Cigarette smoke condensate increases *C. albicans* adhesion, growth, biofilm formation, and *EAP1*, *HWP1* and *SAP2* gene expression

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Abstract

**Background:** Smokers are more prone to oral infections than are non-smokers. Cigarette smoke reaches the host cells but also microorganisms present in the oral cavity. The contact between cigarette smoke and oral bacteria promotes such oral diseases as periodontitis. Cigarette smoke can also modulate *C. albicans* activities that promote oral candidiasis. The goal of this study was to investigate the effect of cigarette smoke condensate on *C. albicans* adhesion, growth, and biofilm formation as well as the activation of *EAP1*, *HWP1* and secreted aspartic protease 2.

**Results:** Cigarette smoke condensate (CSC) increased *C. albicans* adhesion and growth, as well as biofilm formation. These features may be supported by the activation of certain important genes. Using quantitative RT-PCR, we demonstrated that CSC-exposed *C. albicans* expressed high levels of *EAP1*, *HWP1* and *SAP2* mRNA and that this gene expression increased with increasing concentrations of CSC.

**Conclusion:** CSC induction of *C. albicans* adhesion, growth, and biofilm formation may explain the increased persistence of this pathogen in smokers. These findings may also be relevant to other biofilm-induced oral diseases.

**Keywords:** Cigarette smoke, Tobacco, *C. albicans*, Adhesion, Growth, Biofilm, Genes, *EAP-1*, *HWP-1*, *Sap2*

**Background**

One of the most commonly encountered opportunistic microorganisms in humans is *Candida albicans*, a ubiquitous fungus that is a part of the normal microbial flora found on mucosal surfaces such as those of the oral cavity, gastrointestinal tract, and vagina in human beings and domestic animals [1]. This yeast is the most common cause of mucosal and invasive fungal infections observed in humans [2]. Host protection against *C. albicans* infection is complex and includes different subsets of the immune defense system [3-5].

During the development of oropharyngeal candidiasis (OPC), *Candida* adheres to and invades the tissue. The adhesion of this yeast to host tissue is the initial phase of a potential infection that enables microorganisms to survive inside the host and eventually colonize host tissues during the onset of candidiasis [6,7]. Following this irreversible attachment, fungal cells proliferate and optimally interact to ensure their sustainability in the imposed environment. After the proliferative phase comes the production and deposition of a thick extracellular matrix (mature biofilm) that procures chemical as well as physical protection for cells [8,9].

*C. albicans* adhesion and growth leading to biofilm formation are mediated by various genes such as *HWP1* and *EAP1* that encode well-characterized class of cell wall proteins that house a glycosylphosphatidylinositol (GPI) anchorage motif in their C-terminal domains and a signal peptide at their N-termini. These C- and N-terminal domains mediate the adherence of *C. albicans* to various surfaces [10]. *HWP1*, a hyphal-specific adhesion gene encodes the hyphal cell wall protein, and both are essential for biofilm formation [11]. The involvement of *HWP1* in *C. albicans* adhesion can be supported by the *EAPI* gene which encodes a glucan-cross-linked cell wall protein (adhesin Eap1p) and mediates the adhesion of *C. albicans* to different surfaces, including epithelial cells and polystyrene [12]. Similar to other genes, *HWP1* and *EAP1* are downstream effectors of *EFG1* [13], a transcription regulator [14].
efg1 mutant strain has been shown to exhibit defects in growth, biofilm formation, and virulence [15].

C. albicans virulence is also mediated by proteolytic enzymes such as secreted aspartyl proteinases (Saps) [16,17]. The contribution of Saps to mucosal and systemic infections and their involvement in adherence, tissue damage, and evasion of host immune responses has been reported, showing the implication of the Sap2 gene in C. albicans growth in protein-containing media [18]. SAP1 and SAP3 are expressed during phenotypic switching [19,20], while SAP4, SAP5 and SAP6 are expressed upon hyphal formation [20]. SAP9 and SAP10 are involved in the mechanism of adhesion to host cells [21]. This proteolytic enzyme family is therefore involved in C. albicans virulence.

C. albicans infection can be promoted by several factors. Candidiasis has been associated with long-term antibiotics intake, AIDS, leukemia, malignancy, radiation therapy for head and neck cancer, or other risk factors that interfere with immunocompetence [22-24].

Environmental factors such as smoking may also promote Candida infections [25,26]. Tobacco smoke exposure has been shown to promote microbial biofilm formation [27]. Specifically, it has been shown that cigarette smoke interferes with S mutans and C. albicans adhesion, resulting in biofilm formation on dental restoration materials [28], which suggests that cigarette smokers are more susceptible to life-threatening oral infections including candidiasis. The aim of this study was to investigate the effect of cigarette smoke condensate on C. albicans adhesion, growth, and biofilm formation, and on the activation of several genes involved in the virulence of this yeast.

Results

Cigarette smoke condensate promoted C albicans adhesion and growth

C. albicans attachment to the surface of glass slides for 1, 3, and 6 h was measured by means of crystal violet staining. As shown in Figure 1 C. albicans adhesion was significant (p < 0.05) at 3 and 6 h of incubation. All of the tested CSC concentrations promoted the adhesion of C. albicans. Adhesion was related to incubation period, with low adhesion reported at 1 h and high adhesion at 6 h. This result indicates that CSC can increase C. albicans adhesion and that this effect can lead to significant C. albicans growth. For this purpose, we investigated the effect of CSC on C. albicans growth. As shown in Figure 2, high C. albicans growth was obtained in the presence of CSC, compared to that obtained by the controls. Indeed, C. albicans growth significantly (P < 0.05) increased with as low as 10% CSC. Of interest is that the most effective concentrations of CSC were between 20 and 40%; at these concentrations, C. albicans growth was two to threefold higher than that recorded by the controls. Overall data thus demonstrate that cigarette smoke favors C. albicans adhesion and growth.

Cigarette smoke condensate promoted C albicans biofilm formation

Because CSC contributed to increasing C. albicans adhesion and growth, we tested its potential to promote C. albicans biofilm formation. Using SEM analyses and a crystal violet assay, we were able to demonstrate the stimulatory effect of CSC on biofilm formation (Figure 3). SEM analyses revealed a high C. albicans density in the CSC-treated culture (Figure 3A). A high C. albicans density was
observed in the scaffold in the presence of 30% CSC and this density increased with 50% CSC. To confirm these observations, quantitative analyses were conducted using the crystal violet staining method. Figure 3B showed that after 2 days of culture, CSC was able to significantly (p < 0.05) increase biofilm formation. This effect was observed beginning at a concentration of 20% CSC, and at 50% CSC, biofilm formation was greater than that observed in the controls and at 30% CSC.

Cigarette smoke condensate modulated HWP1, EAP1, and SAP2 expression

Based on the data showing that CSC increased C. albicans adhesion, growth, and biofilm formation, we sought to determine whether this took place through the regulation of certain genes. Figure 4 reveals that HWP1 gene expression significantly increased following exposure of C. albicans to CSC. The activation of this gene significantly (p < 0.001) increased according to CSC concentration. As shown in Figure 4, a twofold increase in HWP1 gene expression was recorded with a concentration of 30% CSC, compared to that observed in the controls, and with 50% CSC, this...
increase was over threefold. Similarly, *EAP1* gene, which encodes a glycosylphosphatidylinositol-anchored, glucan-cross-linked cell wall protein involved in adhesion and biofilm formation, was also affected by CSC treatment. Figure 5 shows that CSC significantly increased the expression of the *EAP1* gene and that this increase was dependent on the concentration of CSC; the higher the concentration, the greater was the expression of this gene. The *SAP2* gene was also modulated by CSC. Figure 6 shows that the CSC led to a significant (*p < 0.001*) increase of *SAP2* gene expression. Of interest is the increased *Sap2* gene expression with CSC concentration. Indeed, with 30% CSC, a twofold expression was recorded and with 50% CSC this expression increased threefold compared to that observed in the controls (*C. albicans* not exposed to CSC).

**Discussion**

Smoking is known to induce a variety of changes in the oral cavity. Cigarette smoke affects both saliva [29] and oral microorganisms, including *C. albicans*, a leading cause of oral candidiasis [28]. However, the specific effect of cigarette smoke on *C. albicans* remains to be elucidated. The first question we addressed in this study was: What is the effect of cigarette smoke on *C. albicans* adhesion? We demonstrated that CSC promoted *C. albicans* adhesion, which is in agreement with previously reported studies showing a high rate of oral candida carriage in tobacco smokers compared to non-smokers [30,31]. Furthermore, bacteria exposed to CSC were shown to adhere more to epithelial cells compared to non-exposed specimens [32], which supports our data with *C. albicans*. Increased adhesion of *C. albicans* in the presence of CSC may occur due to changes in the interaction of *C. albicans* with its environment through the expression of high levels of adhesins, as previously suggested [33]. Furthermore, it is important to realize that cigarette smoke comprises a high number of individual compounds [34], including acetaldehyde, benzene, 1,3-butadiene, and isoprene with an elevated mutagenic potential [35]. Thus, it is possible that these compounds...
may have exerted specific effect on C. albicans adhesion, growth and probably biofilm formation. The effect of cigarette smoke promoting cell adhesion was previously reported by Baboni et al., (2009) showing linear dose response adhesion [25]. The mechanism involved in such effect could involve kinase pathways. These pathways can be promoted by CSC compounds at certain concentration, but inhibited when these compounds are high explaining the decrease of C. albicans adhesion/biofilm formation at 40% and 50% of CSC. Further research is mandatory to shed light on the mechanisms leading to the up-regulation of C. albicans adhesion when exposed to cigarette smoke.

C. albicans adhesion is one of the key events leading to candidiasis [36,37]. This adhesion is usually followed by overgrowth and invasion [38]. Consequently, in promoting C. albicans adhesion, CSC may lead to an overgrowth of this yeast. Our study confirms this hypothesis showing a growth increase of C. albicans in the presence of CSC. This concurs with previously published reports showing that smoking can be an important predisposing factor for oral candidiasis [28], which may be enhanced by cigarette smoke through an increased secretion of histolytic enzymes by C. albicans, thus contributing to its virulence [28]. However, the exact pathogenic influence of smoking has yet to be investigated.

C. albicans adhesion and growth are particularly necessary for biofilm formation [39,40]. Because CSC significantly increased C. albicans adhesion and growth in the present study, it is suggested that CSC may also promote C. albicans biofilm formation. Using appropriate conditions to form biofilms, our findings indicate that CSC was indeed capable of promoting biofilm formation. Of interest is that a significant increase of biofilm formation was obtained at both tested concentrations, and that this phenomenon was dependent on CSC concentration. These useful data are comparable to those of other studies showing increased microbial biofilm formation with cigarette smoke [41-43]. By showing the significant stimulatory effect on increasing C. albicans biofilm formation, cigarette smoke can thus be labeled as an infection-promoting agent.

Promoting C. albicans adhesion, growth, and biofilm formation may operate through the modulated expression of certain C. albicans genes [44,45], as supported by our study demonstrating that CSC led to a high expression of the EAPI gene. As a member of the GPI-CWP family in C. albicans [46], Eap1p was originally identified because of its ability to mediate adhesion to polystyrene when the EAPI gene was expressed in a flocculin-deficient Saccharomyces cerevisiae strain. EAPI expression in a C. albicans efg1/efg1 mutant was able to restore C. albicans adhesion to epithelial cells [12]. Deleting EAPI in C. albicans was shown to reduce cell adhesion to polystyrene and to epithelial cells in a gene dosage-dependent manner [12,46]. Indeed, this suggests that exposure to CSC increases EAPI expression, which may in turn contribute to increasing C. albicans adhesion, and ultimately, biofilm formation and pathogenesis.

We also demonstrated that CSC increased HWP1 mRNA expression. HWP1 is a downstream component of the cAMP-dependent PKA pathway and is positively regulated by EFG1 [47]. The transcript level of HWP1 increased with increasing CSC stimulation, which suggests that CSC did affect cAMP–EFG1 pathway activity, resulting in an increase of C. albicans adhesion and growth with biofilm formation. Further investigations are therefore warranted to gain greater insight into the interaction between cigarette smoke and C. albicans leading to infection.

Candida pathogenesis is associated with the production and secretion of histolytic enzymes [48]. Secreted aspartyl proteases (Saps) and phospholipases were specifically reported as being directly related to C. albicans virulence [49]. During infection, Saps are incriminated degrading host proteins involved in tissue barriers and immune defense [18,50,51]. Here, we report that CSC upregulated Sap2 mRNA expression. It is known that Sap gene upregulation contributes to increasing C. albicans transition, and later, its pathogenicity through an augmented secretion of proteinases [52]. Our study thus establishes, for the first time, a clear link between cigarette smoke and C. albicans pathogenesis through the behavior of key genes such as EAPI, HWP1 and Sap2. These genes are known to be involved in controlling Candida adhesion, growth, and biofilm formation [53]; however, the precise interactions between these different genes and cigarette smoke during C. albicans pathogenesis have not yet been fully investigated. Data suggest that gene activation can be involved in C. albicans adhesion, growth and biofilm formation promoted by CSC. This may involve kinase pathways contributing to C. albicans adaptation to the CSC environment as previously suggested [25].

Conclusions
This study demonstrated that CSC upregulates C. albicans adhesion and growth that promote biofilm formation. Of interest is that these effects were supported by the modulation of C. albicans genes EAPI, HWP1, and Sap2. Overall results therefore suggest a possible link between cigarette smoke, C. albicans activation, and oral candidiasis.

Methods
Preparation of cigarette smoke condensate
1R3F cigarettes were purchased from the Kentucky Tobacco Research & Development Center (Orlando, FL) and were used to prepare the cigarette smoke condensate solution, as shown in Figure 7. Each cigarette was placed into one end of a silicone tube linked to an Erlenmeyer flask.
containing 200 ml of 0.09% sodium chloride. On the other end, a second silicone tube linked to the Erlenmeyer was connected to a standard vacuum. The cigarette was attached to the cigarette holder and lit and the smoke was extracted by applying vacuum, pulling the smoke directly into the 0.09% sodium chloride solution. The process was repeated for a total of ten whole cigarettes. The resulting cigarette smoke condensate (CSC) solution was then sterilized by filtration through a 0.22 μm filter and stored at 4°C until use.

Candida strain
C. albicans SC5314 was cultured for 24 h on Sabouraud dextrose agar plates (Becton Dickinson, Oakville, ON, Canada) at 30°C. For the C. albicans suspensions, one colony was used to inoculate 10 ml of Sabouraud liquid medium supplemented with 0.1% glucose, pH 5.6. The cultures were then grown to the stationary phase in a shaking water bath for 18 h at 30°C, after which time the yeast cells were collected, washed with phosphate-buffered saline (PBS), counted by means of a haemocytometer, and adjusted to 10⁶/ml prior to use.

Effect of CSC on C. albicans adhesion
A glass slide (0.5 cm in diameter) was placed into each well of a 12-well plate then covered with 1 ml of sterile artificial saliva solution at room temperature for 30 min under agitation to ensure that the slides were covered by liquid at all times. Before use, the artificial saliva (2.5 g of NaCl, 332.97 mg of CaCl₂, 250 mg of MgCl₂·6H₂O, 189.48 mg of KCl, 3 g of anhydrous potassium acetate (C₂H₃O₂K), 772.00 mg of K₃PO₄·3 H₂O, and 0.1 ml of H₃PO₄ (85 wt. % in H₂O, Sigma Aldrich)) was supplemented with 140 mg of Type II mucin in 1000 ml of the prepared saliva solution, pH 7. Following the saliva coating, the glass slides were gently rinsed with sterile saline, transferred to new wells of sterile 12-well plates, and subsequently dried for 3 h under a sterile culture hood. Each saliva-coated glass slide was then covered with 10⁴ C. albicans cells in 50 μl of Sabouraud medium supplemented or not with CSC at various concentrations (10, 20, 30, 40 or 50%) and incubated for 60 min at 30°C under stable conditions to prevent the medium from leaking from the slide onto the plastic. Following this incubation period, 1 ml of Sabouraud with or without CSC was added to each well and the cultures were further incubated for 1, 3, or 6 h. At the end of each incubation period, each slide was removed from its well and placed into new wells of a 12-well plate, subsequently washed twice with warm PBS, and subjected to crystal violet staining. One milliliter of 1% w/v crystal violet solution in demineralized water was added, and the slides were further incubated at room temperature for 30 min. After incubation, the non-bound dye was removed from the wells by thorough washing with demineralized water, followed by drying at 37°C. Bound crystal violet was dissolved by adding 1 ml of absolute ethanol and incubating on a rocking platform for 15 min at room temperature. The absorbance levels of the dissolved dye were measured at a wavelength of 590 nm by means of an optical density reader (X-Mark Microplate Spectrophotometer, Bio-Rad Laboratories, Mississauga, ON, Canada).

Effect of CSC on C. albicans growth
The yeast was seeded into separate tubes (10⁵ C. albicans per tube) in 4 ml of Sabouraud culture medium supplemented or not with CSC at 10, 20, 30, 40, or 50%. The cultures were maintained at 30°C for 24 h, after which time C. albicans growth was evaluated by means of the (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) (MTT) assay (Sigma-Aldrich, St. Louis, MO) which measures viable cells as a function of mitochondrial activity. Briefly, an MTT stock solution (5 mg/ml) was prepared in PBS and added to each culture at a final concentration of 10% (v/v). The C. albicans cultures were then incubated with the MTT solution at 30°C for 4 h, after which time the plate was centrifuged for 10 min at 1200 rpm and the supernatant was removed.
The remaining pellet in each condition was then washed with warm PBS, and 2 ml of 0.04 N HCl in isopropanol were added to each pellet, with a further incubation for 15 min. Absorbance (optical density, OD) was subsequently measured at 550 nm by means of an xMark microplate spectrophotometer (Bio-Rad, Mississauga, ON, Canada).

Effect of CSC on C. albicans biofilm formation

C. albicans biofilms were obtained by culturing the yeast on a porous collagen scaffold which facilitated C. albicans penetration through the pores and its adhesion to the scaffold through collagen affinity. This also promoted biofilm formation and handling with no cell loss, thus contributing to the maintenance of the biofilm structure. For this purpose, 5 mm × 5 mm samples of porous scaffold (Collatape, Zimmer Dental Inc., Carlsbad, CA, USA) were placed in a 24-well plate, rinsed twice with culture medium, seeded with C. albicans (10^6 cells), and finally incubated for 30 min at 30°C without shaking to allow for adherence. Fresh Sabouraud medium was added to each well in the presence or absence of various concentrations of CSC (10, 30, or 50%). The C. albicans-seeded scaffolds were then incubated for 2 or 3 days at 30°C. Following each culture period, C. albicans growth and biofilm formation was assessed by scanning electron microscopy and the crystal violet assay.

Scanning electron microscopy (SEM) analysis

C. albicans-rich scaffolds (biofilms) were fixed in ethylene glycol for 60 min followed by a rinse with sterile PBS. Dehydration was performed in a series of 5-min treatments with ethanol solutions of increasing concentration (50, 70, 90, and twice at 100%). The dehydrated biofilms were kept overnight in a vacuum oven at 25°C, after which time they were sputter-coated with gold, examined, and imaged under a JEOL 6360 LV SEM (Soquelec, Montréal, QC, Canada) operating at a 30 KV accelerating voltage.

Biofilm staining by crystal violet

Following incubation for 24 h at 30°C, biofilms were stained with 1% crystal violet (100 μl) for 15 min. They were then washed three times with PBS to remove unbound crystal violet dye and were dried overnight at room temperature. The biofilms were then covered with 1 ml of absolute ethanol and were incubated on a rocking platform for 20 min at room temperature to release the stain from the biofilms. Absorbance was recorded at 590 nm. Each biofilm assay was run in triplicate and the means ± standard deviations of four separate experiments were calculated and plotted.

Effect of CSC on C. albicans gene activation/repression

C. albicans (10^5 cells) was cultured in the presence or absence of CSC at various concentrations (30 and 50%) at 30°C for 24 h under agitation. At the end of this incubation period, the cultures were centrifuged 10 min at 13,000 rpm, the supernatants were discarded, and each pellet was suspended in 0.6 ml of lysis buffer (1 M glycerol, 0.1 M EDTA). Glass beads (0.425–0.6 mm in diameter; 0.2 ml) were then added to each suspended pellet prior to sonication (4 × 1 min, followed by 2 min of incubation in ice) by means of a MiniBead-beater (Biospec Products, Bartlesville, OK, USA). Following cell lysis, the total RNA was extracted from each sample by means of the Illustra RNAspin Mini kit (GE Health Care UK Limited, Buckingham, UK). The concentration, purity, and quality of the extracted RNA were determined using the Experion system and the RNA StdSens analysis kit according to instructions provided by the manufacturer (Bio-Rad, Hercules, CA, USA). Appropriate RNAs were used to perform quantitative RT-PCR.

Quantitative real-time RT-PCR:

RNA (500 ng of each sample) was reverse transcribed into cDNA by means of the iScript cDNA synthesis kit (Bio-Rad, Mississauga, ON, Canada). The conditions for the preparation of the cDNA templates for PCR analysis were 5 min at 25°C, 1 h at 42°C, and 5 min at 85°C. Quantitative PCR (qPCR) was carried out. The quantity of mRNA transcripts was measured with the Bio-Rad

| Gene | Primer sequence 5′ to 3′ | Amp size (bp) |
|------|--------------------------|--------------|
| ACT1 | Forward: GCTGGTGAGAGACTTGACCAACCA | 87 |
|      | Reverse: GACAATTCTCTTCTCGCGACTAGTAGTGA | |
| EAP1 | Forward: CTGCCTCAACTCAATCTCAATTTGTCG | 51 |
|      | Reverse: GAACACATCCACCTTCGGGA | |
| HWP1 | Forward: GCTCAACTTATTGCTATCGCTTATTACA | 67 |
|      | Reverse: GACCGTCTACCTGTGGGACAGT | |
| SAP2 | Forward: TCCTGATGTTAATGTGTATTGTCAG | 82 |
|      | Reverse: TGGATCATATGTCCCTTTTGT | |
Cary, NC, USA). SAS version 8.2 statistical package (SAS Institute Inc., Cary, NC, USA). Each reaction was performed in a Bio-Rad MyCycler thermal cycler. For the qPCR, the CT was automatically determined by the ac-

Statistical analysis
Each experiment was performed at least four times, with experimental values expressed as means ± SD. The statistical significance of the differences between the control (absence of CSC) and test (presence of CSC) values was determined by means of a one-

Competing interests
The authors declare that they have no competing interests.

Authors’ contributions
MR and AS conceived the study. AS, KK and HA conducted the experiments. MR, AS, HA, WC, and MR analyzed and interpreted the data. AS, KK and HA drafted the Materials and Methods section. MR completed the manuscript with the help of WC. All of the authors read and approved the final manuscript.

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