Assessing Efficacy of Clinical Disinfectants for Pathogenic Fungi by Single-Cell Raman Microspectroscopy

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Disinfectants are crucial for root canal therapy (RCT), as metabolism of canal-inhabiting microbes can cause refractory infections. To develop effective yet patient- and environment-friendly disinfectant formulations, we quantitatively assessed the metabolism-inhibiting effects of intracanal disinfectants via D2O-probed Single-Cell Raman Spectra (SCRS), using Candida albicans (C. albicans) as a pathogen model. For chlorhexidine gluconate (CHX), sodium hypochlorite (NaClO), and hydrogen peroxide (H2O2), at their MIC of 4, 168, and 60 mg/ml, respectively, despite the complete growth halt, metabolic activity of individual fungal cells was reduced on average by 0.4%, 93.9%, and 94.1% at 8 h, revealing a "nongrowing but metabolically active" (NGMA) state that may underlie potential refractory infections, particularly for CHX. In contrast, at their Metabolic Activity-based Minimum Inhibitory Concentrations (MIC-MA) of 8, 336, and 120 mg/ml, respectively, metabolic activity of all cells was completely halted throughout 8 h exposure. Moreover, combined use of NaClO+H2O2 (combination at 0.5× MIC-MA each) outperforms solo uses of CHX, NaClO, H2O2, or other binary combinations. Furthermore, dynamics of SCRS revealed distinct fungicidal mechanisms of CHX, NaClO, H2O2, and their pairwise combinations. MIC-MA is advantageous in critically assessing antifungal efficacy, and NaClO+H2O2 can potentially serve as a more efficient disinfectant formula for fungal pathogens.

Keywords: heavy water, single-cell technology, Candida albicans, chlorhexidine gluconate, sodium hypochlorite, hydrogen peroxide
INTRODUCTION

Microbial infections in the pulp and periapical tissues could cause pulpitis, apical periodontitis, or even persistent inflammatory reaction. In these endodontic infections, pathogenic fungi such as Candida albicans (C. albicans) are the most frequently isolated eukaryotes (Siqueira and Sen, 2004; Kumar et al., 2015). In particular, C. albicans can readily form biofilms (Alshanta et al., 2019) colonize dentinal walls, and penetrate into dentinal tubules (Siqueira et al., 2002), resulting in persistent infections (Mergoni et al., 2018). Therefore, one key goal of root canal treatment (RCT) is to control and prevent microbial infections in the intracanal areas.

During RCT, mechanical debridement by hand and rotary instruments can leave 35% or more surface area of canals untouched (Tomson and Simon, 2016). Therefore, the use of liquid intracanal disinfectants such as chlorhexidine gluconate (CHX), sodium hypochlorite (NaClO), and hydrogen peroxide (H₂O₂) to ensure thoroughness of the intricate debridement of liquid intracanal disinfectants such as chlorhexidine gluconate untouched (Tomson and Simon, 2016). Therefore, the use of disinfectants is to control and prevent microbial infections in the intracanal areas. Thus, the correct choice of type, concentration, duration, or combination of intracanal disinfectants is required.

An ideal intracanal disinfectant should be effective in inhibiting the growth of all microbial cells, capable of dissolving pulp tissue remnant and smear layer, and, moreover, nontoxic or nonallergic (Galler, 2016). However, frequently, these desirable features are mutually exclusive: e.g., the more potent pathogen-inhibitory effect can be linked to more severe side effects to host tissues (Gomes-Filho et al., 2008). Therefore, the choice of type, concentration, duration, or combination of intracanal disinfectants is often based on empirical notion, due to the inability to rapidly assess the interaction between disinfectants and microbial cells (Chong and Pitt Ford, 1992; Rahimi et al., 2014). Therefore, there is an urgent need for method development to tackle this challenge.

Current methods that assess efficacy of disinfectants and other antimicrobials can be broadly classified as “growth-based” or “non-growth-based”. Growth-based methods typically examine the sensitivity of cell growth curve to antimicrobial via dilution and diffusion, and quantitative parameters such as “minimum inhibitory concentration” [MIC, i.e., the lowest drug concentration under which microbial growth is entirely inhibited (Brauner et al., 2016)] are then derived. As growth inhibition does not necessarily correlate with metabolic inhibition or cell death, the growth-based methods are usually unable to distinguish between bactericidal and bacteriostatic effects and consequentially fail to detect “non-growing but metabolically active” (NGMA) cells (Tao et al., 2017), which are responsible for many latent or recurring infections (due to their ability to resume growth after removal of antimicrobials) and eventually lead to treatment failure (Manina and McKinney, 2013). Moreover, these methods can be time-consuming (frequently exceeding 24 h for fast-growing pathogens as extended duration of drug exposure to detect growth changes is required).

Rather than assessing the efficacy of antimicrobials based on “growth” inhibition, we recently introduced D₂O-probed Single-cell Raman Microspectroscopy (D₂O-Ramanometry), which can serve as a quantitative yet universal method to detect and measure metabolic-activity change of cells in response to drug treatments at the single-cell resolution (Teng et al., 2016; Tao et al., 2017; Bauer et al., 2020). Specifically, we proposed “Minimum Inhibitory Concentration based on Metabolic Activity” (MIC-MA), defined as the minimal dose under which the median D⁳⁰O-D-ratio at 8 h of drug exposure is ≤0 and the Standard Deviation (SD) of the ΔC-D ratio among individual cells is ≤0.005, to evaluate the metabolism-inhibiting efficacy of antimicrobials (Tao et al., 2017). However, this method and concept have not been tested in pathogenic fungi or for disinfectants; thus, it is unclear whether and how such metabolic-activity-based fungus-disinfectant interaction, including its intercellular heterogeneity, can be quantitatively accessed and screened for rational development of efficient disinfecting formula.

To tackle this challenge, here employing C. albicans as a model of fungal pathogen, we quantitatively assessed the metabolism-inhibiting effects of clinical intracanal disinfectants via D₂O-probed Single-Cell Raman Spectra (SCRS), aiming to demonstrate application of the method in screening new formula of intracanal disinfectants of reliable antimicrobial efficacy.

RESULTS

Tracking D₂O Incorporation in C. albicans via Single-Cell Raman Spectra

All living cells consume H₂O in the metabolism process and the H₂O intake rate is proportional to the level of cellular metabolic activity (Berry et al., 2015). Thus, metabolic inhibiting effect of an antimicrobial to a cell can be quantified based on the H₂O intake rate of the cell under drug exposure, which is measured via the extent of Raman shift at the C-D (carbon-deuterium vibration) band in 2,040 to 2,300 cm⁻¹ in the SCRS of a cell to which D₂O is fed (Berry et al., 2015). However, high levels of D₂O, which accelerate cellular D₂O intake and thus allow faster assays, can be cytotoxic or cytostatic to cells (Takeda et al., 1998). Therefore, we started by selecting a proper concentration of D₂O, via comparison among the growth curves of C. albicans under various D₂O levels (Figure 1A). Compared with the control group (D₂O-free conditions), C. albicans growth was not significantly inhibited by a D₂O level below 30% during 10 h of culture (p > 0.05; Figure 1A). After overnight culture, which corresponds to the stationary phase of C. albicans, intensity of the C-D band (2,040–2,300 cm⁻¹) increased along with the elevation of D₂O level in the medium (Figure 1B). The corresponding C-D ratio, defined as ratio of the integrated spectral intensity of the C-D band (2,040–2,300 cm⁻¹) compared to the sum of the C-D band and the predominant C-H band (2,800–3,100 cm⁻¹) (Tao et al., 2017), showed strong positive correlation with media D₂O level (R² = 0.997, p < 0.01; Figure 1C), consistent with the positive link between substrate level and cellular substrate intake rate (i.e., metabolic activity). Therefore, 30% D₂O was chosen for evaluating C. albicans’ metabolic response to the intracanal disinfectants via SCRS.

Under 30% D₂O (in the media), the C-D peak emerged and increased along with duration of D₂O incubation (Figure 1D). The corresponding C-D ratio started growing almost immediately after D₂O introduction, yet in contrast, the growth of OD₁₀₀ wasn’t detectable until ~4 h afterwards (Figure 1E). These results support detecting D₂O incorporation via SCRS under these conditions and
can serve as a proxy for assessing the metabolic activity of *C. albicans*, and suggest that D₂O-probed SCRS can be more sensitive and faster than OD₆₀₀ in detecting *C. albicans* growth, which also incurs metabolic change of individual cells.

**MIC and MIC-MA of CHX, NaClO, and H₂O₂ for *C. albicans***

To assess the metabolic susceptibility of *C. albicans* to each of the three intracanal disinfectants, we determined the corresponding MIC and MIC-MA of CHX, NaClO, and H₂O₂ via broth dilution and D₂O-probed SCRS, respectively (Figures 2A–F; Materials and Methods). In contrast to MIC-MA, which assesses metabolic activity of individual cells, MIC evaluates drug efficacy based on growth inhibition of the whole bacterial population (Materials and Methods).

For CHX, the MIC and MIC-MA are 4 and 8 μg/ml, respectively (Tables S1 and 1). At the MIC of CHX, although fungal growth was entirely inhibited, the temporal dynamics of C-D ratio showed that
it was not lower than the drug-free control and eventually reached nearly an equivalent level after 8 h treatment ($p > 0.05$; Figure 2D). Thus, metabolic activity of *C. albicans* cells was still quite active under the MIC of CHX (4 μg/ml; Figures 2A, D). For the 0.5× MIC and 1.5× MIC groups, the trend within 8 h is similar to the MIC group ($p > 0.05$; Figure 2D). Notably, at the MIC-MA of CHX, the C-D ratio was maintained at the baseline level within 8 h from the start of drug exposure to the end of observation period (Figure 2D), suggesting that the metabolism of *C. albicans* was completely inhibited (ΔC-D-ratios < 0 at 8 h; Figure 2A).

For NaClO and H2O2, the MICs are 168 and 60 μg/ml, respectively (Tables S1, 1), and its MIC-MA are 336 and 120 μg/ml, respectively, in which the averaged ΔC-D-ratios was <0 during an 8-h period (Figures 2B, C). The temporal dynamics of the C-D ratio showed a drug-dose-dependent effect that is reproducible (Figures 2E, F). At their 0.5× MICs of NaClO (84 μg/ml) and H2O2 (30 μg/ml), to a certain extent, the

**TABLE 1** | Comparison of MIC and MIC-MA for the three antimicrobial disinfectants tested.

| Disinfectant (μg/ml) | MIC | MIC-MA |
|---------------------|-----|--------|
| CHX                 | 4   | 8      |
| NaClO               | 168 | 336    |
| H2O2                | 60  | 120    |

**FIGURE 2** | The MIC-MA for each of the three intracanal disinfectants. (A–C) Dose effects of CHX, NaClO, and H2O2 on the ΔC-D-ratio of *C. albicans* cells. (D–F) Temporal dynamics of the C-D ratio of *C. albicans* under increasing doses of CHX, NaClO, and H2O2.
increment of C-D ratio was inhibited as compared to the drug-free control ($p < 0.05$). However, at their MICs of NaClO (168 μg/ml) and H$_2$O$_2$ (60 μg/ml), the increase of C-D ratio was much lower than the drug-free control ($p < 0.05$), and the C-D ratio was maintained at a low level but failed to reach the baseline level during the whole period. Finally, at the MIC-MAs of NaClO (336 μg/ml) and H$_2$O$_2$ (120 μg/ml), the C-D ratio always stayed at the baseline level (Figures 2B, C, E, F); thus, the metabolic activity of C. albicans was entirely inhibited instantaneously and throughout the 8-h duration.

Comparison Between MIC-MAs and MICs Reveals NGMA C. albicans Cells
The MICs of CHX, NaClO, and H$_2$O$_2$ for C. albicans are 4, 168, and 60 μg/ml, respectively (Tables S1 and 1), while the corresponding MIC-MAs are 8, 336, and 120 μg/ml (Tables S2 and 1). Notably, under the MIC-MA level for these three intracanal disinfectants, the majority of C. albicans cells have entered “metabolism quiescence zone”. However, at the MIC of CHX, C-D ratio curve was comparable to the drug-free control, showing the presence of considerable cellular metabolic activity after drug treatment. For NaClO and H$_2$O$_2$, under the MIC treatment, the metabolic activity of the fungal cells was inhibited to a significant degree; however, almost all fungal cells still exhibited a relatively low level of metabolic activity even after 8-h drug exposure (Figure 3A). Therefore, (i) the MIC-MAs of the above three intracanal disinfectants were twice that of MIC; (ii) at their respective MIC level of CHX, NaClO, and H$_2$O$_2$, despite the completely halted growth, metabolic activity of C. albicans cells was inhibited by merely 0.4%, 93.9%, and 94.1% at 8 h (i.e., the inhibitory effect of NaClO and H$_2$O$_2$ on the metabolic activity of C. albicans was much stronger than that of CHX, while no significant difference was found between NaClO and H$_2$O$_2$; Figure 3B), suggesting the presence of “nongrowing but metabolically active” (NGMA) cells that may underlie refractory infections for each of the treatments (particularly for CHX).

Assessing the Efficacy of Disinfectant Combinations on Inhibiting Fungal Metabolism
The distinct C. albicans-inhibitory effects of CHX, NaClO, and H$_2$O$_2$ raise the possibility that rational combination of the disinfectants can potentially further improve the efficacy. To probe this hypothesis, we measured the MIC-MA of multiple combinations of different agents and concentrations.

The CHX (0.5× MIC-MA) and H$_2$O$_2$ (0.5× MIC-MA) combination was unable to completely inhibit C. albicans’ metabolic activity, which was equivalent to the effect of using H$_2$O$_2$ (0.5× MIC-MA) alone ($p > 0.05$) (Figure 4A). The combination of CHX (0.5× MIC-MA) and NaClO (0.5× MIC-MA) was not satisfactory in inhibiting C. albicans metabolic activity either (Figure 4B). However, the metabolism of C. albicans can be completely inhibited by the combination of NaClO (0.5× MIC-MA) and H$_2$O$_2$ (0.5× MIC-MA) (Figure 4C). Notably, further reduction of the level of the two disinfectants, i.e., the NaClO (0.25× MIC-MA) and H$_2$O$_2$ (0.25× MIC-MA) combination, failed to completely inhibit the metabolic activity (Figure 4C). Thus, among the various singular or combinatorial recipes tested here, the combination of NaClO and H$_2$O$_2$ exhibits the most efficient inhibitory effect on fungal metabolism (Figure 4D).

Raman Barcodes for Stress Response Provided Mechanistic Insights into Drug Efficacy
Each SCRS sampled from a drug-responding C. albicans population consists of thousands of Raman bands. Thus, by identifying those marker Raman bands that are both specific and shared among the six stress-response programs (Materials and Methods), we derived RBSC [Raman barcode of cellular-response to stress (Teng et al., 2016)], which consists of 48 elementary Raman bands that collectively characterize the temporal pattern of C. albicans’ response to each of the treatments (Table S3). Among them, four bands that represent

**FIGURE 3** | Metabolic activity of C. albicans under the MIC of each of the three intracanal disinfectants. (A) Temporal dynamics of the C-D ratio of C. albicans under the MIC doses. (B) The degree of metabolic-activity inhibition of C. albicans at 8 h of exposure under the MICs of CHX, NaClO, or H$_2$O$_2$. Each dot represents a cell.
carbohydrates (1,048 and 1,147 cm\(^{-1}\)) and proteins (758 and 1,005 cm\(^{-1}\)), respectively, were shared among the marker bands for all the six stressors; thus, they are part of a general cellular response.

Under CHX, the most prominent change was nucleic acid (1,578 cm\(^{-1}\)) (Figure 5A). Compared with the control group, the intensity of nucleic acid band was generally elevated upon exposure to CHX. It is possible that the density of nucleic acids gradually decreases with the growth of cells in normal-growing cells, while CHX can alter cell membrane integrity by electrostatic binding with the negatively charged cell wall, which results in the leakage of low-molecular-weight components (Bernardi and Teixeira, 2015). These may have caused the relative increase in intensity of nucleic acid band. Under NaClO or H\(_2\)O\(_2\), temporal changes of protein bands for NaClO (1,206 cm\(^{-1}\)) (Figure 5B) and for H\(_2\)O\(_2\) (1,582, 1,572, and 1,561 cm\(^{-1}\)) (Figure 5C) were the most pronounced. Compared with the control group, the intensity of protein bands gradually reduced along with the duration of exposure. NaClO and H\(_2\)O\(_2\) can produce hypochlorous acid (HClO) (Siqueira, 1997) and O\(_2\) (Huang and Pik, 2014) respectively, whose strong oxidizing effect can lead to destruction of proteins and other substances.

As for the combinations, the protein bands of 890 cm\(^{-1}\) (Figure 5D) for CHX+NaClO and of 1,613 and 1,572 cm\(^{-1}\) (Figure 5E) for CHX+H\(_2\)O\(_2\) were reduced. It is possible that CHX precipitates proteins (Bernardi and Teixeira, 2015) and that the strong oxidizing effect of NaClO or H\(_2\)O\(_2\) on protein (Siqueira, 1997; Huang and Pik, 2014) eventually leads to the decrease of protein content or density in cells. Under NaClO+H\(_2\)O\(_2\), the intensity of protein (1,561 and 1,104 cm\(^{-1}\)) and nucleic acid (898 cm\(^{-1}\)) both decreased (Figure 5F), indicative of the oxidation effect that results in decreased proteins and nucleic acids (Siqueira, 1997).

**DISCUSSION**

Proper administration of intracanal disinfectants is vital for both long-term efficacy of antimicrobial RCT and reducing side effects that compromise patient experience. For example, NaClO is one of the most widely used intracanal disinfectants due to its ability to dissolving necrotic tissue and antibacterial activity (Siqueira et al., 1999). However, the proper concentration to administer under a...
clinical setting is controversial, as overly high drug concentrations can reduce biocompatibility and promote irritation of periodontal and periapical tissues (Tanomaru Filho et al., 2002; Gomes-Filho et al., 2008). CHX has also been suggested as an effective antimicrobial agent for RCT; however, cytotoxic effect, allergic reaction, and extrinsic tooth or tissue staining may ensue with usage (Bernardi and Teixeira, 2015). H2O2 remains a frequently used agent in RCT despite its potential for serious complications, which include air emphysema or even systemic gas embolus (Akuji and Chambers, 2017). Therefore, it is critical to develop a methodological scheme that can (i) quantitatively assess the antimicrobial effect based on halting of pathogen metabolic activity, instead of just growth, for avoiding later or recurring infections (Lopatkin et al., 2019), and (ii) rapidly screen new formula of intracanal disinfectants of reliable antimicrobial efficacy.

The susceptibility of fungal pathogens to these three intracanal disinfectants was compared via MIC. For C. albicans ATCC 64342 (also an oral isolate), Ferguson et al. reported the MICs of CHX, NaClO, and H2O2 as <0.63 μg/ml, <10 μg/ml, and 234 μg/ml, respectively (Ferguson et al., 2002). These MICs are comparable to our measurements for C. albicans ATCC 10231 here, with the variation likely due to the change of C. albicans strains and distinction in medium composition, amount of inoculum, incubation temperature, facilities, technical skills, etc. (Mouton et al., 2018). However, MIC’s reliance on growth inhibition can be time-consuming (frequently exceeding 24 h for common pathogens (Berkow et al., 2020), and also results in the inability to detect NGMA cells of C. albicans whose metabolic activity may cause reinfections after clinical latency (Manina and McKinney, 2013; Manina et al., 2015; Lempp et al., 2020).

Our research group originally proposed the “MIC-MA” parameter using D2O-Ramanometry to evaluate the metabolism-inhibiting efficacy of drugs, which tackles the drawbacks of the growth-inhibition-based methods (Tao et al., 2017). Here, we employed MIC-MA to further assess the metabolism-level inhibition of CHX, NaClO, and H2O2 on pathogenic fungi at single-cell resolution. Metabolic activity of the C. albicans cell population was inhibited by merely 0.4%, 93.9%, and 94.1% at their respective MIC level of CHX, NaClO, and H2O2. In contrast, the MIC-MA dose of 8 μg/ml for CHX, 336 μg/ml for NaClO, or 120 μg/ml for H2O2 can each completely halt the metabolic activity of C. albicans. The MIC-MAs of the above three intracanal disinfectants are approximately twice of MICs. This result is consistent with previous literature that shows that the MIC-MAs of NaF or CHX on four prevalent members of oral microbiota (Streptococcus mutans, Streptococcus gordonii, Streptococcus sanguinis, and Lactobacillus fermentum) are 2–3 times that of MICs (Tao et al., 2017). Moreover, the C. albicans-metabolism inhibition of CHX features a distinct threshold in the dose effect (Figure 2A), yet those of NaClO and H2O2 are quite different as semi-linear dose dependency (Figures 2B, C). In particular, at its MIC dose when cellular growth is fully arrested, for CHX, the vast majority of the disinfectant-exposed C. albicans cells are still alive (metabolically active). Thus, by distinguishing NGMA C. albicans cells and quantifying the degree of heterogeneity in metabolic phenotypes (Zhang et al., 2018; Bauer et al., 2020), MIC-MA would be an advantageous parameter to MIC in assessing disinfectant–pathogen interaction.

Based on growth inhibition, Kuruvilla et al. showed that CHX and NaClO combined in the root canal resulted in the greater reduction of microorganisms than either alone (Kuruvilla and Kamath, 1998). In addition, an antibacterial synergistic effect between CHX and H2O2 (Helming and Chandler, 1998; Steinberg et al., 1999) and those between NaClO and H2O2 were reported (Cerioni et al., 2009). Based on metabolic inhibition, we found that the combined formula of NaClO and H2O2 at 0.5× MIC-MA of each can elicit a level of metabolic inhibition of C. albicans that is equivalent to solo use of either NaClO or H2O2 at their
respective MIC-MA level. Since a lower level of each ingredient can reduce side effect (Tanomaru Filho et al., 2002; Gomes-Filho et al., 2008), this NaClO+H2O2 formula should be more efficient. Moreover, no apparent synergistic effect in metabolic inhibition was observed when combining CHX and either NaClO or H2O2. Therefore, growth- and metabolic-based assessment can produce linked yet distinct findings.

Moreover, as a signature for the mode of action, RBCS, which is a barcode of temporal pattern of 48 elementary Raman bands, was derived for each of the pairs of fungi-drug interaction. Compared to other single-cell stress-response profiling methods such as morphological analysis (Choi et al., 2014), fluorescence imaging-based biosensing (Shintaku et al., 2014), or transcriptomics (Islam et al., 2011), RBCS can be advantageous as (i) it rapidly yields a comprehensive and landscape-like view of molecular events of stress response in a label-free, non-disruptive, and simple manner [without the need for preexisting biomarkers (Xu et al., 2017; He et al., 2021)]; (ii) it can predict global gene expression profiles, and vice versa, as the SCRS and transcriptomes (e.g., via RNA sequencing) can be connected linearly through a shared low-dimensional subspace (Germond and Ichimura, 2018; Kobayashi-Kirschvink et al., 2018).

Notably, in order to derive a clinically relevant personalized disinfectant level, drug dosages above MIC-MAs, which are much lower than the recommended concentrations (2% CHX, 1%-5.25% NaClO, 3% H2O2), should be measured not just for pure culture of C. albicans as was here, but also for the microbiota of an infected root canal system, which likely are much more resistant to the disinfectants in the polymicrobial biofilm stage (Ricucci and Siqueira, 2010) (Swimberghe et al., 2019). The heterogeneity of drug response thus can be measured via SCRS, the metabolite-conversion network can be profiled via algorithms such as Intra-Ramanome Correlation Analysis [IRCA (He et al., 2021)], and mechanism can be decoded via Raman-activated cell sorting [RACS (He et al., 2019); e.g., RAGE (Xu et al., 2020) and flow-mode RACS (Wang and Xin, 2020)], to establish the links between disinfectant-susceptibility phenotype and genomes or transcriptome at single-cell resolution. Nevertheless, by developing a workflow for single-cell Raman-based interaction assay for C. albicans and disinfectants used in RCT, this study paves the way for culture-free, rapid, mechanism-based assessment of personalized disinfecting efficacy and screening of treatment regimens for fungal infections.

MATERIALS AND METHODS

Fungal Strain, Media, and Disinfectants

Candida albicans ATCC 10231 (C. albicans), one of the most commonly used fungal strain for drug susceptibility testing (Fidalgo et al., 2010), was obtained from China Center of Industrial Culture Collection. This strain was inoculated on Sabouraud dextrose agar plates at 37°C for 12 h. Grown colonies were picked from the plate and incubated in RPMI 1640 culture medium (pH 7.0 ± 0.1) in an aerobic incubator at 37°C. The 20% Chlorhexidine gluconate (Macklin, Shanghai, China) and 3% hydrogen peroxide (Huanbomiao, Hebei, China) and the 5.25% sodium hypochlorite (Weizhenyuan, Fujian, China) were purchased. All media were stored at 4°C.

Sensitivity of C. albicans to D2O Concentration

C. albicans cells were 1:100 diluted from the stationary-phase culture and exposed to a final concentration of 0%, 10%, 20%, 30%, 40%, or 50% D2O. To track fungal growth under the D2O concentrations, the cells were cultivated in a Bioscreen C (Lab systems, Helsinki, Finland). The working volume in the Bioscreen plate was 300 µl/well and the temperature was controlled at 37°C and optical density (OD) was controlled at 600 nm. OD of the samples was automatically read at regular intervals of 30 min, over a 10-h period (before every measurement, the sample was gently shaken for 10 s). In addition, the cells were sampled at various time points for acquisition of SCRS to probe the intake of D2O by C. albicans cells. Three biological replicates were carried out.

Measuring the MICs of Each of the Three Intracanal Disinfectants for C. albicans

Several colonies from a 24-h-old C. albicans culture grown on Sabouraud dextrose agar plate were transferred to 5 ml of sterile water and vortexed for 15 s until evenly distributed. The measurement of MIC was performed using the broth dilution method according to Clinical Laboratory Standards Institute (CLSI) guidelines. By adjusting cell density, cell suspensions were prepared to reach 0.5 McFarland standard (1× 10^6 to 5× 10^6 cells/ml). Then, a working suspension was prepared via a 1:100 dilution followed by a 1:20 dilution of the stock suspension with RPMI 1640 to obtain a final cell density of 5× 10^5 to 2.5× 10^6 cells/ml. Then, the MIC value was determined by measuring the change of OD_{600} before and after a 24-h disinfectant exposure. Three biological parallels were carried out.

Measuring the MIC-MAs for Each of the Three Intracanal Disinfectants for C. albicans

C. albicans cells were incubated at 37°C in media with the various formulas of intracanal disinfectants and 30% D2O. Samples were collected at 0, 0.5, 1, 2, 3, 4, 6, and 8 h after exposure, respectively, for acquisition of SCRS. The concentrations of disinfectants were initially set as 0, 1/2× MIC, MIC, and 2× MIC. Then, the change of C-D ratio of ~30 individual C. albicans cells randomly sampled from the population was profiled before and after drug treatment for 8 h. The approximate MIC-MA value was found when the mean value of C-D ratio at 8 h minus 0 h (ΔC-D ratio) of drug exposure is ≤0 and the SD is ≤0.005.

Calculation of the Metabolism-Inhibiting Degree of the Disinfectants (%)

The C-D ratio values of the control group (i.e., the absence of disinfectants) were employed as a reference to calculate the percentage decrease of the C-D ratio values after exposure to the intracanal disinfectants, so as to quantify the degree of metabolic inhibition. The following formula was used: 1 – x/x₀,
where $x_0$ is the C-D ratio of the control group at 8 h, while $x_1$ is the C-D ratio of the test group 8 h after exposure to a particular formula of the intracanal disinfectants.

**Assessment of the Efficacy of Combinations of Intracanal Disinfectants**

The combination of disinfectants tested includes MIC and 0.5× MIC of CHX and $H_2O_2$, MIC and 0.5× MIC of CHX and NaOCl, and MIC and 0.5× MIC of NaOCl and $H_2O_2$, respectively. The negative control did not include any disinfectants. Samples were collected at 0, 0.5, 1, 2, 3, 4, 6, and 8 h after exposure respectively for SCRS acquisition. Then, the effect of *C. albicans* metabolism inhibiting was determined by measuring the change of C-D ratio value. Three biological replicates were carried out for each condition.

**Raman Microspectroscopy Analysis**

Samples pretreatment and SCRS acquisition were performed as we previously described with slight modification (Teng et al., 2016; Tao et al., 2017). In brief, SCRS were obtained using a Clinical Antimicrobial Susceptibility Test Ramanometry instrument (CAST-R; Qingdao Single-Cell Biotech Inc, China) or a modified confocal Raman-fluorescent microscope based on LabRam HR system (Horiba Ltd., U.K.). The acquisition time for each cell was 1 s.

Pre-processing of raw SCRS data was performed using R (version 3.5.1), including background subtraction and area normalization. Spectra were cropped to a spectral region of interest ranging from 600 cm\(^{-1}\) to 1800 cm\(^{-1}\) for chemometrics analysis.

**Raman Barcodes for Stress Response and Chemometrics Analyses**

After a series of basic processing of the raw Raman spectra (600 cm\(^{-1}\) to 1800 cm\(^{-1}\)), in order to get the marker bands, the Random Forest model was firstly used to classify SCRS under different disinfectant treatments via default parameters [R package “randomForest”, ntree = 5,000, using default mtry of $\sqrt{p}$ where $p$ is the number of Raman bands]. The rank lists of Raman bands in the order of “band importance” by Random Forests were determined over 50 iterations of the algorithm. Then, the SCRS datasets were reordered based on the rank list and used as the input data for calculating the minimum number (Nmin) of Raman bands for discriminating between the control and the stressed cells via ROC (receiver operating characteristic) analysis, based on the largest AUC (area under the ROC curve). Finally, Top Nmin ranking bands that show significant difference between the control and the disinfectant-exposed cells were designated as the marker bands for each of the disinfectant treatments (Wilcoxon rank sum test; $p < 0.01$).

**DATA AVAILABILITY STATEMENT**

All the Single-cell Raman Spectra produced in this study are publicly accessible at: http://pub.single-cell.cn/index.php/Publication/view_anti/P_SCRS0101.

**AUTHOR CONTRIBUTIONS**

FL, LR, PZ, and FY conceived and designed the research. FL carried out the experiments with the supervision of LR, RC, XS, JX, PZ, and FY. FL, LR, RC, XS, and PZ analyzed data. FL drew the figures. FL and LR drafted the manuscript. FL, XS, JX, PZ, and FY critically revised the manuscript. All authors contributed to the article and approved the submitted version.

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**SUPPLEMENTARY MATERIAL**

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcimb.2022.772378/full#supplementary-material
Changes in Metabolic Activity at the Single-Cell Level. *Front. Microbiol.* 9. doi: 10.3389/fmicb.2018.02243

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