in untreated control cells where the cellular processes were well defined (Fig. 1a). In FB_1-treated cells (Figs 1b–d), there was a retraction of cellular processes that was more pronounced with increasing concentrations of the toxin; this may have been associated with the progression towards cell death. Treatment of SNO cells with 4 μM and 8 μM FB_1 resulted in blebbing or vesiculation of the plasma membrane (Figs 1b and 1c). The treated cells broke up into smaller bodies with no swelling (Figs 1b and 1c). Blebbing of the plasma membrane into smaller membrane-bound like apoptotic bodies is a common structural feature of apoptotic cells.[28,29] Thus, at the lower concentrations of FB_1, apoptosis was the likely mechanism of cell death, since the integrity of the plasma membrane was maintained. At higher concentrations of FB_1 (16 μM), the SNO cells exhibited gross pathology such as swelling (Fig. 1d); the plasma membrane is a target of FB_1 and may therefore have been the major site of damage.[30] Loss of membrane integrity prevents cells from regulating osmotic pressure, causing them to swell and rupture (Fig. 1d). Since necrosis is the death of cells through external damage, usually mediated via the destruction of the plasma membrane or the biochemical supports of its integrity,[31] it is likely that necrosis was the mechanism of death at higher concentrations of FB_1. Loss of cells due to cell death mechanisms (apoptosis or necrosis) may initiate a compensatory mitosis that could contribute to the onset of cancer.[32,33]

Transmission electron microscopy revealed that untreated SNO cells contained several Golgi bodies and mitochondria (Fig. 2a); a single large prominent nucleolus was present in the nucleus (Fig. 2b). Nuclear and nucleolar alterations were some of the toxic effects in FB_1-treated SNO cells. Nuclear alterations of FB_1-treated cells included enlargement, irregular profiles, and invagination of the nuclear membrane that often led to microsegregated nuclei (Fig. 2c). Nucleolar effects included an increase in the size and number of nucleoli (Fig. 2c). Mitochondria were elongated (Fig. 2d) and/or had swollen cristae (Fig. 2e). Endoplasmic reticuli (ER) were more abundant and some cisternae of ER were vesiculated (Fig. 2e) or swollen (Fig. 2f). Other cellular alterations of FB_1-treated cells included the presence of numerous and occasionally swollen cellular processes (Fig. 3d).

Using ICC, FB_1 was identified by the presence of 10 nm gold probes. Both the untreated SNO cells and method controls showed no positivity for FB_1, whilst gold-labelled FB_1 was immunolocalised in the treated cells (Figs 3a–d). Fumonisin B_1 was present in the cytoplasm (Fig. 3a). Within nuclei of FB_1-treated cells (Fig. 3b), label was associated with the nuclear membrane, nucleoplasm and nucleolus. There was an abundance of FB_1 in mitochondria that showed pathological changes (Fig. 3c), but FB_1 label was also localised to a limited extent in mitochondria that appeared normal. Label also was present within swollen processes or membrane blebs of FB_1-treated cells (Fig. 3d).

The presence of the gold probes within the cytoplasm, mitochondria and nucleus of treated cells (Figs 3a–c) shows that FB_1 gained entrance into the cell. Studies with ^{14}C-labelled FB_1

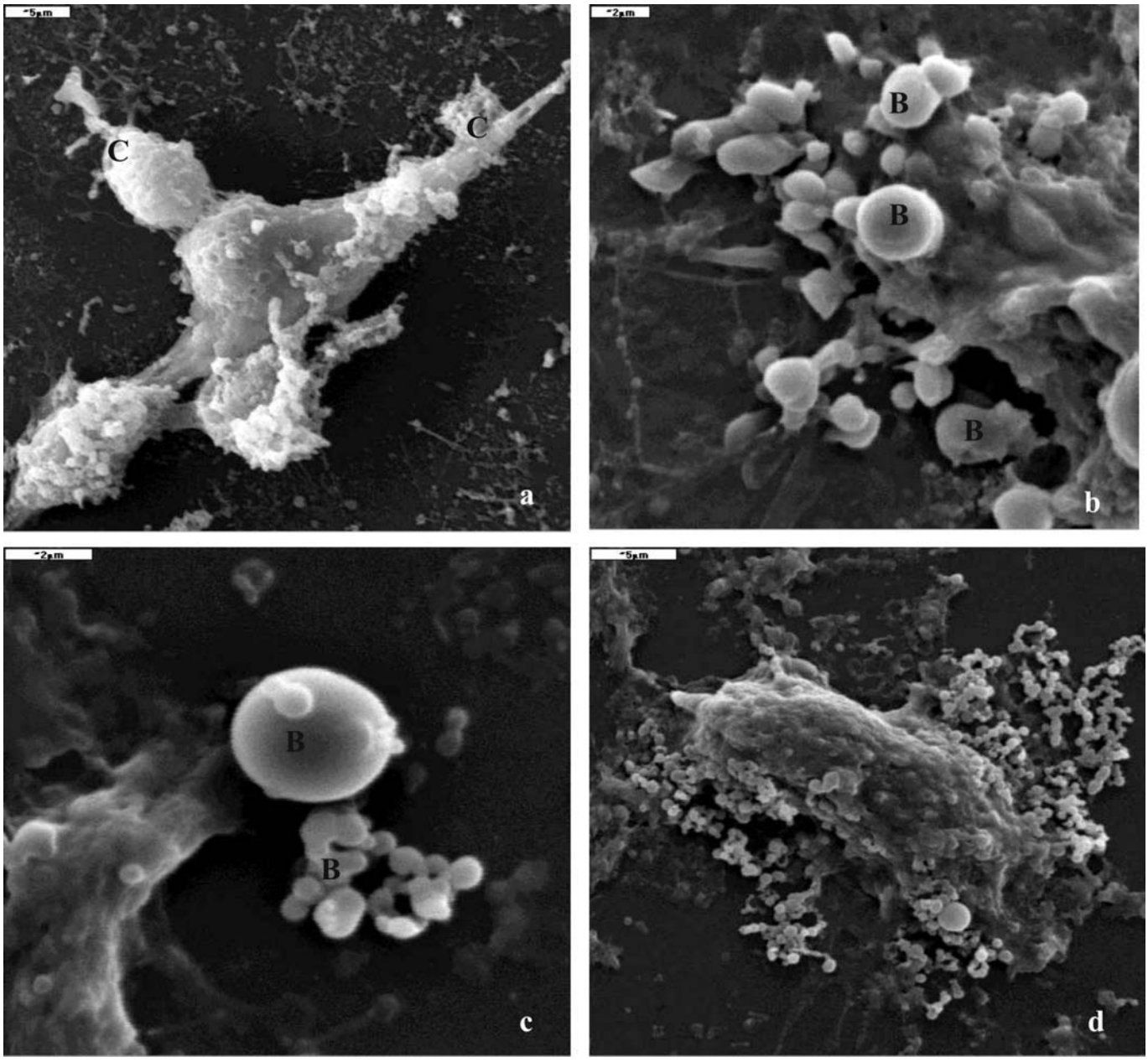

Fig. 1. The effects of FB_1 on SNO cells as shown by SEM. **a**, Control cells were anchored to the coverslip by well-defined cellular processes (C). **b**, **c**, Treatment of cells with increasing concentrations of FB_1 (B = 8 μM, C = 16 μM) resulted in retraction of cellular processes and blebbing of the plasma membrane (B). **d**, Cells treated with 32 μM FB_1 were rounded and the cellular processes were almost totally retracted.

conducted by Cawood *et al.*[13] showed that FB_1 bound tightly to microsomes and plasma membranes of rat liver after 1 h incubation, and FB_1 remained in the membranes even after extensive washing of these fractions. Fumonisin B_1 is a highly polar molecule consisting of four carboxyl groups, one amine group and several hydroxyl groups. Even allowing for complete chelation of the carboxyls with elements such as calcium, it does not seem possible that FB_1 could freely permeate membranes. Two possibilities exist for the passage of FB_1 through the cell membrane, namely, that a trans-membrane transport system existed or that it was metabolically modified to allow permeation. Because of the unusual structure of FB_1, it is tempting to suggest that it entered the cell via some sort of endocytic process, possibly mimicking sphingolipid-type membrane-binding agents. Continuity between the outer membrane of the nuclear envelope and the sacs of the ER were frequent, presumably providing FB_1 access to the nucleus and nucleolus. Alternatively, FB_1 in the soluble portion of the cell may have diffused into the nucleus via the nucleopore, bearing in mind the strong resemblance of the toxin to membrane lipids.

The higher levels of FB_1 found in nuclei and nucleoli in this study correlated well with the ultrastructural observation that FB_1 targeted the nucleolus, resulting in enlargement and microsegregation of the nucleolus. In addition, some of the ultrastructural alterations observed, particularly swelling of organelles, were manifestations of membrane damage, which may have been caused by the action of FB_1 on these cells. Cellular membranes are postulated to be one of the principal targets for the fumonisins *in vivo*.[30] Fumonisin B_1 has been shown to exert its effects through a disruption in sphingolipid metabolism.[34,35] Fumonisin B_1 is a competitive inhibitor of the enzyme, ceramide synthase, which catalyses the acylation of sphinganine (Sa) in the *de novo* biosynthesis of sphingolipids and the

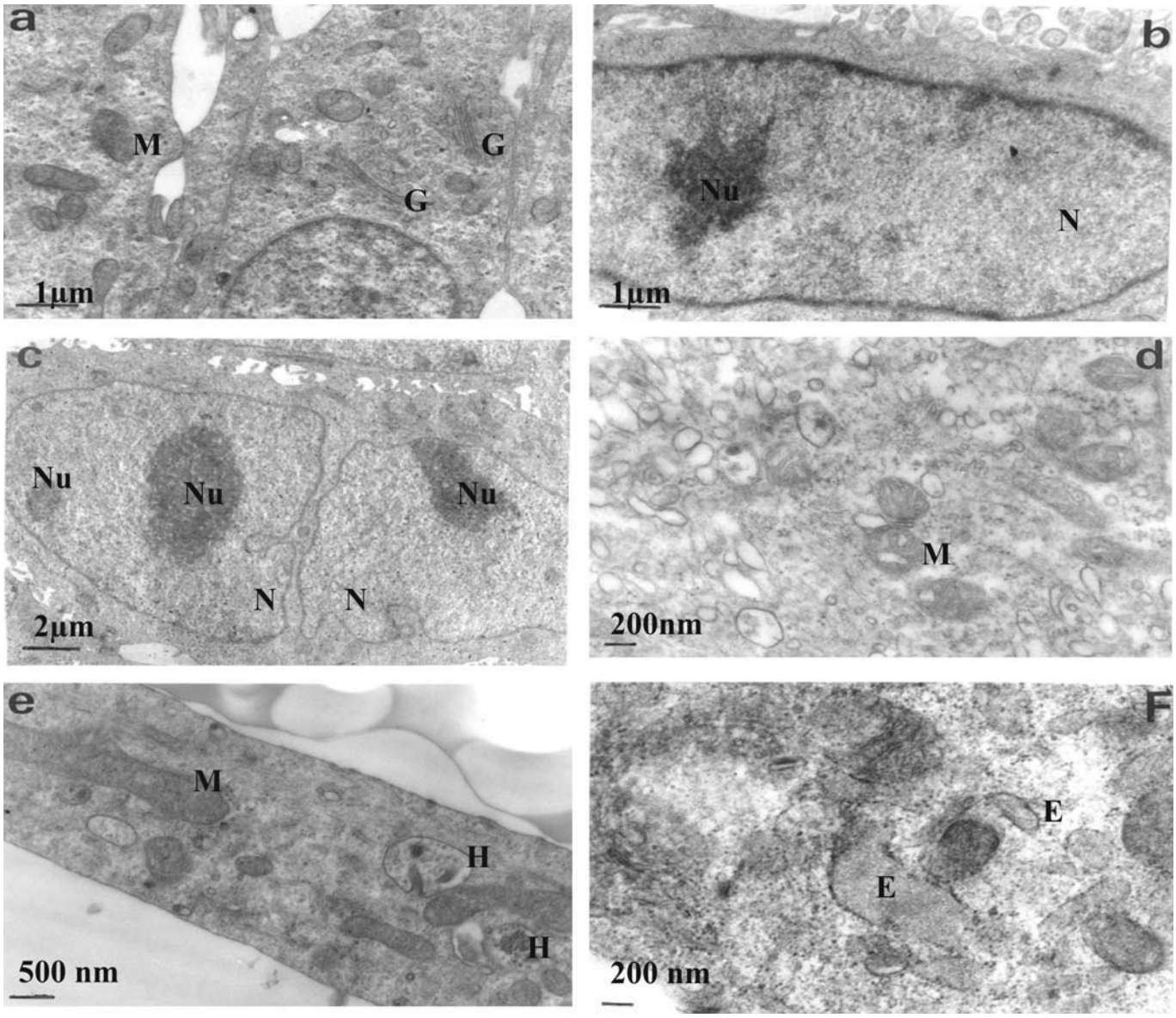

Fig. 2. **a**, Several Golgi bodies (G) were present in untreated SNO cells. Mitochondria (M) and ER showed normal morphology. **b**, An untreated epithelial cell containing a large nucleus (N) with a single prominent nucleolus (Nu). **c**, Microsegregated nucleus (N) and nucleolus (Nu) in an FB_1-treated cell (16 µM). Distinct granular and fibrillar components can be seen. **d**, Mitochondria (M) with swollen cristae in an FB_1-treated cell (16 µM). **e**, An increased number of heterophagosomes (H) were present in FB_1-treated cells (16 µM). Mitochondria (M) were elongated and showed signs of membrane damage. **f**, Cisternae of ER (E) were swollen in some of the toxin-treated SNO cells (16 µM).

re-utilisation of sphingosine (So) derived from sphingolipid metabolism.[36] As a result, FB_1 causes an increase in the amount of free Sa and a decrease in the formation of complex sphingolipids such as So and ceramide.[37] Complex sphingolipids have been implicated in cell–cell interactions.[34] Therefore, a blockage of *de novo* sphingolipid synthesis might weaken intercellular interactions and make membranes leaky. This would allow increased penetration of plasma components into underlying tissues.[38]

Fumonisin B_1 has also been implicated in the disruption of a variety of cellular responses including mitogenesis[39] and cytotoxicity.[40] Other *in vitro* investigations have demonstrated that FB_1 inhibited cell proliferation and induced either cell necrosis or apoptosis in SNO cells,[25] LLC-PK_1 cells,[40] cultured turkey lymphocytes,[41] chicken macrophages[42] and CV-1 African green monkey kidney cells.[43] A 4-h exposure of FB_1 caused significant cytoplasmic blebbing and varying degrees of nuclear disintegration *in vitro*.[42]

Long-term studies on FB_1 exposure in rodents[32] and nonhuman primates[33] showed no oesophageal lesions in either animal model. In our indigenous population, however, maize is the staple diet and often heavily contaminated maize (maize not suitable to be milled into flour) is brewed into a traditional beer. This coupled to chronic FB_1 exposure, alcohol consumption, smoking, nitrosamines and scalding hot food or drinks may all be aetiological agents in OC in South Africa.

Conclusion

The cellular pathology observed suggests that FB_1 specifically targeted mitochondria, the nucleus and nucleolus in SNO cells. The high levels of label (gold probes) found in these organelles further suggests that ultrastructural alterations occurred as a result of FB_1 toxicity. The known mechanism of FB_1-induced toxicity is through disruption of sphingolipid metabolism. It may, however, also be possible that FB_1 exerts its biological effects through binding to macromolecules in these organelles.

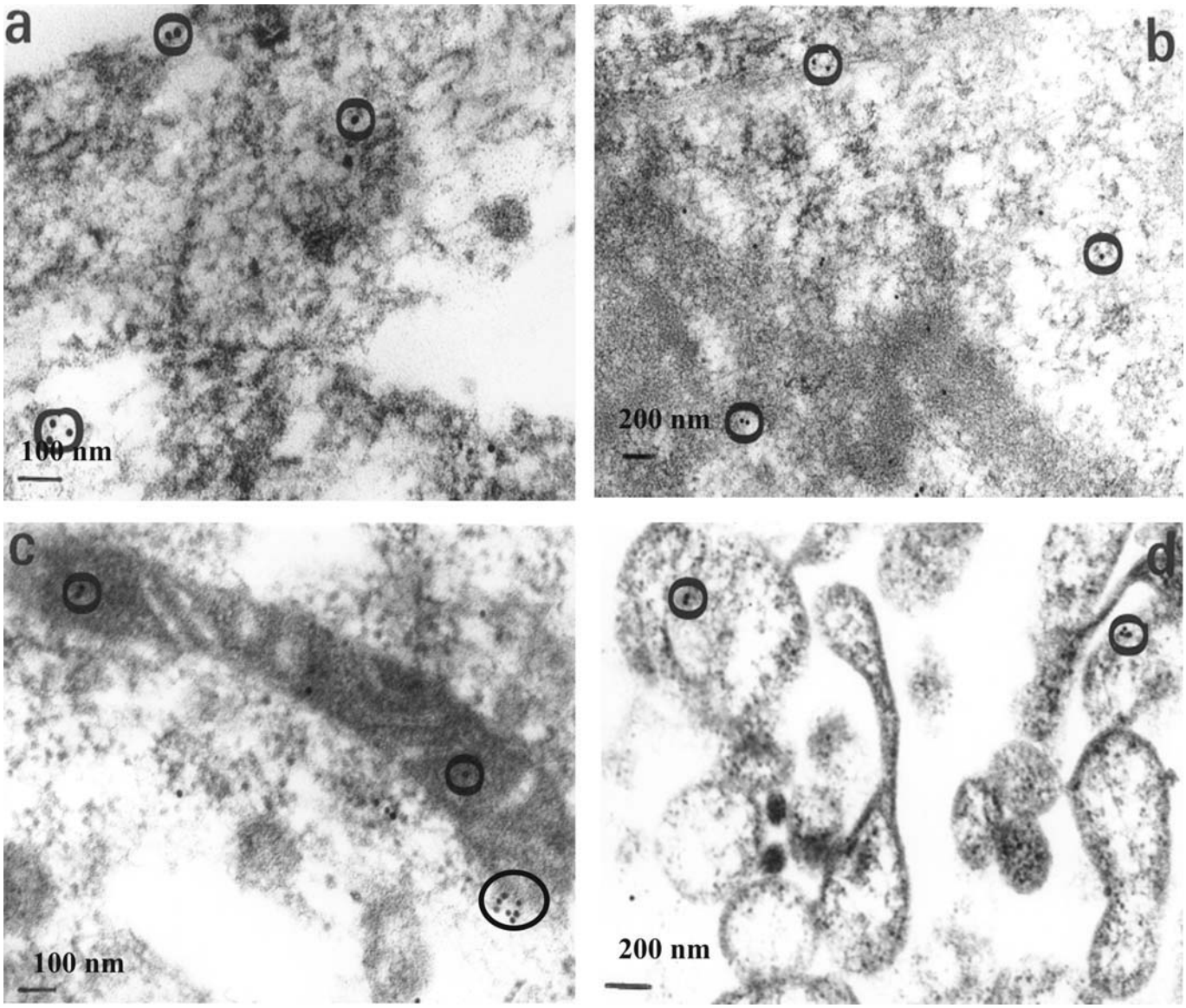

Fig. 3. **a**, Gold-labelled FB_1 was present in the cytoplasm of 16 µM-treated cells. **b**, Large quantities of gold label (circled) were present in the nucleolus and nucleoplasm of FB_1-treated cells. **c**, Positively-stained cells containing FB_1 in an elongated mitochondrion. **d**, Membrane blebs that detached from the cell contained labelled FB_1.

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