Original Article

Comparative Cytotoxicity and Biocompatibility of Two Aluminum Chloride Local Hemostatic Agents

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Abstract

The objective of this study was to evaluate the cytotoxicity and biocompatibility of two aluminum chloride containing local hemostatic agents, Racestyptine and a local hemostatic agent prototype, Dent-Chula100®. To determine cytotoxicity, primary human gingival and pulpal fibroblasts were treated with the local homeostatic agents at concentrations of 0.1, 1.0, 2.5, 5.0 or 10.0 % (v/v) for 15 minutes. The MTT assay was used, with untreated cells serving as control. The biocompatibility test was performed using the lower incisors of twelve 8-week old male Sprague Dawley rats. The free gingival margin of the lower central incisors was temporarily displaced for 15 minutes using retraction cords soaked with Racestyptine or Dent-Chula100®. Cords soaked with normal saline served as a control. Seven days after treatment, the lower jaws were dissected. The H&E stained tissue sections were histopathologically examined for four pathologic index scores; degree of sulcular epithelium damage, collagen fiber appearance and orientation, inflammation and vascular reaction. Two-way ANOVA and Kruskall-Wallis tests were performed for statistical analysis of the *in vitro* and *in vivo* studies, respectively ($p < 0.05$). Our data revealed that Dent-Chula100® at 5.0 % - 10.0 % significantly reduced gingival fibroblast cell viability compared with the control group ($p < 0.05$), while Racestyptine at 2.5 % - 10.0 % significantly decreased cell viability ($p < 0.05$). Both Dent-Chula100® and Racestyptine at 2.5 % significantly reduced pulpal fibroblast cell viability ($p < 0.05$). The histopathological data indicated that the periodontal tissues did not incur any significant damage

after exposure to Dent-Chula100® or Racestyptine. In conclusion, the cytotoxicity of Dent-Chula100® and Racestyptine was dose-dependent. The *in vivo* study revealed that both Dent-Chula100® and Racestyptine were biocompatible with gingival tissues.

Key words: Aluminum chloride; Biocompatibility; Cytotoxicity; Local hemostatic agent

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Introduction

Local hemostatic agents have been widely used in prosthodontic and operative dentistry because of their effectiveness, safety and convenience.[1,2] Ideally, local hemostatic agents should provide not only a sufficient hemostatic effect, but also be biocompatible with oral tissues.[3,4] To reduce the cost of dental treatment and strengthen Thailand's biomaterials research, our group has developed a local hemostatic agent prototype, Dent-Chula100®.

A previous study has reported that Dent-Chula100® did not affect the physical and mechanical properties of the light-cured resin composite filling materials evaluated.[5] Following ISO 4049:2009, Dent-Chula100® was demonstrated to not affect the solubility, water absorption, depth of cure or flexural strength of the light-cured resin composite materials. However, the cytotoxicity and biocompatibility of this local hemostatic agent have not been investigated. Therefore, the purpose of this study was to examine the cytotoxic effect of Dent-Chula100® on primary human gingival and pulpal fibroblasts. The biocompatibility of this local hemostatic prototype on gingival tissue was also investigated using an animal model.

Materials and Methods

The hemostatic agent Racestyptine (Septodont, PA, USA; 25 % aluminum chloride, oxyquinol and hydroalcoholic excipient) was used as the reference material compared with our local hemostatic agent prototype Dent-Chula100® (Research Unit of Herbal Medicine, Biomaterial and Material for Dental Therapy, Faculty of Dentistry, Chulalongkorn University, BKK, Thailand; 25 % aluminum chloride, 30 % ethanol and distilled water). The Racestyptine's expiration date was at least 6 months after the experiments were performed.

In vitro study

pH measurement

The pH of the local hemostatic agents was measured with a pH meter (Orion 420A pH Meter, Orion Research Inc., Boston, MA, USA). The measurements were repeated 3 times. The results were expressed as mean and standard deviation.

Cell culture

Following a protocol approved by the Human Research Ethics Committee of the Faculty of Dentistry, Chulalongkorn University, Thailand (HREC-DCU 2012-043), pulpal and gingival tissues were obtained from non-carious, impacted third molars and adjacent non-inflamed healthy gingiva of 18 - 25 year-old healthy donors. The tissues were washed 3 times with phosphate buffered saline solution (PBS), cut into 1 - 2 mm^3 pieces and placed into 35-mm culture dishes. The explants were incubated with growth media (Dulbecco's modified Eagle's medium (DMEM) supplemented with 10 % fetal bovine serum, 10,000 IU/ml penicillin G sodium, 100,000 µg/ml streptomycin sulfate, 25 µg/ml amphotericin B, and 1 % L-glutamine) at 37°C in a 5 % CO_2 atmosphere. The media was replaced every two days. When the cells reached confluence, the cells were subcultured using 0.25 % trypsin - EDTA solution. All materials were obtained from Sigma-Aldrich, St. Louis, MO, USA. In the present study, the experiments were performed using

cells from the third - fifth passages. Three donor lines were evaluated.

MTT viability assay

The MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay was performed as previously described.[6] Briefly, 5 x 104 cells per well were seeded in 24-well plates for 24 h. The cells were then washed twice with PBS and treated with the local hemostatic agents at concentrations of 0.1, 1.0, 2.5, 5.0 or 10.0 % (v/v) in growth media for 15 minutes. The pH of each final concentration of the hemostatic agents in DMEM is shown in Table 1. Cells incubated with growth medium alone served as the control group. Subsequently, the cells were washed 3 times with PBS and incubated with growth media for 24 h.

To assess cell viability, the cells were washed twice with PBS and incubated with 0.5 mg/ml MTT solution for 10 minutes. The precipitated formazan crystals were dissolved using dimethyl sulfoxide (DMSO). The optical density was determined by measuring the light absorbance at 570 nm with an Epoch Microplate Spectrophotometer (BioTek Instrument, Winooski, VT, USA). The background DMSO absorbance was subtracted from the sample absorbance. The experiments were repeated 3 times.

Table 1 *pH of the hemostatic agents in DMEM*

Concentration of the hemostatic agent in DMEM (V/V)	pH	
	Dent - Chula100®	Racestyptine
100.0	1.79 ± 0.01	0.97 ± 0.09
10.0	3.70 ± 0.02	3.56 ± 0.06
5.0	4.00 ± 0.02	3.85 ± 0.03
2.5	4.80 ± 0.02	4.50 ± 0.07
1.0	6.85 ± 0.01	6.44 ± 0.03
0.1	7.22 ± 0.07	7.20 ± 0.03

Note: pH of DMEM = 7.23 ± 0.04

In vivo study

Following a protocol approved by the Animal Research Ethics Committee of the Faculty of Dentistry, Chulalongkorn University, Thailand (Protocol No. 1132001), twelve male Sprague Dawley rats (8 weeks old, average weight 350 g) were obtained from the National Laboratory Animal Center, Nakhon Pathom, Thailand. The rats were kept on a 12-h light/12-h dark cycle, fed a standard pellet diet and allowed access to water and food ad libitum. The animals received general anesthesia consisting of 80 mg/kg Zoletil® (chloral hydrate tiletamine and chloral hydrate zolazepam; Virbac Laboratories, Carros, France). The twenty-four lower incisors were washed with normal saline and randomly divided into 3 groups: normal saline (control), Racestyptine-, and Dent-Chula100®-treated groups. Fifteen mm of retraction cord (No. 000, Ultrapack, Ultradent Products, Inc., UT, USA) were soaked with 100 µl of the respective group's material for 10 minutes. The free gingival margins of the incisors were temporarily displaced by the soaked retraction cord for 15 minutes and the cords were then removed.

Seven days after treatment, the animals were sacrificed. The lower jaws were dissected, fixed in 10 % neutral formalin buffer and demineralized in 10 % formic acid. The tissues were dehydrated using graded ethanol-acetone solutions, followed by paraffin embedding. Five-µm thick serial sections were prepared in the sagittal (labio-lingual) plane. Three sections were selected from each specimen. The first section was obtained from the center of the tooth. The 2nd and 3rd sections were obtained 100 µm mesially and distally to the first section, respectively. The sections were stained with hematoxylin and eosin (H&E), scanned and captured using the OLIVIA program (Olympus, Tokyo).

Histomorphometric analysis

Histomorphometric analysis was performed on the stained sections using the Image Pro-Plus program, version 6.0 (Media Cybernetics, USA). The examined area was localized as shown in Fig. 1. The *a* line was parallel to the root surface. The apical border (*b* line) and occlusal border (*c* line) of the localized area were set by the lines drawn through the lowest point of the junctional epithelium and the highest point of the free gingival margin, respectively. The *b* and *c* lines were perpendicular to the *a* line (Fig. 1).

Four histopathological indexes (Table 2) consisting of degree of sulcular epithelium damage, collagen fiber appearance and orientation, inflammation and vascular reaction were modified from Akca *et al.*[7] and used to evaluate each stained section.

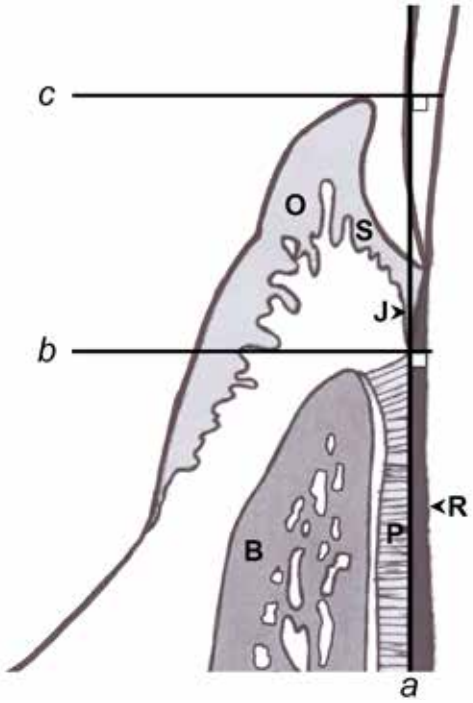

Figure 1 *Schematic illustration of the localized area of the gingival sulcus and surrounding periodontium. J = junctional epithelium, S = sulcular epithelium, O = oral epithelium, B = alveolar bone, P = periodontal ligaments, R = root surface.* The *a* line is parallel to the root surface, *b* line = apical border of the localized area that was set at the lowest point of the junctional epithelium, *c* line = occlusal border of the localized area that was set at the highest point of the free gingival margin, *b* and *c* lines are perpendicular to the *a* line.

Table 2 Histopathological index *[modified from Akca et al.[7]]*

Score	Degree of sulcular epithelium damage
0	No damage (epithelium is attached to the connective tissue.)
1	Slight damage (epithelium attachment to connective tissue is disrupted.)
2	Moderate damage (epithelial desquamation is evident.)
3	Severe damage (epithelium is completely detached from the connective tissue.)
Score	**Collagen fiber appearance and orientation**
0	No alteration in connective tissue (collagen fibers intact and orientation normal)
1	Minor alteration in the connective tissue (collagen fibers intact but orientation disrupted)

Table 2 Histopathological index [modified from Akca et al.[7]] (continue)

Score	**Collagen fiber appearance and orientation** (Continue)
2	Moderate alteration in connective tissue (collagen fibers disrupted and fragmented, but identifiable; orientation of fibers disrupted)
3	Severe alteration in connective tissue (collagen fibers are not identifiable; the region appears as an amorphous mass)
Score	**Inflammation** (Cells were counted under a 40X object magnification)
0	No inflammation
1	Slight inflammation [minor inflammatory cells in the region, < 10 cells/localized area (40X)]
2	Moderate inflammation [identifiable inflammatory cells in the region, 10 - 50 cells/localized area (40X)]
3	Severe inflammation [abundant inflammatory cells in the region, > 50 cells/localized area (40X)]
Score	**Vascular reaction** (Cells were counted under a 40X object magnification)
0	Normal, no vasodilation, no congestion
1	Small amount of congested blood vessels [< 10 vessels/localized area (40X)]
2	Moderate amount of congested blood vessels [10 - 20 vessels/localized area (40X)]
3	Large amount of congested blood vessels [> 20 vessels/localized area (40X)] or dilated blood vessels

Statistical Analysis

The data were collected and analyzed using the SPSS program for Windows, version 17.0 (SPSS, Chicago, IL, USA). For the *in vitro* study, the results were expressed as mean ± standard deviation. The percentage of cell viability was statistically analyzed by two-way analysis of variance (ANOVA) and the Bonferroni multiple comparison test. For the *in vivo* study, the results were expressed as median and interquartile range. The scores of each index were analyzed by the Kruskal-Wallis test to evaluate the differences between groups. Values of $p < 0.05$ were considered as significant.

Results

Cytotoxicity of the local hemostatic agents on gingival fibroblasts

The mean pH of Dent-Chula100® and Racestyptine was 1.79 ± 0.01 and 0.97 ± 0.09, respectively (Table 1). Racestyptine significantly decreased the gingival fibroblast cell viability at concentrations of 2.5 %, 5.0 % and 10.0 % (v/v), while Dent-Chula100® significantly decreased cell viability at concentrations of 5.0 % and 10.0 % (v/v), compared with the untreated group ($p < 0.05$, Fig. 2). Moreover, at concentrations of 2.5 %, 5.0 % and 10.0 % (v/v), the viability of Dent-Chula100®-treated gingival fibroblast group was significantly higher than that of the Racestyptine-treated group ($p < 0.05$, Fig. 2).

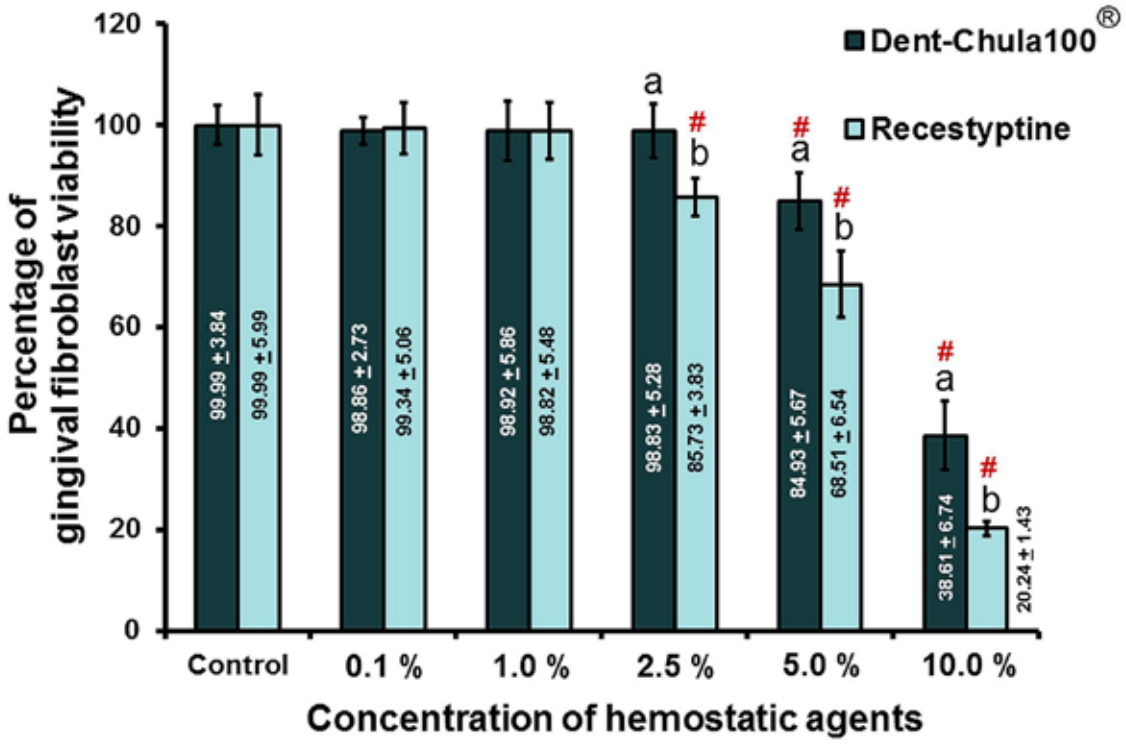

Figure 2 *Percentage of cell viability of human gingival fibroblasts treated with the local hemostatic agents for 15 minutes and incubated with growth medium for 24 h (MTT assay). Bars labeled with # indicates statistically significant difference between treated and control groups ($p < 0.05$, $N = 3$). Bars labeled with different letters (a, b) indicates statistically significant difference between the Dent-Chula100® and Racestyptine-treated groups ($p < 0.05$, $N = 3$).*

At concentrations of 0.1 % and 1.0 % (v/v), the pulpal fibroblast viability in both local hemostatic agent groups was not significantly different from the untreated group. However, at higher concentrations, a significant difference was detected. Dent-Chula100® and Racestyptine significantly decreased pupal cell viability at concentrations of 2.5 %, 5.0 % and 10.0 % (v/v) compared with the control ($p < 0.05$, Fig. 3). However, there were no significant differences between the Dent-Chula100® and Racestyptine groups at any concentration (Fig. 3).

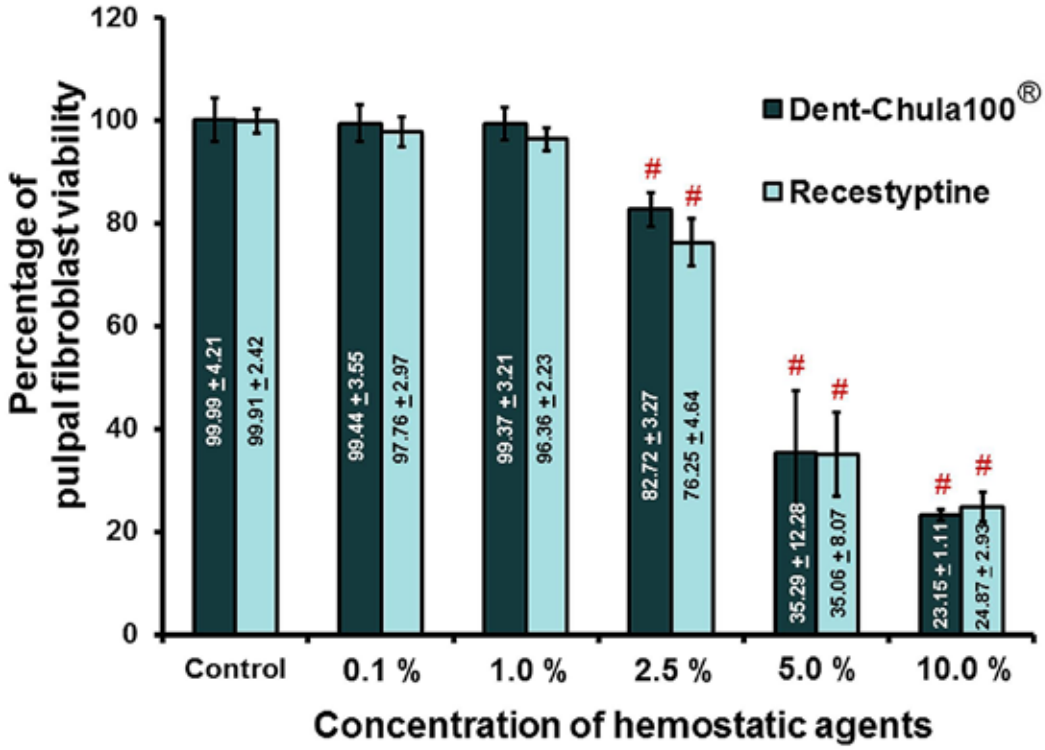

Figure 3 *Percentage of cell viability of human pulpal fibroblasts treated with the local hemostatic agents for 15 minutes and incubated with growth medium for 24 h (MTT assay). Bars labeled with # indicates statistically significant difference between treated and control groups ($p < 0.05$, $N = 3$). Pulpal fibroblast cell viability was not significantly different between the Dent-Chula100® and Racestyptine-treated groups at any concentration ($N = 3$).*

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Gingival and periodontal tissue response to the local hemostatic agents

No animals died during or after the experiment. Seven days after treatment, clinically healthy gingiva was observed in all groups. The histopathologic assessment was evaluated based on 4 criteria; degree of sulcular epithelium damage, collagen fiber appearance and orientation, inflammation and vascular reaction.

The percentages of normal sulcular epithelium observed in the control group, Dent-Chula100® and Racestyptine groups were 50 %, 37.5 % and 37.5 %, respectively. The scores of the degree of sulcular epithelium damage were not significantly different between the control, Dent-Chula100® and Racestyptine groups (Table 3). In all the groups, 100 percent normal collagen fiber appearance and orientation was observed (Table 4, Fig. 4). The Dent-Chula100® and Racestyptine groups demonstrated slightly more inflammation and amount of congested blood vessels than those of the control group. However, the scores of the inflammation and vascular reaction of the control, Dent-Chula100® and Racestyptine groups were not significantly different (Tables 5 - 6, Fig. 4). Taken together, these results indicate that the control group had a slightly healthier tissue appearance than that of the Dent-Chula 100® and Racestyptine groups.

Table 3 *Sulcular epithelium damage*

Groups	Degree of sulcular epithelium damage					
	Score = 0 (N)	Score = 1 (N)	Score = 2 (N)	Score = 3 (N)	Median	Interquartile range
Control (N = 8)	4	3	1	0	0.5	1
Dent-Chula100® (N = 8)	3	4	1	0	1	1
Racestyptine (N = 8)	3	3	2	0	1	1.25

Table 4 *Collagen fiber appearance and orientation*

Groups	Degree of sulcular epithelium damage					
	Score = 0 (N)	Score = 1 (N)	Score = 2 (N)	Score = 3 (N)	Median	Interquartile range
Control (N = 8)	8	0	0	0	0	0
Dent-Chula100® (N = 8)	8	0	0	0	0	0
Racestyptine (N = 8)	8	0	0	0	0	0

Table 5 *Inflammation*

Groups	Degree of sulcular epithelium damage					
	Score = 0 (N)	Score = 1 (N)	Score = 2 (N)	Score = 3 (N)	Median	Interquartile range
Control (N = 8)	5	3	0	0	0	1
Dent-Chula100® (N = 8)	4	4	0	0	0.5	1
Racestyptine (N = 8)	3	5	0	0	1	1

Table 6 *Vascular reaction*

Groups	Degree of sulcular epithelium damage					
	Score = 0 (N)	Score = 1 (N)	Score = 2 (N)	Score = 3 (N)	Median	Interquartile range
Control (N = 8)	2	1	3	2	2	1.5
Dent-Chula100® (N = 8)	0	2	3	3	2	1.25
Racestyptine (N = 8)	0	2	2	4	3	1

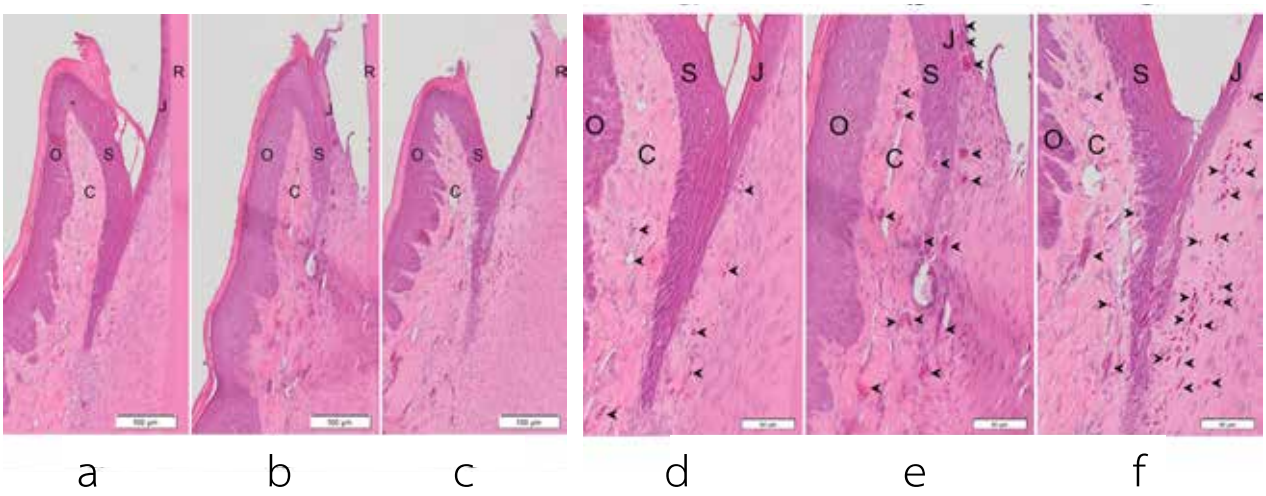

Figure 4 *Representative histopathological images of the gingival sulcus of the control group (a, d), Racestyptine group (b, e) and Dent-Chula100® group (c, f) 7 days after treatment at 10X magnification (a - c) and 40X magnification (d - f). Normal appearance of the sulcular and junctional epithelium is present in all groups. Normal appearance of the collagen fiber orientation is also seen. Inflammatory cells are rarely seen in any group. Dilated and congested capillaries are present in the Racestyptine and Dent-Chula100®-treated groups. J = junctional epithelium, S = sulcular epithelium, O = oral epithelium, C = connective tissue, R = root surface, black arrowheads = congested capillaries, scale bar = 100 µm (a - c), 50 µm (d - f).*

Discussion

Local hemostatic agents have been used in operative and prosthodontic dentistry for hemostasis and in retraction cord used in the temporary displacement of the free gingival margin. Both clinical and animal studies have shown that gingival retraction methods tend to produce transient damage to the gingival sulcus epithelium, junctional epithelium and the underlying gingival tissue.[7,8] Consequently, gingival fibroblasts can be directly exposed to local hemostatic agents via the disruption of the epithelial layer by the retraction procedure.[9-13] In addition to its use in retraction cord for the temporary displacement of the gingival margin, local hemostatic agents are also used to control pulp bleeding before direct pulp capping and pulpotomy procedures.[14-16] Therefore, the cytotoxicity of the hemostatic agents on human gingival fibroblasts and dental pulp fibroblasts was evaluated in our study.

The clinical application of gingival retraction cord is usually not longer than 10 minutes.[17] However, local hemostatic agents can remain in the gingival sulcus after removing the retraction cord. To confirm the toxicity and biocompatibility of the two hemostatic agents in our study, we used an exposure time of 15 minutes in our *in vitro* and *in vivo* studies.

Cytotoxicity evaluation is necessary to assess the biocompatibility of dental materials[6]. According to ISO 10993-5, the MTT assay is an acceptable method to evaluate the cytotoxicity of a biomaterial. This assay measures the conversion of a yellow water-soluble MTT dye into a purple formazan crystal that is produced by active mitochondria.[6] This colorimetric method is recommended as an economic, accurate and reliable test for cytotoxicity determination.[18,19] The MTT test not only evaluates cell - biomaterial interaction, but also relates to the number of viable cells.[6,20] In our study, the MTT assay was used to determine the viability of cells after exposure to local hemostatic agents. Aluminum chloride, the active ingredient in Dent-Chula100® and Racestyptine, has been reported to be an effective and commonly used hemostatic agent.[17,21,22] The hemostatic effect of aluminum chloride is due to its aluminum ions, which act primarily by precipitating tissue proteins and inhibiting the transcapillary movement of plasma proteins.[2,21] Moreover, aluminum ions have no systemic contraindications and few effects on vasoconstriction.[2,23]

The cytotoxicity of Dent-Chula100® and Racestypine likely results from their active ingredient, aluminum chloride.[4,7,16] Acidity is an important property of aluminum chloride. To prepare a hemostatic agent, aluminum chloride is dissolved in alcohol. This reaction turns the neutral pH of alcohol into a powerful acidic solution. Thus, higher concentrations of aluminum chloride results in increased acidity of that solution. Indeed, we observed that cell proliferation was not affected at aluminum chloride pH levels near physiologic pH. In contrast when the aluminum chloride pH dropped precipitously at concentrations of 2.5 % and above, cell cytotoxicity rose in kind. The results of our study indicated that the local hemostatic agents, at concentrations of 2.5, 5.0 and 10.0 %

(v/v) reduced the cell viability of gingival and pulpal fibroblasts. These results are consistent with those of Kopac *et al*. who found that 10 %, 50 % and 100 % (v/v) of 25 % aluminum chloride in culture medium reduced cell viability.[3,4]

In previous animal and clinical studies, aluminum chloride has been reported to have a deleterious effect on tissue.[7,8,13] Used as a hemostatic agent, aluminum chloride caused hydrotropic degeneration, hyperemia and an inflammatory cell infiltration.[13] However, aluminum chloride is still the most popular gingival retraction agent.[2,9] In our study, histomorphometric analysis showed that 25 % aluminum chloride was biocompatible with the sulcular epithelium and underlying connective tissue compared with normal saline.

We found that inflammatory cells were rarely detected in the tissue sections of any group 7 days post-treatment. From our review of the literature, studies on the effect of aluminum chloride on the inflammatory cell response are inconclusive.[7-9] Kopac *et al*. reported that Racestypine-treated gingival tissue in animals showed a severe inflammatory cell infiltration 7 days after treatment.[8] In contrast, an animal study by Acka *et al*. found that aluminum chloride only induced a slight inflammatory cell infiltration into the gingival tissue.[7] Moreover, a clinical study by de Gennaro *et al*. revealed that there was no significant difference in gingival inflammation between the untreated control group and aluminum chloride-treated group 7 days post-treatment.[9] A possible explanation for this variation is the differences in immune responses among rats, dogs and humans.[7]

A vascular reaction was the only sign of inflammation present in our histologic results. A vascular reaction is an early sign of acute inflammation.[24] Vasodilation and increased blood vessel permeability occurring after a chemical stimulus results in decreased blood flow and dense packing of red blood cell in capillaries.[23-25] In our study, more congested and dilated capillaries were found in the Dent-Chula100® and Racestyptine groups than that of the control group; however, the difference was not significant.

In our *in vivo* study, the power analysis of the four histopathological indices; degree of sulcular epithelium damage, collagen fiber appearance and orientation, inflammation and vascular reaction, were varied. For the inflammation and vascular reaction indexes, the power analysis, 0.85 and 1.00, respectively, were acceptable. However, the power analysis of the collagen fiber appearance and orientation and degree of sulcular epithelium damage were low (0 and 0.27, respectively). The reason for this is that none or very slight differences were observed between the groups. To achieve the appropriate power of all 4 indices, 180 animals for the experimental, commercial and control groups were required. Therefore, to minimize the number of animals used in our study, the sample size was set as eight for each group.

Dent-Chula100® has demonstrated *in vivo* biocompatibility at 7 days post-treatment and did not affect the physical and mechanical properties of light-cured resin composite filling materials.[5] Future clinical studies on the safety and effectiveness of Dent-Chula100® are still required.

Conclusion

In our *in vitro* study, Dent-Chula100® had a cytotoxic effect on human gingival fibroblasts and pulpal fibroblasts at 2.5 % and 5 % v/v, respectively. However, Dent-Chula100® and Racestyptine were biocompatible with gingival tissues seven days after treatment. Our data suggests that Dent-Chula100® is a safe local hemostatic agent.

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