Available online at www.sciencedirect.com

ScienceDirect

journal homepage: www.intl.elsevierhealth.com/journals/dema

Cytotoxicity and anti-inflammatory effects of zinc ions and eugenol during setting of ZOE in immortalized human oral keratinocytes grown as three-dimensional spheroids

Jung-Hwan Lee[a,b,c], *Hae-Hyoung Lee*[b,c], *Kyoung-Nam Kim*[a], *Kwang-Mahn Kim*[a,*]

[a] *Department and Research Institute of Dental Biomaterials and Bioengineering, BK21 PLUS Project, College of Dentistry, Yonsei University, Seoul, Republic of Korea*
[b] *Department of Biomaterials Science, College of Dentistry, Dankook University, Cheonan, Republic of Korea*
[c] *Institute of Tissue Regeneration Engineering (ITREN), Dankook University, Cheonan, Republic of Korea*

ARTICLE INFO

Article history:
Received 5 February 2015
Received in revised form
22 October 2015
Accepted 15 January 2016

Keywords:
Cytotoxicity
Zinc oxide–eugenol
Immortalized human oral keratinocytes
3D spheroid culture system
Eugenol
Zinc ion

ABSTRACT

Objectives. The objective of this study is to assess the cytotoxic and anti-inflammatory effects of ZOE cement during setting in two-dimensional (2D) or three-dimensional (3D) cultures of immortalized human oral keratinocytes (IHOKs) with determining the extract components responsible for these effects.

Methods. Extracts of mixed ZOE at different stages of setting were analyzed by a digital pH meter, ICP-MS, and GC–MS. Serial concentrations of extract and their mixture of $ZnCl_2$, $ZnSO_4{\cdot}H_2O$, and eugenol liquid were added to the 2D and 3D IHOK cultures to determine the half maximal effective concentration in investigating the cause of cytotoxicity by means of WST assay and to investigate mRNA expression levels of inflammatory cytokines by RT-PCR.

Results. Zn^{2+} and eugenol (4–19 ppm) were detected in the extracts. In the early setting stage, significant cytotoxicity was observed in the 2D and 3D IHOK cultures ($P < 0.05$). The EC50 of Zn^{2+} from $ZnCl_2$ was 5–44 ppm in both cultures, whereas the EC50 of eugenol was not detectable under 100 ppm. Along with the lower levels of inflammatory cytokine gene expressions in the extract, treatment of the 2D IHOKs with Zn^{2+} alone and treatment of the 3D IHOKs with Zn^{2+} plus eugenol resulted in significantly lower expression levels of IL-1β, IL-6, and IL-8 ($P < 0.05$).

Significance. The cytotoxic effect of ZOE on IHOKs was greater during the setting stage owing to the presence of Zn^{2+}. The anti-inflammatory response to ZOE was induced by a combination of Zn^{2+} and eugenol. Cytotoxic and anti-inflammatory effects differed between the 2D and 3D IHOK cultures.

* *Corresponding author at*: Department and Research Institute of Dental Biomaterials and Bioengineering, Yonsei University, College of Dentistry, 50-1 Yonsei-ro, Seodaemun-gu, Seoul 03722, Republic of Korea. Tel.: +82 2 2228 3082; fax: +82 2 364 9961.
E-mail address: kmkim@yuhs.ac (K.-M. Kim).

http://dx.doi.org/10.1016/j.dental.2016.01.003

1. Introduction

Zinc oxide–eugenol (ZOE) cement has been widely used in dentistry for temporary restorations or cementations because of its easy handling, low cost, excellent cavity-sealing ability, antibacterial properties, and sedative effects on sensitive teeth [1]. ZOE is a material created by combining zinc oxide (ZnO) powder with eugenol liquid extracted from the oil of cloves. An acid–base reaction occurs when hydrated ZnO is mixed with eugenol, resulting in the formation of a long crystal matrix of zinc eugenolate chelate [2]. However, ZOE is cytotoxic and inhibits the polymerization of resin cement [3,4]. Zinc oxide–non-eugenol (ZONE) cement has been developed as an alternative to ZOE and is more compatible with resin cement in terms of bonding strength, resin polymerization, and lower cytotoxicity [5].

A few reports have documented the adverse effects of ZOE, such as increased inflammation in oral mucosal tissue and the recession of gingiva adjacent to the treated tooth [6,7], possibly owing to direct or indirect contact between the cement and the mucosa. Direct contact results in soft-tissue irritation and is therefore avoided; indirect contact with dental material extracts has been reported to have adverse effects on the oral mucosa or gingiva [8,9]. In addition, extracts from set ZOE cement have exhibited soft-tissue cytotoxicity in vitro [10]. In its freshly set state, ZOE is more cytotoxic than in its final set state, and the degree of cytotoxicity decreases substantially as setting takes place. In the clinical situation, the setting time can be accelerated by exposing ZOE to saliva before the final stage of setting; consequently, the cytotoxicity of the ZOE extract is also increasing up. However, no studies have reported data on cytotoxicity during setting to mimic clinical circumstance.

Eugenol, a component of ZOE extracts, has been considered to be a primary factor inducing adverse effects on the oral mucosa [11,12]. The production or acceleration of erythema as well as ulceration has been associated with eugenol exposure [13]. One study showed a strong correlation between cytotoxicity and the release of eugenol from ZOE extracts [14]; however, the pattern of eugenol release relative to toxicity was not consistent, suggesting that other factors might be involved (e.g., the presence of zinc ions [Zn^{2+}]). Although the use of ZOE cement has been reported to have adverse inflammatory effects on the oral mucosa [15,16], eugenol has also been reported to have anti-inflammatory effects on human dental pulp cells and human skin mucosa [17,18]. Because the adverse (or, conversely, the therapeutic) effects of eugenol were found to depend on the concentration applied [17], it is necessary to determine the concentrations of all components of the ZOE extract to evaluate their cytotoxic and anti-inflammatory effects on oral keratinocytes, which make up the outer layer of cells of the oral mucosa [19,20].

An evaluation of the biocompatibility of dental materials (e.g., through in vitro cytotoxicity assays) is an essential step in determining whether such materials are suitable for clinical use [21,22]. However, previous studies of ZOE cements have involved animal-based cell cytotoxicity tests with preset cements and thus lack clinical relevance to human oral cells [10,14,23,24]. Cytotoxicity levels are known to depend on the type of cell line involved, and the levels differ between animal and human cells [10,25]. Because ZOE cement would be exposed to human oral saliva, the ZOE extracts would affect human oral keratinocytes in the mucosa during setting.

The biocompatibility of ZOE cement is commonly evaluated using two-dimensional (2D) cell monolayer structures, in keeping with methods established by the International Organization for Standardization (ISO), using a mouse fibroblast cell line (L929) [22,26]. However, these structures are not physiologically relevant to complex three-dimensional (3D) tissues. The results of cytotoxicity assays involving 2D structures may overestimate susceptibility, thereby exaggerating the cytotoxic effects of ZOE and limiting the clinical applications of ZOE cements [27,28]. Recently, studies have shown that tissue-engineered 3D oral mucosal models are biocompatible owing to their physiological similarity to the structure in humans [28,29]. However, high cost and complex preparation have limited the use of such models in evaluating the cytotoxicity of dental materials [30]. As an alternative, 3D spheroid culture systems have been developed in which small cell aggregates are cultured in suspension. Because of their high degree of clinical and biological relevance to natural tissues, spheroid cultures are being widely used to evaluate the cytotoxicity and effectiveness of drugs in vitro [31,32].

Cytokines are potent local mediators of inflammation and are produced by a variety of cells [33]. The cytokines expressed in epidermal epithelial cells (keratinocytes) have been investigated as potential markers of cellular injury that induce soft-tissue damage through inflammation. These cytokines include interleukin (IL)-1β, IL-6, and IL-8 [34]. Owing to the presence of eugenol, ZOE is known to have anti-inflammatory effects [35]. However, its protective effects against inflammatory cytokines in oral keratinocytes have not been studied.

The aim of this study was to assess the cytotoxic and anti-inflammatory effects of ZOE using 2D and 3D cultured immortalized human oral keratinocytes (IHOKs) and to determine the component(s) of the ZOE extract responsible for inducing such effects. According to our first null hypothesis, the cytotoxic and anti-inflammatory effects observed in the spheroidal 3D model do not differ significantly from those observed in the 2D monolayer culture system. Our second null hypothesis states that the eugenol extracts from ZOE significantly induce cytotoxic and anti-inflammatory effects in human oral keratinocytes.

2. Materials and methods

2.1. *Extract of ZOE cement*

We selected IRM® (intermediate restorative material) (Lot No. 131022, Dentsply, Tulsa, OK, USA) from among the various commercially available ZOE cements owing to its high cytotoxicity [10]. The IRM® was stored under conditions recommended by the manufacturer, and IRM® extracts were prepared in keeping with international standards [36,37]. Briefly, the IRM® powder and liquid were mixed for 2 min on a mixing pad according to the manufacturer's instructions (23 °C, relative humidity 20%). The mixed cement solutions were incubated at 37 °C in a humidified incubator (VS-9160C)