The Role of Cigarettes Smoke Condensatein Enhanced *Candida albicans* Virulence of Salivary Isolates Based on Time and Temperature

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Abstract

Candida albicans is a commensal of the oral cavity and the main agent of oral candidiasis. Cigarette smoke is reported to bea predisposing factor for biofilm formation and morphological changes of *C. albicans*. This study aimed to analyze the role of cigarette smoke condensate (CSC) on biofilm formation and morphological changes of *C. albicans* saliva isolates. *C. albicans* isolated from a smoker's saliva was cultured on CHROM-agar and standardizedusing a 0.5 McFarland (1×10^8 CFU/ml). The biofilms were assayed using spectrophotometric density (620 nm wavelength). The data analyzed by one-way analysis of variance (ANOVA) alsoPearson'scorrelatinwasused. The biofilm mass and morphological changes of *C. albicans* cells were observed by light microscopy (magnificationx 1000). The results showed that non-Indonesian tobacco (non-kretek) CSC strongly induced the formation of biofilms compared to kretek CSC, particularly at 24, 48, and 72 hours ($p < 0.05$) compared to 12hours ($p < 0.01$). This was in accordance with the biofilm mass observed by light microscopy and consistent with the transition of morphological changes from blastospora to pseudohypha and hypha ($p < 0.05$). Kretek and non-kretek CSC increased the biofilm formation and morphological transition changes of *C. albicans* saliva isolates.

Introduction

Indonesian tobacco (kretek) and non-kretek cigarettes are thetobacco products most frequently consumed by Indonesian people compared to pipes and electronic cigarettes. Kretek cigarettes contain tobacco and additional ingredients such as eugenol and aroma, while non-kretek cigarettes contain tobacco without additional ingredients.[1-3]

Cigarette smoke contains around 4000 chemicals comprising: gas components (92%); a mixture of chemicals such as carbon monoxide, hydrogen cyanide, and nitrogen oxide; and solid or particulate components (8%) comprising tar, nicotine, phenol, cadmium, indole, carbarzole, and cresol.[4] Tar, nicotine and carbon monoxide effect oral health.[5]

Cigarette smoke is reported to bea predisposing factor fororal infections such as dental caries and oral-mucosa linfections triggered by *Candida albicans* virulence[6] and as the main agent in the pathogenesis of oral candidiasis.[7] In addition, cigarette smoke causes immune system decline that can facilitate *C. Albicans* biofilm formation, invasion, and colonization of host cells.[8]

Alanazi et al. reported that the smell of cigarette smoke can be a source of nutrition to support *C. albicans* growth thereby increasing adhesion to the mucosal epithelial cells, the initial phase of host cellpenetration.[9,10]

The virulent metabolic properties of *C. Albicans* allow it to adapt to environmental and pH changes inthe oral cavity.[11] The pathogenic properties of *C. Albicans* are associated with adhesion, co-aggregation, immune system

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disorders, phenotypic changes, antibiotic resistance, an Immuno modulation.[12] Blastos poramorphological transition from true pseudohypha and hypha isreported as an important virulent factor for survivaland concentrationin host cells.[13] This is the adaptive phase of *C. Albicans* where it changes from commensal to pathogen.[7]

Semlaliet al. reported that cigarette smoke condensate (CSC) increased the biofilm formation of *C. albicans.*[14] Genotypically, the expression of the biofilm gene and the morphological changes occur when the *C. Albicans* biofilm protein is expressed in blastospora and hypha cells.[15] In general, the biofilm formation of *C. albicans* in the oral cavity occurs inthree phases: initial (0–11 hours), intermediate (12–30 hours), and mature (38–72 hours).[16] *In vitro* studies have shown that biofilm formation of *C. albicans* occurs through adhesion, initiation, maturation, and spread.[17] Violet crystals are reported to be able to detect biofilm formation.[18] This research was evaluated role of kretek and non-kretek CSC to enhancement the biofilm formation and morphological changes of *C. Albicans* based on time and temperatur.

Methods

This research was approvedby the Faculty of Dentistry, Syiah Kuala University, Banda Aceh-Indonesia (ethical clearance No.053/KE/FKG/2016). *C. Albicans* was isolated from a smoker's saliva; kretek CSC was isolated from kretek cigarettes produced by PT HM Samporna Tobacco (Dji Sam Soe®); and non-kretek CSC was isolated from PT Philip Morris Indonesia (Marlboro®) cigarettes. The production method of kretek and non-kretek CSC was adapted from Semlali et al. Whoused *C. Albicans* laboratory isolate and tobacco modified from the Kentucky Tobacco Research & Development Center (Orlando, FL).

*Candida albicans*Cultures

C. albicans salivary isolate from a smoker was cultured for 48 hours on a specific medium of CHROM-agar (CAC-Kat No. CA220, CHROM agar, Paris, France). One colony was inoculated in to a 5 ml suspension of peptone medium (Thermo Fisher Scientific Inc., Oxford, UK), re-incubated for 48 hours, and synchronized using a

0.5 McFarland standard (0.5 equal to 1×10^8 CFU/ml). Gram staining was used to determine *C. albicans* morphologies.[19]

Preparation of Cigarette Smoke Condensate

Ten kretek and ten non-kretek cigarettes were placed sequentially into two silicone pipes attached to two Erlenmeyer flasks containing 200 ml of 0.09% sodium chloride solution, and cigarette smoke was sucked directly into the flasks.[14]

The resulting CSC liquids were filtered using 0.22 μm Whatman®filter paper (Merck, Darmstadt, Germany) and stored at 4^0C until required.[19]

Biofilm Assay

Biofilm was examined by violet crystals as reported by Bachtiar et al.[20] A 96-well microplate (Corning Inc., Corning, NY) was coated with 100μl of smoker's saliva for 10 min and then twice rinsed for 1 min with phosphate buffer saline (PBS) while rocking at 200rpm. Next,100μl each of the incubated CSC solutions (100%, 50%, and 25% concentrations) were placed into the microplate, closed for 10 min, and then discarded.

Additionally, 100 μlof *C. Albicans* saliva isolates in peptone were incubated for 12, 24, 48, and 72 hours, respectively. The peptone containing *C. albicans* was suctioned off and discarded. The activity of *C. albicans* biofilm formation was measured using 150 μlof 1% crystal violet (Merck, Darmstadt, Germany) solution fed into each microplate well, Incubated in room temperature for 15 min, twice washed with PBS, and the second for 5 min.

Finally, the crystal violet was extracted by adding 100 μlof 98% ethanol for 5 min at 300 rpm. The final violet crystal extraction added 96% ethanol for 5 min at 300 rpm.[20]

Optical density of the biofilm serial duplo was measured by an Elisa reader (Bio-Rad Laboratories, Hercules, CA) at 620 nm wave length.

Observation of Biofilm Mass

After sensitization with kretek and non-kretek CSC, 100 μl of glycerol was added to each microplate well of *C. Albicans* and left for 24 hours to maintain biofilm moisture. After adding immersion oil (Merck, Germany) to each microplate well the biofilm mass was examined

using light microscopy (magnification x 400) as adapted from Anastasiadis et al.[21]

Observation of *Candida albicans* Morphological Changes

Observation of the morphological transitions of *C. albicans* cells used cultured slides.[22] Medium CHROM-agar was cut into 1x1 cm portions and placed onto glass slides with cultured *C. Albicans* placed over them, then covered with glass covers. Subsequently inserted in a sterile plate under neath was moistened with 5 ml kretek and non-kretek CSC in cotton and incubated for 48 hours at predetermined temperatures: blastopore 30^0C; pseudohypha 35^0C; and hyphae 37^0C. Gram staining was used to observe the transition of morphological changes of *C. albicans*.[23] One of the *C. albicans* colonies was placed ona sterile glass slide and smeared with physiological 0.9% NaCl

(Rindatrabakti, JawaTimur, Indonesia). This was spread as thinly as possible and prepared glass slide was dipped into 2% violet crystals for 20 seconds, rinsed with PBS, dipped into iodine for 1 min, then dissolved with 96% alcohol for 10 seconds.[24] The slide culture was rinsed with PBS, dipped into safranin (Merck, Darmstadt, Germany) solution for 20 seconds, washed with PBS, and dried.[25] The transition of *C. albicans* cellmorphological changes at the prescribed temperatures was observed using light microscopy (magnification x 1000).

StatisticalAnalysis

Biofilm formation data was analyzed using one-way analysis of variance (ANOVA) with a significance probability of $p < 0.05$ and Pearson's correlation ($p < 0.01$). Morphology changes were analyzed descriptively.

Results

The activity of *C. Albicans* biofilm formation increased from 12 to 24 hours and decreased from 48 to 72 hours. Non-kretek CSC had a facilitating growth on biofilm formation at 24, 48, and 72 hours while kretek CSC influenced formation at 12 hours. After sensitization with kretek and non-kretek CSC, *C. albicans* biofilm formation was increased after 24 hours incubation (Figure 1).
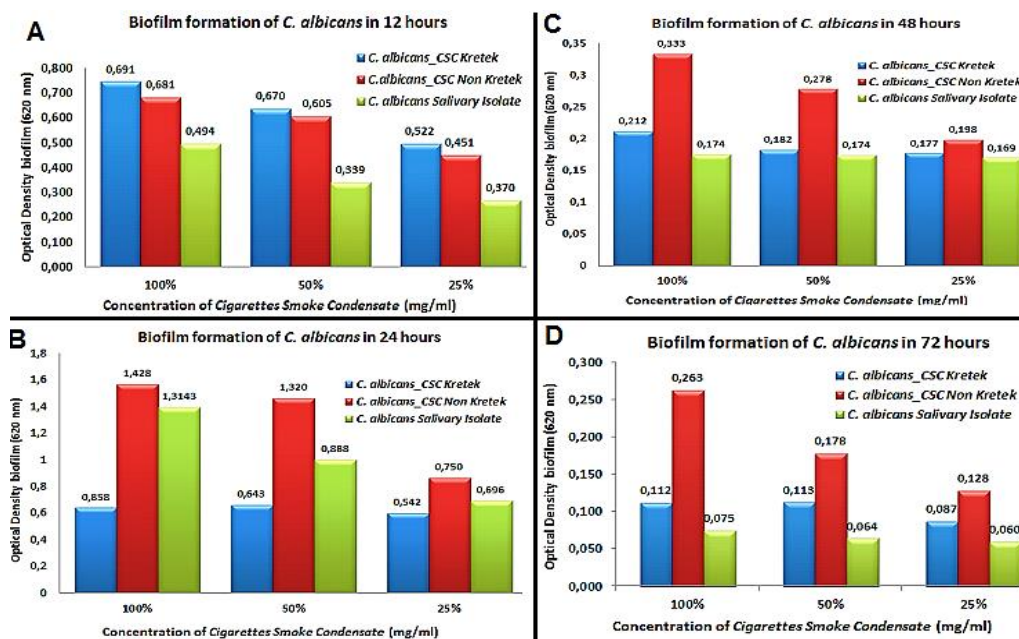

Figure 1. Optical densities for biofilm formation of *Candida albicans* in saliva isolates after sensitization with kretek and non-kretek cigarette smoke condensate (CSC) for (A) 12 hours, (B) 24 hours, (C) 48 hours, and (D) 72 hours.

This was correlated with the biofilm mass profile observed with lightmicroscopy (magnitude 1000x) (Figure 2). Kretek and non-kretek CSC accelerated biofilm maturity and morphology changes from blastospora to pseudohypha and hypha (Figure 3).
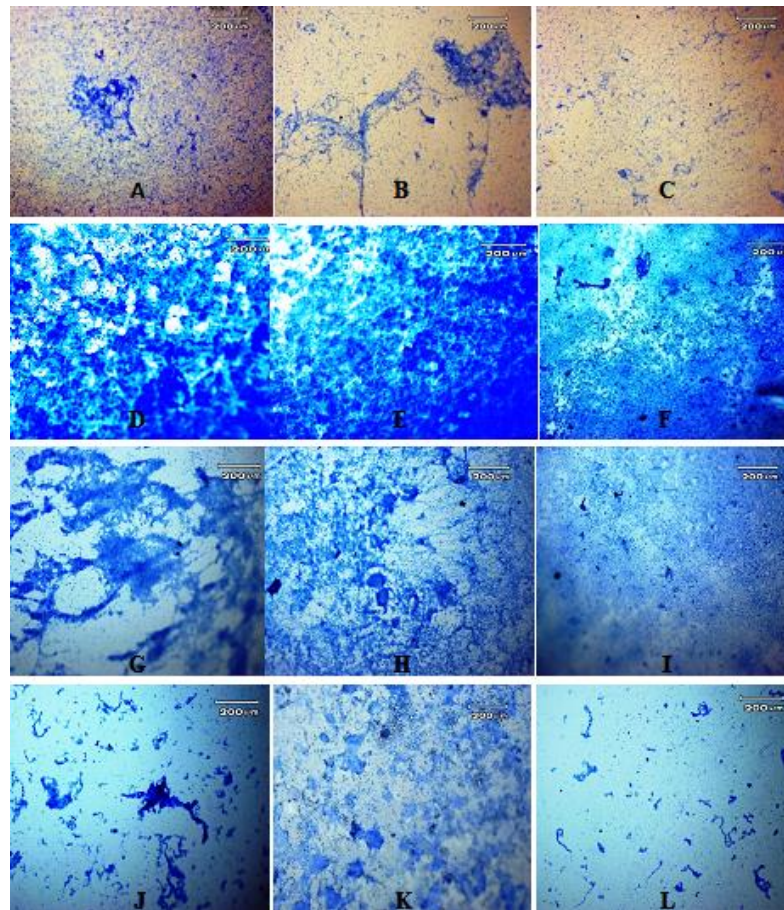

Figure 2. Profiles of mass biofilms(blue) on 96-well microplate after sensitization with kretek and non-kretek cigarette smoke condensate (CSC):(A–C) 12 hours; (D–F) 24 hours; (G–I) 48 hours; and (J–L) 72 hours. Mass profiles of *Candida albicans* biofilms sensitized by: (A, D, G, J)C. *albicans* kretek CSC;(B,E,H,K) C. *albicans* non-kretek CSC; and (C, F, I, L)C. *albicans* isolate saliva.
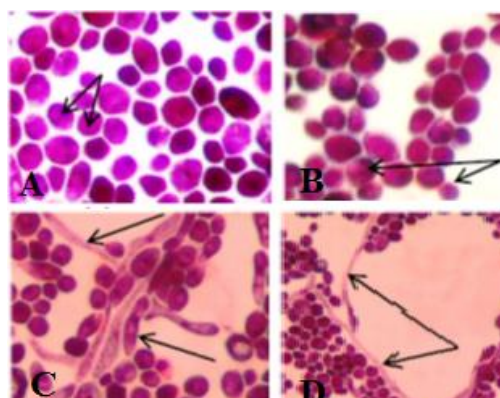

Figure 3. Cell morphology profiles of *Candida albicans* after sensitization with cigarette smoke condensate (CSC) and incubated for 48 hours. Arrows indicate (A) blastospora cells (30^0C); (B) budding cells (30^0C); (C) pseudohypha (35^0C); and (D) hypha (37^0C). Using light microscopy(magnification x 1000).

Table 1 shows the morphology changes after sensitization with kretek and non-kretek CSC. At 30^{0}C, *C. albicans* salivary kretek and non-kretek CSC was exhibited pseudohypha. At 35^{0}C, *C. albicans*, sensitized by kretek CSC, was dominated by pseudohypha formation, where as non-kretek CSC was dominated by hypha formation. Where as, *C. albicans* ATCC (American Type Culture Collection) not yet hypha on temperature of 30^{0}C and have shown hypha.

Table 1. Morphology changes of *Candida albicans* after sensitization with kretek and non-kretek cigarette smoke condensate.

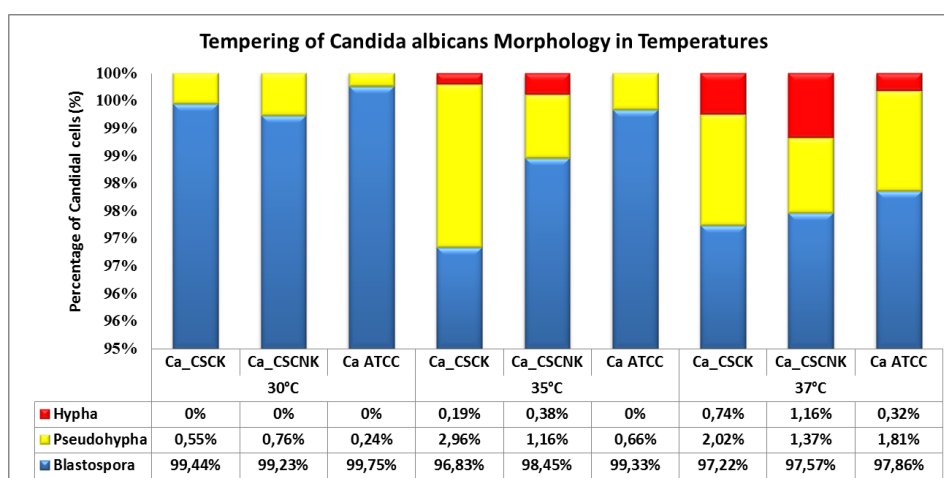| *Candida albicans*	Incubation temp(°C)	Blastospora (cells/plate)	Pseudohypha (cells/plate)	Hypha (cells/plate)
*C. albicans*_CSCKretek	30°C	1080	12	5
*C. albicans*_CSCKretek	35°C	980	5	5
*C. albicans*_CSCKretek	37°C	840	34	9
*C. albicans*_CSC Non-kretek	30°C	904	8	4
*C. albicans*_ CSC Non-kretek	35°C	1808	11	6
*C. albicans*_ CSC Non-kretek	37°C	1656	12	13
C. albicans ATCC 10261	30°C	971	1	0
C. albicans ATCC 10261	35°C	396	8	1
C. albicans ATCC 10261	37°C	407	9	2

Figure 4. Percentage changes of cell morphology of *Candida albicans* at various temperatures.
Note: Ca_CSCK (*C.albicans* kretek cigarette smoke condensate (CSC)). Ca_CSCNK (*C.albicans* non-kretek CSC). Ca_ATCC (*C.albicans* American-type culture collection).

Figure 4 shows that the percentage of blastospora growth is dominant at all temperatures followed by pseudohypha and hypha. The presence of *C. albicans* hypha in smoker's salivary isolate at 35^{0}C compared to the *Candida albicans* ATCC showed that kretek and non-kretek CSC influenced the increased blastos pora to pseudohypha and hypha morphology transitions. The standard deviation valuesat all temperatures showed small error bar values (Figure 5) indicating that temperature wasthe determinant of influence of kretek and non-kretek CSC to change the morphology of *C. albicans*. ANOVA tests showed that the morphology transitions of blastospora at 30^{0}C, 35^{0}C, and 37^{0}C were not significantly different

(p>0.05) where as transition of blastospora to pseudohypha and hypha were significant between 30^0C, 35^0C, and 37^0C (p<0.05).
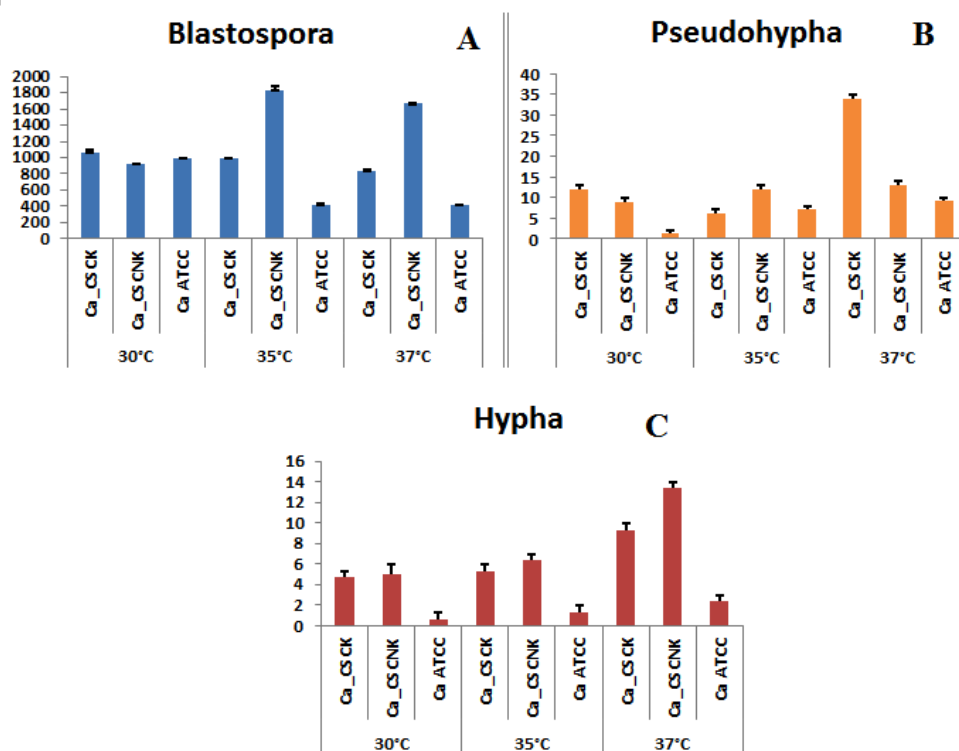

Figure 5. Influence of kretek and non-kretek cigarette smoke condensate (CSC) on the morphology changes of *Candida albicans*: (A)blastospora, (B) pseudohypha, and (C) hypha. Bar = average temperature. Error bar =deviation standard values. All temperatures show a decrease.

Discussion

*C. albicans*is highly sensitive to environmental change and the transition from blasto spora to pseudohyphae and hypha is aform of adaptation to environmental change. This fungus also expresses a number of virulent factors like secreted aspartyl proteinases and hyphal wall protein 1 (HWP1) that are involved in adhesion and biofilm formation against host cells. Phenotypic switching is an effort to avoid the human body's defense system.[26,27]

Cigarette smoke is a reported environmental factor that often affects increased virulence of*C. albicans*.[28] Cigarette smoke is a predisposing factor for*C. albicans* infection by suppressing the immune system and destroying mucosal epithelial cells. Both of these events can provide benefits for *C. albicans* in the oral cavity.[29]

The results show that kretek and non-kretek CSC may increase the biofilm formation of *C. Albicans* salivary isolate (p <0.05) (Figures 1 and 2). Similarly, the effect of kretek and non-kretek CSC on the transition of morphological changes of *C. Albicans cells* from blastospora to pseudohypha and hypha is shown in Figures 3 and 4, and Table 1. It can be assumed that cigarette smoke has potential toincrease *C. albicans* virulence. This virulence activityis the result of the active ingredients of cigarette smoke such as acetaldehyde, benzene, and isoprene because these three substances are mutagenic to host cells and support the growth and adhesion of *C. albicans* in host cells.[14] Nitrosamines formed from burning cigarettes are reported to support the growth of *C. albicans*.[30] In addition, cigarette smoke contains aromatic hydrocarbon compounds, a source of growth nutrients for *C. albicans*.[9]

In a previous study, CSC significantly improved the ability of *C. albicans* adhesion and biofilm formation (p<0.05).[31] Semlali'set al.'s research correlated closely with the results study, whereby CSC increased biofilm formation at12, 24, 48, and 72 hours, although with different

intensities.[14] Řičicová et al. reported that the most dominant phases of biofilm formation by *C. albicans* occurs at 24–72 hours.[32] CSC may affect biofilm formation more rapidly starting from 3–6 hours of incubation and increasing biofilm formation by candida from 24–48 hours.[14]

Incubation for 12 hours (Figure 1A) showed that the formation of *C. albicans* biofilms sensitized by kretek CSC was dominant compared to *C. Albicans* sensitized by non-kretek CSC. This was correlated with the proliferation stage of *C. albicans* cells. Biofilm formation of *C. albicans* is characterized by: early adhesion of candida cells (0–2 hours), germination and micro-colony formation (2–4 hours), filament formation (4–6 hours), development of monolayers (6–8 hours), proliferation (8–24 hours), and maturation (24–48 hours).[33] At complete maturation, the biofilm serves as a protective agent against antimicrobials.[34]

C. albicans of kretek CSC was able to modulated the biofilm formation at 24, 48, and 72 hours (Figure 1) more effectively than non-kretek CSC ($p < 0.05$). Similarly, at 37°C, transition changes for hypha-formation were higher in *C. albicans* sensitized with non-kretek CSC compared to kretek CSC (Figure 4). This may have been the result of eugenol, contained in the kretek CSC, which is capable of inhibiting the development of *C. albicans* by interfering with the adhesion of candida cells and causing a disturbance of the quorum sensing system between micro organisms. In addition, eugenol activity may inhibit the synthesis of chitin, nucleic acid, and proteins and inhibit the production of energy by ATP (AdenosineTriphospate).[35] In the current study, it is assumed that eugenol interferes with the transition from the pseudohypha form to the hypha form.[36,37] In addition, kretek CSC can decrease *C. Albicans* biofilm formation and it is suspected that the eugenol effect disables the intracellular enzyme synthesis stage by causing leakage of *C. albicans* cell cytoplasm and interfering with biofilm formation.[38] Zhou et al. also reported that eugenol might prevent quorum sensing between microorganisms atan early phase of biofilm formation.[3]

Generally, enhancement of biofilm formation by *C. albicans* sensitized by kretek and non-kretek CSC occurred after 24 hours incubation and declined from 48 hours and 72 hours ($p < 0.05$). The results of the current study differ from those reported Semlali et al. where biofilm formation peaked at 48 hours after culturing in CSC solution. These differing results may be due to the administration of the CSC. In the current study, *C.albicans* saliva isolates were used as subjects desensitized with CSC, while in the study by Semlali et al., *C.albicans* was cultured with CSC.[14] Therefore, it can be assumed that the CSC preparation using the sensitization model can accelerate biofilm maturation compared to culture modeling.

Based on Gram staining, *C.albicans* cultured for 48 hours at 30°C, 35°C, and 37°C exhibited blastospora, pseudohypha, and hypha (Figure 3). The *C. albicans* CSC kretek and non-kretek cultured for 48 hours at 30°C, 35°C, and 37°C was dominated by blasto spora (budding cells) (Table 1). Blastosporas are reported as the first form of *C. albicans* cell morphology before transitioning to pseudohypha and hypha.[39] Barbieri et al. Reported the blasto spora isr elatively large and better than virulent compared pseudohypha.[40]

The current research showed that the percentage transition of blastospora to pseudohypha and hypha in *C. albicans* cells sensitized by kretek and non-kretek CSC and incubatedat 35°C and 37°C is better than without sensitization by kretek and non-kretek CSC (Figure 4). It is assumed that cigarette smoke in the form of CSC is able to modulate the transition of morphological changes of *C. albicans*. Alanazi et al. confirmed that CSC solution can trigger *C. albicans* transition from blastospora to pseudohypha and hypha, and this change becomes an important virulence factor when performing adhesions, invasions, and infections of host cells.[9] Temperature and pH are other interrelated factors for morphological changes of *C. albicans* cells. Increased temperature is associated with increased transition activity of morphological changes from blastospora to pseudohypha and hypha.[41]

The current research showed that all incubation temperatures had small error valuesfor morphology changes of blastospora to pseudohypha and hypha (Figure 5). Theoretically, molecular aspects of phenotypic change are believed to be more common in virulent strains than in normal flora strains.[42]

This finding is analogous to the current study; *C. Albicans* isolated from smokers during inducted by CSC influenced the phenotype change (biofilm and morphology) and virulence compared tonon-sensitization bykretek and non-kretek CSC.

Conclusion

Based on the results of the current research, it was concluded that kretek and non-kretek CSC could increase the virulence of *C. albicans*, such as biofilm formation, and accelerate the transition of morphological changes from blastospora to pseudohypha and hypha, and non-kretek CSC had a stronger effect than kretek CSC on increasing the biofilm formation and morphological transition of *C. albicans* ($p < 0.05$).

Acknowledgement

Declaration of interest

The authors report no conflict of interest.

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