Cytotoxicity and Antibacterial property of New Resin-based Sealer

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국문초록

새로운 레진 계통의 근관봉함재의 독성과 항균 작용에 대한 연구

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이 연구는 기존의 레진 근관봉함재를 보완하여 개발한 근관봉함재(Adseal; 새로운 레진 계통의 근관봉함재)를 이미 상품화된 레진 계통의 근관봉함재(AH 26, AH Plus), 산화 아연 유지놀 계통의 근관봉함재(TubliSeal EWT , Pulpcanal sealer EWT), 수산화 칼슘 계통의 근관봉함재(Sealapex)와 비교하여 세포독성과 항균작용을 평가하고자 한다. 세포독성 실험은 L929 쥐의 섬유아세포를 사용하여 세포의 viable ratio를 계산한 후, Giemsa stain으로 염색하여 세포의 양상을 관찰하였고, 항균작용 실험은 *Enterococcus faecalis, Porphyromonas endodontalis, Porphyromonas gingivalis, Prevotella intermedia, Fusobacterium nucleatum* 와 *Fusobacterium necrophorum* 를 사용하여 agar diffusion test 로 평가한다. Adseal은 다른 근관봉함재에 비해 훨씬 낮은 세포독성을 보였고, AH Plus, AH 26, TubliSeal EWT, Sealapex, Pulpcanal sealer EWT의 순으로 세포독성의 정도가 높아짐을 알 수 있었다. 또한 Adseal은 *Enterococcus faecalis* 에서는 낮은 항균작용을 보이지만, Black-pigmented bacteria 에서는 높은 항균작용을 보이는데, 모든 근관봉함재는 서로 다른 종에 따라 어느 정도의 항균효과를 가지고 있음을 알 수 있었다.

주요어 : 근관봉함재, 세포독성, 항균작용, Adseal

Ⅰ. Introduction

The main objective of a successful endodontic treatment is the proper cleaning and shaping of the root canal, as well as a total obturation of the canal space with an inert, dimensionally stable and biologically compatible material. A large variety of root canal filling materials have been used through the years. To achieve an effective seal and promote healing, a root canal sealer should possess certain characteristics. Grossman identified several characteristics of the ideal sealer. One of these is that it must have bactericidal or bacteriostatic activity. Several antibacterial agents have been added to root canal sealers to improve this effect. In addition, it should be completely compatible with the periapical tissue, being neither toxic nor inflammatory[1,2].

There have been many reports showing that resin-based sealers have better physical characteristics than zinc oxide-eugenol-based sealers, and that AH 26, in particular, which is composed mainly of epoxy resin, is quite effective because of its close adaptation to the root canal walls and its very low contraction rate during setting. As far as the cytotoxicity of resin-based sealers is concerned, there have been conflicting results. However, several researchers have reported that the cytotoxicity is quite strong at the time of mixing but weakens over time[3].

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Recently, domestic research teams have succeeded in developing an improved resin-based sealer. However, it has yet to undergo the necessary tests before it can be introduced to the market.

In general, the biocompatibility of a root canal sealer is assessed using a three-step approach[4]. A first step is to screen a candidate material using a series of *in vitro* cytotoxicity assays. Second, if the material is demonstrated not to be a cytotoxic agent *in vitro,* it can be implanted in subcutaneous tissue and the local tissue reaction can be evaluated. Finally, the *in vivo* reaction of the target tissue with the material must be evaluated in either animals or humans.

In this regard, this study aimed to evaluate the cytotoxicity and antibacterial effect of a new resin-based sealer and compare it with those of other commonly used resin-based, zinc oxide-eugenol-based, and calcium hydroxide-based sealers.

Ⅱ. Materials and methods

Cytotoxicity test

Five root canal sealers (Sealapex ; Kerr ; USA , Tubli-Seal EWT ; Kerr ; USA , Pulpcanal sealer EWT ; Kerr ; USA , AH 26 ; De trey/ Dentsply; Germany , AH Plus ; De trey/ Dentsply; Germany) and the new resin-based sealer (Adseal ; Meta ; Korea) were tested. The components of Adseal are listed in Table 1.

L929 mouse fibroblast cells were used in this study. The culture medium used was Eagle's minimum essential medium (MEM) supplemented with 10% fetal calf serum (FCS) and antibiotics (100lU/ml penicillin and 50㎍/ml streptomycin). The cultures were incubated at 37℃ in a 100% humid 5% CO_2 atmosphere.

The sealers were prepared under aseptic conditions according to the manufacture's instructions and 0.1ml of each sealer was placed in the center of 5cm petri dishes with a micropipette. The sealers were allowed to set for 6hrs, 24hrs, 48hrs, 1wk, and 5wks at 37℃ under ultraviolet light to prevent bacterial contamination. Each dish was covered with a 5ml suspension of the fibroblasts at a concentration of 40,000 cells/ml. Five milliliters of the same cell suspension was dispensed in dishes, which serve as a control.

For each sealer and each observation period, five samples and their respective controls were prepared. All dishes were incubated at 37℃ and the incubation was concluded after 24, 48 and 72hrs. At the end of the incubation period the culture medium was removed and 0.5 ml of a 0.25% (wt/vol.) trypsin solution in PBS was added to each dish to detach the cells. The cell suspension in the dish was mixed with MEM. The viable cells were stained with trypan blue and a small amount of this suspension was dropped into a hemocytometer chamber and all viable cells were counted using optical microscopy.

The number of cells counted in the experiment dishes was calculated as a percentage using the following formula

$$\text{percentage of viable cells} = \frac{A}{B} \times 100$$

where A is the number of viable cells in the experimental dish and B is the number of viable

Table 1. Components of Adseal (Meta;Korea)

	Base	Catalyst
Adseal	Oligomer	Poly (bis-4-amino benzoate)
	Ethylene glycol mono salicylate	Triethanol amine
	Calcium phosphate	Calcium phosphate
	Zirconium oxide	Zirconium oxide
	Bismuth subcarbonate	Bismuth subcarbonate
	Calcium oxide	

cells in the control dish. This procedure was repeated twice for each sealer and each incubation period. One sample of each group was stained with Giemsa stain and the morphology was examined using optical microscopy.

Antibacterial property test(agar diffusion test)

Test microorganism

The antibacterial effect of the sealers were evaluated against *Enterococcus faecalis*(ATCC29212), *Porphyromonas endodontalis*(ATCC35406), *Porphyromonas gingivalis*(ATCC33279), *Prevotella intermedia*(ATCC25611), *Fusobacterium nucleatum*(VPI10197) and *Fusobacterium necrophorum* (ATCC25286). These are commonly isolated from an infected root canal and are employed widely for testing the antimicrobial activity of endodontic materials[5,6,7].

Agar diffusion test

The bacteria were grown from frozen stock cultures in a brain heart infusion (BHI) broth at 37℃. 200 ㎕ of the bacterial suspension (5×10^7) were spread on BHI agar plates. The anaerobic bacteria except *Enterococcus faecalis* were spread over the columbia agar plate supplemented with hemin (5mg/L) and menadione (0.5mg/L) and enriched with rabbit blood.

Freshly mixed specimens of each tested material were prepared by pouring into uniform wells (5mm diameter) punched in the agar. After incubation at 37℃ for 24hrs, the agar plates were examined for any inhibition of bacterial growth. The diameter of the halo formed in the bacteria lawn was measured in millimeters in two perpendicular locations for each sample.

Ⅲ. Results

Cytotoxicity test

The results of this study are summarized in Table 2 and Fig. 1. The experimental results were statistically analyzed by the Kruskal-Wallis test ($P<0.001$), and the Friedman's 2-way ANOVA ($P<0.05$). In the control dishes, the cells appeared viable and morphologically normal. Table 2 shows that Adseal was significantly less cytotoxic compared to the other sealers in the early stage.

Zinc oxide-eugenol-based sealers continuously show low viable rates in comparison with resin-

Table 2. viable cell ratio (percentage) by quantitative method

		6hrs	24hrs	48hrs	1wk	5wks
Sealapex	24hrs	0.5	1.34	7.63	31.25	56.25
	48hrs	0	0	6.75	7.5	43.21
	72hrs	0	0	6.2	4.8	14,57
Tubliseal	24hrs	6	16.65	33.3	50	49.98
EWT	48hrs	2.32	1.43	5	32.14	14.28
	72hrs	2.5	0	3.49	5.14	0
Pulpcanal	24hrs	0	3.75	2.8	0.63	31.83
sealer EWT	48hrs	0	0.6	0	0	2.25
	72hrs	0	0.69	0	0	0.86
AH 26	24hrs	1.25	5.56	33.65	87.5	84.77
	48hrs	7.15	1.4	33.75	45	71.6
	72hrs	3.75	0	17.7	7.1	41.66
AH plus	24hrs	1.5	50	62.25	88.89	87.5
	48hrs	6.93	69.05	54.2	54.28	61.13
	72hrs	7.5	46.26	42.34	81.39	59.5
Adseal	24hrs	66	64.78	72.33	78.16	88.89
	48hrs	42.71	42.02	48.26	47.22	47.14
	72hrs	50.68	65.09	59.91	49.1	46.51

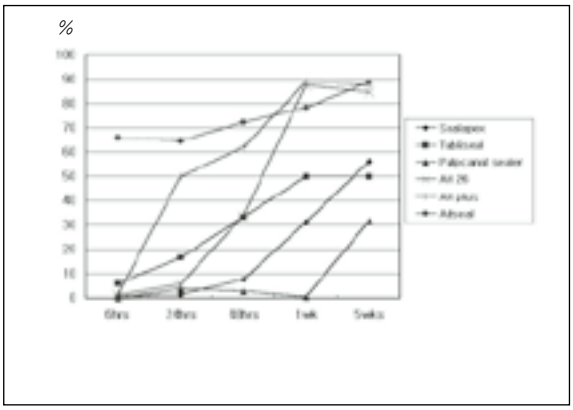

Fig. 1. Viable rate by cytotoxicity test (%)

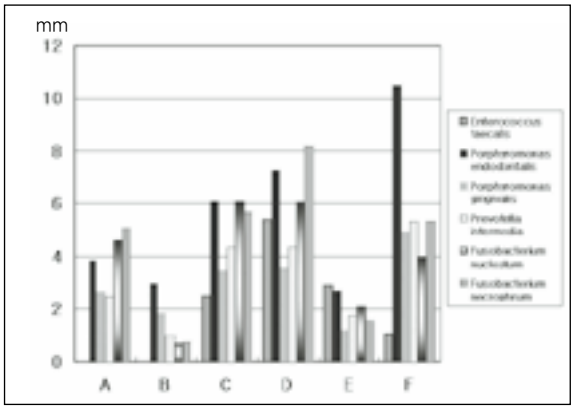

Fig. 2. Inhibition zone by agar diffusion test (mm)
(A : Sealapex, B : Tubliseal EWT, C : Pulpcanal sealer EWT, D : AH 26, E : AH plus, F : Adseal)

Table 3. Inhibition zone (mm) by the agar diffusion test

	Sealapex	Tubliseal EWT	Pulpcanal sealer EWT	AH 26	AH plus	Adseal
Enterococcus faecalis	0	0	2.5	5.39	2.875	1.04
Porphyromonas endodontalis	3.83	2.95	6.08	7.25	2.7	10.5
Porphyromonas gingivalis	2.66	1.83	3.45	3.54	1.20	4.91
Prevotella intermedia	2.5	1	4.33	4.33	1.75	5.33
Fusobacterium nucleatum	4.6	0.70	6.08	6.04	2.08	4
Fusobacterium necrophorum	5.08	0.75	5.70	8.20	1.54	5.33

based ones. In particular, the Pulpcanal sealer EWT had continuously shown severe cytotoxicity before it reached a viable rate of 31.83% in the fifth week. The Tubliseal EWT consistently showed a moderate viability rate.

Sealapex initially showed a very low viability rate. However, the viability rate began to increase after a 48hrs incubation period until it was higher than those of the zinc oxide-eugenol-based sealers in the fifth week.

In the case of resin-based sealers, AH plus exhibited a cytotoxic effect for a shorter period than the AH 26. However, both had far lower viability rates than Adseal. Among those sealers tested, Adseal had the highest viability rate throughout this study.

Fig. 3 shows the cell appearance of each sealer observed by optical microscopy and Giemsa stain at the early stages (6hrs setting and 24hrs incubation). It clearly demonstrates that the cell appearance of Adseal is different from those of the other sealers.

Antibacterial properties test

For all the tested sealers, different antibacterial effects according to the species were observed. Table 3 and Fig. 2 shows the mean inhibition diameter obtained. The experimental results were statistically analyzed by the Kruskal-Wallis test ($P<0.001$).

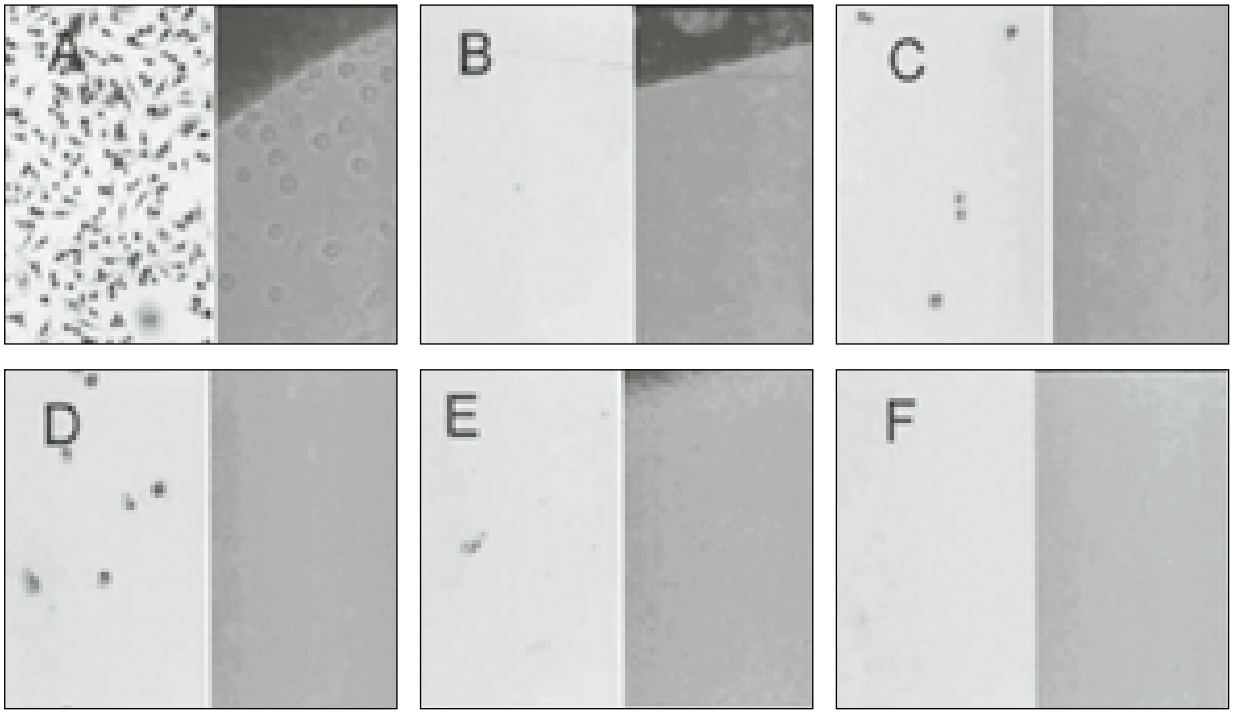

Fig. 3. Cell appearance after 6hrs setting and 24hrs incubation (Giemsa stain and inverted microscope X400)
Here, each sealer has two pictures. The left shows the cell appearance observed by the Giemsa Stain, and the right by inverted microscopy. Many more cells appear in the case of Adseal than they are in the cases of the other sealers.
(A: Adseal, B: AH 26, C: AH plus, D: Tubliseal EWT, E: Pulpcanal sealer EWT, F: Sealapex)

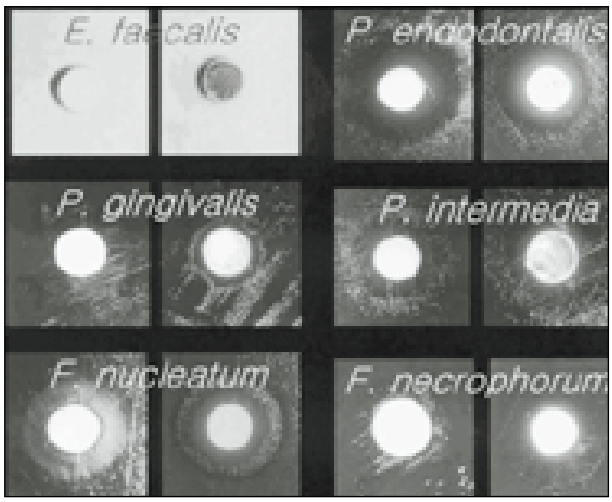

Fig. 4. Inhibition zone by Agar diffusion test (Adseal and AH 26)
This shows inhibition zones of Adseal (left) and AH 26 (right) against each microorganism. Adseal barely works against *E. faecalis*, but it shows very broad inhibition zones against black- pigmented bacteria in general and *P. endodontalis* in particular. These results of the experiment lead us to conclude that in comparison with AH 26, Adseal has excellent antibacterial effects against microorganisms tested excluding *E. faecalis*

AH 26 was more potent than any other sealer against *Enterococcus faecalis*. Adseal had a low inhibitory effect. Sealapex and Tubliseal EWT barely works against *E. faecalis*. However, Adseal's activity was as great as observed with AH 26 against black-pigmented anaerobic rods. In addition, it had a very high inhibition effect particularly against *P. endodontalis* in comparison with the other sealers tested. The Pulpcanal sealer and AH26 showed a high inhibition effect against the Fusobacterium species while Adseal had a moderate antibacterial property.

Fig. 4 shows the inhibition zones of Adseal and AH 26 against each microorganism. It indicates that although Adseal had a lower antibacterial effect against *E. faecalis* than AH26 had, it showed excellent effects against the other species

Ⅳ. Discussion

A good sealer should be biocompatible and well

tolerated by the periradicular tissues. All sealers exhibit toxicity when freshly mixed. However, their toxicity is greatly reduced after setting. All sealers are resorbable when exposed to tissues and tissue fluids. Subsequent tissue healing or repair generally appears to be unaffected by most sealers, provided there are no adverse breakdown products of the sealer over time.

It has been established that endodontic failures are related to the presence of bacteria in the root canal. Anaerobic bacteria, mainly black pigmented Gram-negative bacteria and Fusobacterium, have been linked to the signs and symptoms of endodontic disease. Consequently, research in endodontics evaluating the antibacterial effects of root canal sealers must be directed toward these microorganisms. In addition, *E. faecalis* is one of the most resistant species in the oral cavity and may cause endodontic infections that are difficult to treat[5,6].

A percentage of viable cells are generally interpreted to represent the level of cytotoxicity of the test materials. A new resin-based sealer,

Adseal, had a relatively high value for all time periods tested combinations. In addition, the Pulpcanal sealer EWT, a zinc oxide eugenol - based sealer, had the lowest viability rate. In Table 2, the Pulpcanal sealer EWT and Tubliseal EWT showed moderate viability rates in the fifth week after they initially showed severe cytotoxicity. However, compared to the resin-based sealers, they showed a very high cytotoxicity in the fifth week. The toxic effects of zinc oxide eugenol-based sealers have been extensively studied. The cause of the cytotoxicity may be free eugenol remaining in the zinc oxide and eugenol mixture[8].

Sealapex had very low viability rates at the early stage like the Pulpcanal sealer EWT, but 48 hours later, its cytotoxicity began to decrease to a very low level. According to Gordon et al[9], the cytotoxicity by calcium hydroxide is due to its strong alkalinity. The culture substrate surrounding Sealapex showed a much larger amount of disintegrating small particles in comparison to the other materials. This might be attributed to the capability of calcium hydroxyl ions to diffuse and precipitate from the set sample in the medium[10,11]. It showed only slight toxicity in the fresh state. However, it exhibited strong toxicity in the set state. The hardened Sealapex was not completely hardened and collapsed in the medium, thereby turning the color of the medium. Gordon and Alexande reported similar results in that the pH of Sealapex decreases over time[9].

In this experiment, AH 26 and AH Plus also showed severe cytotoxicity initially. It has been said that the formaldehyde released after the reactions is responsible for cytotoxicity, and has recently been reported that epoxy-bis-phenol resin also contributes to the cytotoxicity[3,12]

Unlike AH 26 and AH plus, Adseal showed the lowest cytotoxicity among the sealers tested. Adseal showed an improved biocompatibility having a much lower cytotoxicity than the conventional resin-based sealers. We assume that this was possible with the addition of calcium phosphate. The calcium phosphate materials are highly biocompatible and are osteoconductive. Its high biocompatibility suggests that the inadvertent extrusion beyond the apical foramen should be well tolerated by the periapical tissues. Furthermore, it appears to be devoid of dimensional changes during setting and provides superior adaptation to the canal surface. Therefore, it allows a better seal of the apical foramen and the accessory canals located in the apical third of the root[13,14]

Most materials formerly or currently used are antibacterial to some extent. The results of this study have shown that the various sealers differ in their antibacterial properties. Adseal showed a high level of antimicrobial effects against microorganisms except for *E. faecalis* while AH 26, another resin-based sealer, had a high level of effects against all those microorganisms.

There is no exact correlation between the antimicrobial effects observed *in vitro* and what happens clinically within the root canal or periradicular tissues. However, the *in vitro* tests are only able to indicate which materials have the potential to inhibit microbial growth and metabolism in the local microenvironment of a root canal.

This study demonstrated that Adseal showed higher viability rates than the rest of the sealers tested while having every bit as satisfactory an antibacterial effect. In addition, our other study, where the sealers were implantated in subcutaneous tissue, although there were no significant differences, has confirmed that Adseal showed the lowest inflammatory effect at the early stage among the sealers tested.

Sealers that have very strong antibacterial effects are also toxic to the hosts. Sealers with strong antibacterial effects are not necessarily needed, but sealers that never promote the growth of bacteria are essential. In this regard, Adseal should be evaluated as a sealer with a certain desirable degree of antibacterial effects and at the same time, doing little harm to hosts.

Ⅴ. Conclusion

This study evaluated the cytotoxicity and antibacterial effects of a newly developed resin-based sealer, Adseal.

1. Adseal showed a high biocompatibility having a much lower cytotoxicity than the other sealers tested.
2. Zinc oxide-eugenol-based sealers continuously show low viable rates in comparison with resin-based ones. In the case of resin-based sealers, AH plus exhibited a cytotoxic effect for a shorter period than the AH 26. Sealapex initially showed a very low viability rate. However, the viability rate began to increase after a 48hrs incubation period.
3. Adseal had a high antibacterial effect against Black pigmented bacteria, a moderate effect against *Fusobacterium* and a very low effect against *E. faecalis*.
4. All sealers are antibacterial to some extent. For all the tested sealers, different antibacterial effects according to the species were observed.

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