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THE EFFECT OF *(-)-EPIGALLOCATECHIN-3-GALLATE* GREEN TEA ON NEUTROPHIL COUNT AND INFECTED CELLS BY *CANDIDA ALBICANS* IN A MURINE MODEL OF ORAL CANDIDIASIS

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ABSTRACT

Background: Oral candidiasis is an infection caused by commensal fungi of Candida species in the oral cavity that serves as an opportunistic pathogen with *Candida albicans (C. albicans) as* the most frequent (80%) etiology. The prevalence of oral candidiasis tends to increase due to the increasing population of immunocompromised patients and resistance to anti fungal. This requires alternative treatment to enhance the effectivity of anti fungal medications such as green tea (*Camellia sinensis*) with *(-)-epigallocatechin-3-gallate* (EGCG) as the main polyphenol component and most potent (59-65%). The effect of EGCG green tea in obliterating oral candidiasis through neutrophil count and infected cells by *C. albicans* is not clearly determined yet up to now. Objective: To analyze the effect of EGCG green tea in a murine model of oral candidiasis through neutrophil count and infected cells by *C. albicans*. Method: True laboratory experimental study with randomized post test only control group design, using Wistar male rats. The rats were grouped into 1 control and 3 intervention groups. EGCG was given with the dosage of 0, 1, 2 and 4 mg/kgBW/day as the intervention. Result: immunohistochemistry and Hematoxyllin-eosin stain showed that EGCG green tea increased neutrophil count and decreased infected cells by *C. albicans*. Conclusion: EGCG green tea has been significantly increased neutrophil count and decreased infected cells by *C. albicans* in a murine model of oral candidiasis.

KEYWORDS: *(-)-epigallocatechin-3-gallate*, green tea, neutrophil count, infected cells by *C. albicans*, murine model, oral candidiasis.

INTRODUCTION

Oral candidiasis is an infection caused by commensal fungi of *Candida spp.* in the oral cavity that serves as an opportunistic pathogen.[1-3] The most frequent (80%) etiology of oral candidiasis is *Candida albicans* (*C. albicans*).[3-6] *C. albicans* becomes virulent in the oral cavity and is related to the defect on the immune system, namely immunocompromised conditions.[7-8] The prevalence of oral candidiasis tends to increase due to the increasing population of immunocompromised patients as high as 50-95% (Diabetes Mellitus, immunosuppresant drugs or long term antibiotics usage, Human Immunodeficiency Virus (HIV)/Acquired Immunodeficiency Syndrome (AIDS), transplant organ recipients, and hyposalivation).[6, 9-12] Aside from those, the increased prevalence of oral candidiasis is also induced by the high number of resistant cases to anti fungal medications (32->50%).[6, 13-16] All of those lead to the increasing failure of anti fungal medications (azole), which can lead to systemic candidiasis occurs with mortality of 30-50%.[5-6, 17-19] Therefore alternative treatments are needed to enhance the effectivity of anti fungal drugs. One of the alternative therapy is herbal drugs, including green tea (*Camellia sinensis*) which is already declared as safe for consumption or generally recognized as safe by the Food and Drug Administration Safety (FDA) of United States of America.[20]

Neutrophils or polymorphonuclear lymphocytes (PMNLs) are essential innate immune cells which determine the host's resistance against various bacterial and fungal infections.[21-22] Neutrophils have emerged as an important component of effector and regulatory circuits in the innate and adaptive immune systems.[23] Th17 cells as component of adaptive immune systems, play a role in host defense to various extracellular pathogens, including fungi, bacteria, and some parasites. Th17 cells' main role in antifungal immunity is at sites of infection in the skin and mucosa through the release of proinflammatory factors, recruitment of neutrophils, and production of antimicrobial peptides.[24]

Green tea has been known to have many benefits for human life, including being an immunomodulator and antifungal. The main polyphenol component (59-65%) and the most potent of green tea is (-)-Epigallocatechin-3-gallate (EGCG). The antifungal effects of EGCG were mainly studied against yeasts such as Candida spp. and moulds such as

dermatophytes.[16, 25-28] The effect of EGCG green tea in obliterating oral candidiasis on neutrophil count and infected cells by *C. albicans* is not clearly determined yet up to now. Considering the essential role of neutrophil in oral candidiasis, this study was aimed to analyze the effect of EGCG green tea in obliterating oral candidiasis on neutrophil count and infected cells by *C. albicans***.**

MATERIALS AND METHODS

This study was true laboratory experimental study with randomized post test only control group design. Male Wistar rats (n=23) (age 12 weeks; approximately 150-200 g; Animal Model Unit, Biochemistry Laboratory, Faculty of Medicine, Airlangga University) were used in this study. After acclimation for 7 days, rats were randomized into 4 groups of five or six animals, housed in large cages. During the experiment, food composition was complete and equilibrated, free from antifungal agents. The research complied with ethical clearance from Faculty of Dentistry – Airlangga University, No: 139/KKEPK.FKG/IX/2014, on 18 September 2014.

This study used EGCG was purchased from Xi'an Rongsheng Biotechnology Co., Ltd. (No. 82, Keji Road, Xi'an Hitech Industries Development Zone, Shaanxi Province, P. R. China). *Candida albicans* was obtained from the Oral Biology Laboratory, Faculty of Dentistry, Airlangga University. A single colony from Sabouraud glucose agar was grown in yeast extract-peptone glucose medium YPG (yeast extract, 2%; bactopeptone, 1%; glucose, 2%) for 18 hours at 30°C in a shaker. The culture was harvested by centrifugation at 2500g, then cells were washed three times in phosphate buffer saline (PBS) and adjusted to a final concentration of $3x10^8$ CFU/mL. The viability of the inoculum was confirmed by quantitative cultures of serial 10 fold dilutions on Sabouraud dextrose agar plates. To enhance the infection rate, rats were immunosuppressed with dexamethasone (Dexamethasone, Tianjin Tianyao Pharmaceuticals Co., Ltd) and treated with tetracycline hydrochloride (Tetrasanbe; Sanbe Farma Laboratories, Bandung, Indonesia). One week before infection, rats received 3.2 mg/kgBW/day of dexamethasone with 120 mg/kgBW/day of tetracycline, were intraperitoneally (i.p.) administered to rats (day 1 until 7). On the day of infection, dexamethasone was raised to 6.4 mg/kgBW/day**,** tetracycline was reduced to 12 mg/kgBW/day, and maintained throughout the experiment (day 8 until 22). We used modification model of oral candidiasis in immunosuppressed rats that was reported by Martinez et al.[7] and Chami et al.[29], with modification dosage of dexamethasone and

tetracycline from Thong & Ferrante[30] and Brummer et al.[31] The rats were orally infected three times at 48 h intervals (days 8, 10 and 12) with 0.1 mL of saline suspension containing $3x10^8$ viable cells of C. albicans. Oral infection was achieved by means of a cotton swab rolled twice over all parts of the mouth. Just before inoculation, the animals were sampled to confirm the absence of C. albicans in the oral cavity, and 72 h (day 15) after the last inoculation all groups were sampled in the same manner to check for the presence of the fungi in the oral cavity before the beginning of the treatment.[7, 29, 32] Just before treatment, the animals were sampled to confirm the presence of C. albicans in the oral cavity. Then, the animals were randomized and assigned to groups of four. Treatment was administered for seven consecutive days (from day 15 to day 22). The EGCG at 1, 2, and 4 mg/kgBW/day, respectively, were intraperitoneally administered to rats. The control group (n=5) received sterile saline by the i.p. route. Control and intervention groups were sacrificed at day 23 with combination of Ketamine HCl (Ketamil Injection) and Xylazine (Xyla) with 100 mg/kgBW and 10 mg/kgBW per intramuscular because at that time, was also conducted blood sampling from intracardial (3 mL) and gingival tissue excision. Samples were collected at day 15 by rolling a sterile cotton swab over the oral cavity, which was then suspended in 1 mL of sterile saline. 25µl samples from this suspension were dropped in duplicate, after serial ten fold dilution on Sabouraud agar plates containing 0.05% chloramphenicol. All plates were incubated at 30°C for 24 h.[7, 29] At day 23, i.e. 24 h after the administration of the last dosage of EGCG or saline, the animals were sacrificed. The gingival tissues were removed, fixed in normal buffer formalin 10% solution for at least 48h. Gingival sections were embedded in paraffin and 5µm thick serial transverse sections were stained with hematoxylin-eosin stain to assess the neutrophil count. The infected cells by *C. albicans* stained with immunohistochemistry (Novolink™ Polymer Detection Systems). The data was processed with Pearson correlation using the Statistical Analysis System (SAS).

RESULTS

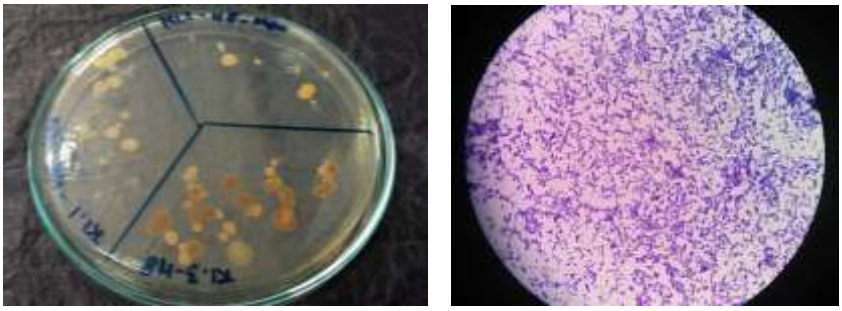

Figure 1. Identification of Candida Before Inoculation C. albicans (Day 8)

Prior to initiating the study, oral cavity cultures of each rat were performed, and no *C. albicans* organisms were found (Figure 1).

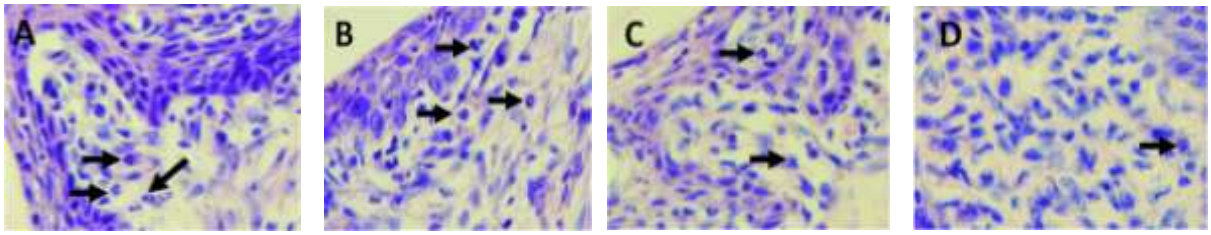

Figure 2. Hematoxyllin-eosin stain showed neutrophil count in gingival tissue (arrows) in each EGCG dosage group at day 23 (400 times magnification). Figure A is control group. EGCG dosage of 1, 2 and 4 mg/kgBW/day (Fig. B-D).

The neutrophil count in gingival tissue after being evaluated with Hematoxyllin-eosin stain showed an increased neutrophil count compared to other treatment groups (Figure 2).

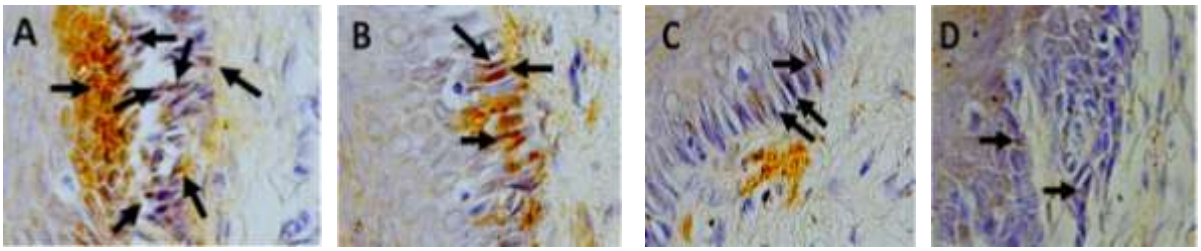

Figure 3. Immunohistochemistry method showed infected cells by *C. albicans* in gingival tissue (arrows) in each EGCG dosage group at day 23 (400 times magnification). Figure A is control group. EGCG dosage of 1, 2 and 4 mg/kgBW/day (Fig. B-D).

The infected cells by C. albicans in gingival tissue after being evaluated with Immunohistochemistry method showed a decreased infected cells by C. albicans compared to other treatment groups (Figure 3).

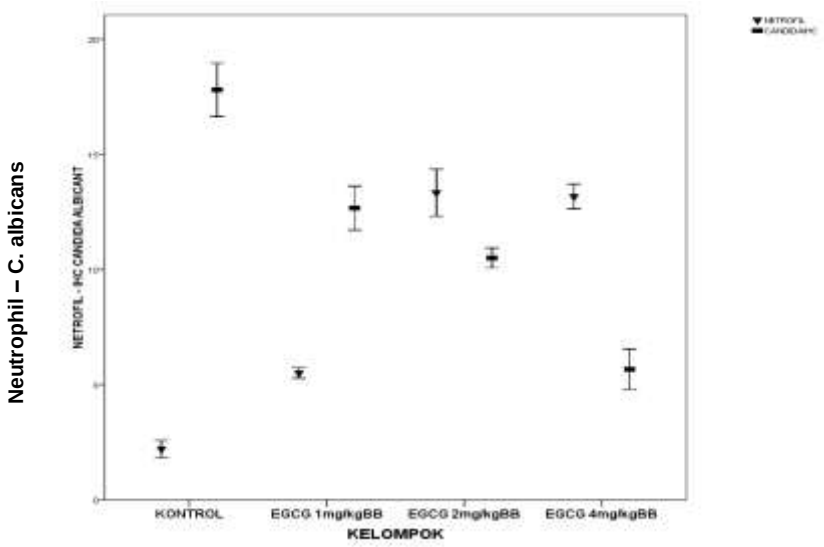

Figure 4. The results showed significant increased neutrophil count and decreased infected cells by *C. albicans*

Table 1. The Pearson correlation between neutrophil count and number of C. albicans

Correlations			
		NETROFIL	**CANDIDAIHC**
NETROFIL	Pearson Correlation	1	**-.769****
	Sig. (2-tailed)		.000
	N	23	23
CANDIDAIHC	Pearson Correlation	-.769**	1
	Sig. (2-tailed)	.000	
	N	23	23
**. Correlation is significant at the 0.01 level (2-tailed).			

Figure 4 and table 1 showed significant correlation between neutrophil count and infected cells by *C. albicans.*

DISCUSSION

Neutrophils or polymorphonuclear lymphocytes (PMNLs) are essential innate immune cells which determine the host's resistance against various bacterial and fungal infections.[21-22] Neutrophils have emerged as an important component of effector and regulatory circuits in the innate and adaptive immune systems. Neutrophils engage in bidirectional interactions with different components of both the innate and adaptive immune systems and can differentially influence the response depending on the context.[23]

Th17 cells' main role in antifungal immunity is at sites of infection in the skin and mucosa through the release of proinflammatory factors, recruitment of neutrophils, and production of antimicrobial peptides. Neutrophils have 3 main mechanisms to directly kill invading microbes: phagocytosis, degranulation and activation of the oxidative burst, and neutrophil extracellular traps. Microbes are taken up by phagocytosis and are then destroyed by reactive oxygen species with antimicrobial potential, which are produced in a process called oxidative or respiratory burst. Through degranulation, neutrophils release proteins with lytic and antimicrobial function, such as cathepsins, defensins, myeloperoxidase, and bactericidal/permeability-increasing protein. Finally, neutrophils can release so-called neutrophil extracellular traps, which act as a mesh to trap and kill microorganisms independently of phagocytic uptake. The traps consist of a web of DNA and histones and contain granule-derived proteins with antimicrobial activity.[24]

EGCG green tea administration in this study has been significantly proven to increase neutrophil count in gingival tissue in each EGCG dosage group. The increasing EGCG green tea dosage leads to the increasing neutrophil count (Figure 4, Table 1). This might happened because Th17 cells' as adaptive immune system main role in antifungal immunity is at sites of infection in the skin and mucosa through the release of proinflammatory factors, recruitment of neutrophils, and production of antimicrobial peptides.[24] The effect of EGCG green tea through neutrophil count is not clearly determined yet up to now, but in this study showed increased neutrophil count.

Tea is the most consumed drink in the world after water. Green tea is a 'non-fermented' tea and contains more catechins than black tea or oolong tea. Catechins are in vitro and in vivo strong antioxidants. In addition, its content of certain minerals and vitamins increases the antioxidant potential of this type of tea.[33] Green tea has been known to have many benefits for human life, including being an immunomodulator and antifungal. The main polyphenol component (59-65%) and the most potent of green tea is EGCG. The antifungal effects of EGCG were mainly studied against yeasts such as Candida spp. and moulds such as dermatophytes.[16, 25-28]

EGCG green tea administration in this study has been significantly proven to decrease infected cells by *C. albicans* in each EGCG dosage group, thus it can obliterate oral candidiasis (Figure 4, Table 1). It was consistent with several studies that showed beside as main active catechin, EGCG has been known as the main polyphenol component (59-65%)

and the most potent of green tea, has effect as an immunomodulator and antifungal.[16, 25-28] Thus, another several studies showed that EGCG green tea as antifungal was observed through various variable such as Minimum Inhibitory Concentration[13, 28, 34-35]; inhibition effect on *C. albicans dihydrofolate reductase* (DHFR), a key enzyme in the biosynthesis of purines, pyrimidines and several amino acids, was pH-independent[36-37]; EGCG also the most potent at retarding the formation and maintenance of Candida biofilm and to disrupt a preformed biofilm.[28, 38] Correlation with neutrophil count and infected cells by *C. albicans* after EGCG green tea administration (Table 1) showed that the increased neutrophil count and the decreased infected cells by *C. albicans* in a murine model.

CONCLUSION

This study proved that EGCG green tea has been significantly increased neutrophil count and decreased infected cells by *C. albicans* in a murine model of oral candidiasis.

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