Comparative effect of osmium tetroxide and ruthenium tetroxide on *Penicillium* sp. hyphae and *Saccharomyces cerevisiae* fungal cell wall ultrastructure

Orlando Torres-Fernández 1, Nelly Ordóñez 2

1 Laboratorio de Microscopía Electrónica, Instituto Nacional de Salud, Bogotá, D.C., Colombia.
2 Laboratorio de Patología, Instituto Nacional de Salud, Bogotá, D.C., Colombia.

The fungal cell wall viewed through the electron microscope appears transparent when fixed by the conventional osmium tetroxide method. However, ruthenium tetroxide post-fixing has revealed new details in the ultrastructure of *Penicillium* sp. hyphae and *Saccharomyces cerevisiae* yeast. Most significant was the demonstration of two or three opaque diverse electron dense layers on the cell wall of each species. Two additional features were detected. *Penicillium* septa presented a three-layered appearance and budding *S. cerevisiae* yeast cell walls showed inner filiform cell wall protrusions into the cytoplasm. The combined use of osmium tetroxide and ruthenium tetroxide is recommended for post-fixing in electron microscopy studies of fungi.

**Key words:** fungal cell wall, fungal ultrastructure, osmium tetroxide, ruthenium tetroxide, *Penicillium* sp., *Saccharomyces cerevisiae*.

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The two-step fixing procedure, glutaraldehyde followed by osmium tetroxide (OsO_4_) (1), continues to be the preferred method for preserving fine structures in biological samples for electron microscopy studies. Although ruthenium tetroxide (RuO_4_) was proven effective as an alternative post-fixative (2), this reagent decomposes rapidly and has poor tissue penetration (3,4). However, RuO_4_ is an excellent fixative for revealing the existence of some non-OsO_4_-fixed cellular compounds. RuO_4_ has been used mainly to visualize extracellular saturated lipids in ultrastructural studies of stratum corneum from skin. These lipids constitute the dermal permeability barrier (5,6).

The ability of RuO_4_ to preserve previously unknown ultrastructural details in microsporidian (Protozoa) spores has been demonstrated recently. The
Microsporidian spore wall consists of a chitin-protein complex compound with saturated lipids probably embedded in it (7,8). Microsporidia and fungi are structurally and phylogenetically related microorganisms (9). The microsporidian spore wall and the fungal cell wall, viewed by electron microscopy, appear to be transparent when post-fixed by the conventional osmium tetroxide method. Since post-fixation with RuO₄ reveals new microsporidian spore wall details and establishes distinct ultrastructural differences among microsporidian species (7,8), it possibly also will reveal new ultrastructural features of the fungal cell. The present study was designed to evaluate the comparative effect of OsO₄ and RuO₄ post-fixing on fungal ultrastructure, particularly on their cell walls.

The fungal cell wall is a very important constituent and protective structure (10,11). The wall, more than any other cellular organelle, taxonomically defines fungi and distinguishes them from other organisms. Knowledge of fungal cell wall structure is of interest for taxonomy (12) and in the search for potential target sites for new chemotherapeutical agents (13).

Materials and methods

Saccharomyces cerevisiae yeasts, maintained in distilled water at 4 ºC for several weeks, and Penicillium sp. mycelia, growing on decaying tangerine, were used for this study. For the electron microscopy of yeasts, a yeast pellet was obtained by centrifugation and processed by dehydration and embedding, with centrifugation after each step. Mycelial mats were processed including the whole plant substrate. All fixatives were buffered in 0.13 M phosphate buffer (14). The same buffer was used for rinsing after each fixing step. The samples were first fixed for 24 hours in 3% glutaraldehyde, then washed for 12 hours. Three groups of samples from each fungal species were post-fixed separately in the following solutions: 1) 1% OsO₄, 1 hour; 2) 0.2% RuO₄, 1 hour; 3) 1% OsO₄, 1 hour followed by 0.2% RuO₄, 1 hour. After OsO₄ or RuO₄ post-fixing, the samples were washed several times for 2 hours. They were then dehydrated in a graded ethanol series (50, 70, 96 and 100%) 20 min/change, and infiltrated with resin:solvent (Epon-Spurr: propylene oxide) mixtures (1:2; 1:1; 2:1) at 12 hour intervals until embedding in pure resin Epon-Spurr mixture (15). Embedded capsules were then polymerized at 68 ºC for 48 hours. Thin sections were stained with uranyl acetate and lead citrate and then examined with a Zeiss EM 109® electron microscope. Representative thin sections were observed unstained.

Results

Both hyphae and yeasts exhibited a different appearance after OsO₄ or RuO₄ post-fixing. The fungal OsO₄ post-fixed cell wall showed an electron-lucent thick band sometimes bordered by a thin electron-dense layer (figures 1, 3, 5, 7). RuO₄ or OsO₄ followed by RuO₄ changed the fungal cell wall’s ultrastructural image, both on hyphae (figures 2, 6) and yeasts (figures 4, 8-12). RuO₄ post-fixing revealed fungal cell walls with two or three graded electron-dense layers (figures 2, 4, 6). Two other prominent features were observed. First, septa of Penicillium hyphae appeared as three layers composed of two grey layers separated by a translucent layer. The grey layers were seen as an invagination of the cell wall’s inner layer (figure 2). The second feature concerned filiform protrusions emerging from the inner layer of budding S. cerevisiae yeasts’ cell walls, and intruding into the cytoplasm (figures 4, 12). These protrusions seemed to originate from plasma membrane invaginations (figures 9-11) which were then penetrated by cell wall material. The cell wall showed a more homogeneous appearance in the double post-fixed samples (OsO₄ plus RuO₄), indicating that the internal fungal cell ultrastructure was better preserved. In budding yeasts, however, the cell wall protrusions were not always distinguishable from the cytoplasm (figure 8).

Discussion

The apparent electron transparencies on the fungal cell wall may be due to deficient post-fixing by the conventional OsO₄ method. The polysaccharides chitin and glucan are the main fungal cell wall components (10,11). Because RuO₄ is a stronger oxidizing agent than OsO₄ (3,4), it probably reacts better with polysaccharide hydroxyl groups (7). The fungal cell wall also contains saturated fatty acids that perhaps confer hydrophobicity (10,12). RuO₄ post-fixing revealed saturated lip-
Figures 1 and 2. Cell walls and septa of *Penicillium* sp. hyphae. Figure 1. OsO₄ post-fixed specimen: the cell wall and the septum are transparent. Note the border (arrows). Scale bar, 0.4 µm. Figure 2. RuO₄ post-fixed specimen: the cell wall and the septum show a three-layered image. W: cell wall; S: septum. Scale bar, 0.3 µm.

Figures 3 and 4. *Saccharomyces cerevisiae* yeasts. Figure 3. OsO₄ post-fixed yeast: the cell wall is translucent. Scale bar, 0.5 µm. Figure 4. Part of a budding yeast post-fixed with RuO₄: the cell wall is electron-dense. Note the filiform protrusions (P) intruding into the cytoplasm. W: cell wall. Scale bar, 0.2 µm.
**Figures 5 and 6.** *Penicillium* sp. hyphae. **Figure 5.** OsO$_4$ post-fixed hyphae showing electron-transparency of the cell wall and septum. Scale bar, 1.5 µm. **Figure 6.** RuO$_4$ post-fixed specimen showing electron-dense cell wall. Scale bar, 0.8 µm.

**Figures 7 and 8.** Budding yeasts of *S. cerevisiae*. **Figure 7.** OsO$_4$ post-fixed yeast displaying electron-lucent cell wall. Scale bar, 0.5 µm. **Figure 8.** Yeast post-fixed by OsO$_4$ followed by RuO$_4$ displaying electron-opaque cell wall. Note the Woronin body (arrow). Scale bar, 0.6 µm.
Figures 9 and 10. Cell wall of a *S. cerevisiae* yeast post-fixed with RuO₄. Note the beginning of plasma membrane invaginations (arrows). Figure 9. Scale bar, 0.3 µm. Figure 10. Scale bar, 0.2 µm.

Figures 11 and 12. Budding yeasts of *S. cerevisiae* post-fixed with RuO₄. Figure 11. The filiform protrusions (arrows) emerge from the cell wall. Scale bar, 0.6 µm. Figure 12. The filiform protrusions (P) emerge from a thicker cell wall. Scale bar, 0.5 µm.
ids which OsO₄ did not (5,6). The electron opacity observed on the fungal cell wall following post-fixing with RuO₄ therefore may be explained by the ability of this fixative to react with polysaccharides and saturated lipids. Differences in the electron density in each of the cell wall layers suggested different polysaccharide and lipid distributions.

Other electron microscopy methods have previously been used for fungal cell wall staining. Candida albicans yeasts have shown a two-layered electron-dense cell wall in samples fixed with glutaraldehyde-osmium tetroxide and stained using the periodic acid-thiocarbohydrazide-Ag-proteinate (PATAg) technique (16). PATAg is a specific cytochemical method for revealing polysaccharides by electron microscopy (17). In other studies, KMnO₄ fixing has produced light electron-density staining in the wall of young cells from some fungal species (18,19). Recently, Ustilago maydis hyphae, fixed with KMnO₄ followed by OsO₄, were shown to have cell walls with several distinct layers with differing electron densities. This treatment stained only the outer surface layer of yeast cell walls, which is not the case with mycelial walls from the same species (20). Several authors have obtained sharp ultrastructural yeast images by conventional methods but these images do not show adequate contrast in the cell wall (21-23). On the other hand, the yeast cell wall post-fixed with OsO₄ was stained after enzyme treatment (24,25); however, this procedure requires removing carbohydrates from the cell wall and proteins within the cell (25).

High fungal cell wall contrast has been obtained with the method described here. The results agree with previous descriptions of the microsporidian spore wall (7,8). RuO₄ penetration of the mature cell wall revealed three-layered Penicilli um septa. This method also enabled visualization of the filiform cell wall protrusions that extend into the cytoplasm in budding S. cerevisiae. Previously, these ultrastructural features had been unnoticed. The filiform cell wall protrusions seem to originate in the plasma membrane invaginations which are then penetrated by the cell wall material. Other authors have reported that RuO₄ reacts with superficial polysaccharide fibrils from reverting yeast proto-plasts without penetrating intact cells (4,26). A recent review on preparation of yeasts cells for transmission electron microscopy includes OsO₄ and KMnO₄ but not RuO₄ post-fixing (25). Our results demonstrated RuO₄ reactivity with intact and mature fungal cell walls. Post-fixing procedures, including RuO₄ combined with OsO₄, are therefore recommended for ultrastructural studies of clinically important fungi.

References


