치의학석사 학위논문

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지도교수 안 석 준

이 논문을 치의학 석사 학위논문으로 제출함

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송영두의 석사 학위논문을 인준함

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Abstract

Antimicrobial effect of licorice root extracts against mutans streptococci isolated from Korean

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Introduction: The purpose of this study was to analyze optimal concentration of licorice root extracts to inhibit growth of the clinical isolates of mutans streptococci from Koreans.

Methods: Minimum inhibition concentration (MIC), minimum bactericidal concentration (MBC) and time-kill kinetic assays were evaluated using the clinical strains of *S. mutans* (KCOM 1054, KCOM 1111, KCOM 1113, KCOM 1116, KCOM 1126, KCOM 1128, KCOM 1136, KCOM 1197, KCOM 1202, KCOM 1207, KCOM 1217) and *S. sobrinus* (KCOM 1157, KCOM 1196, KCOM 1221) isolated from Koreans and obtained from the Korean Collection for Oral Microbiology.

Results: The MIC values of licorice root extracts against the clinical strains of mutans streptococci ranged from 4 to 8 μg/ml. The MBC values of licorice root extracts against the clinical strains of mutans streptococci ranged from 8 to 16 μg/ml. A time-kill assay indicated that the antimicrobial effects of licorice root extracts mainly result from the bactericidal effects. The all data suggest that

regardless of *S. mutans* or *S. sobrinus*, the optimal concentration of licorice root extracts over 8 μg/ml can be used *in vivo* for the prevention of dental caries in Koreans.

목 차

I. Introduction

The dental caries is the most common oral disease with the major causative bacterial species being *Streptococcus mutans* and *Streptococcus sobrinus*, which inhabit the dental plaque in the human oral cavity [1].

Many antimicrobial agents have been used to prevent dental caries. Chlorhexidine (CHX) is generally accepted as the gold standard for antimicrobial agents in the dental field due to its clinical efficacy on a wide range of microorganisms in the oral cavity. However, the regular use of CHX as an anti-caries agent is not easy because of its common side effects, such as the formation of extrinsic stain on the tooth and tongue [2,3]. As a result, there is a considerable interest in the development of new antimicrobial agents for the control of dental caries. Recently, several plants have been studied for their potential in the prevention of dental caries [4].

Licorice (*Glycyrrhiza glabra, G. inflata* and *G. uralensis*) can be a one of the candidates. Traditionally, the licorice had been used to lengthen one's life-span, for improving health, cures for injury and swelling, and detoxification in China and Rome [5].

Licorice root extracts contain a considerable amount of triperpenoid saponins, mostly glycyrrhizin [6]. Glycyrrhizin is converted by human intestinal bacteria to glycyrrhetic acid, which has been reported to induce severe hypertension, hypokalemia, and mineralocorticoid excess through preventing renal conversion of cortisol to cortisone [7]. Therefore, it is important to prepare a licorice extract without containing glycyrrhizin for safe use in human. We had prepared roots of Glycyrrhiza uralensis using a specific resin filter to obtain a crucial extract containing no detectable glycyrrhizin.

Generally, just type strains or a few wild type strains of mutans streptococci have been used to test the antimicrobial activity of various natural extracts [8]. However, previous studies have reported that the antimicrobial activity differs according to the type of

mutans streptococci strains [9]. This means that the use of clinical Korean isolates of mutans streptococci needs to be determined to prevent dental caries in Koreans. In addition, it's better to use licorice from Korea for the control of dental caries of Korean. The purpose of this study was to analyze optimal concentration of licorice root extracts to inhibit growth of the clinical isolates of mutans streptococci from the Korean oral cavity.

II. Material and methods

Preparation of the deglycyrrhizined licorice root extract (DG-LRE). Licorice root derived from *Glycyrrhiza uralensis* was purchased from an herbal drug market (Youngju, Korea). The deglycyrrghizined licorice root extract was prepared as previously described [10]. Briefly, the dried roots were cut into thin slices, mixed with distilled water (distilled water:dried root ratio of 20:1 [v/w]) and heated for 2 h in a round bottom flask. The distilled water was removed and 95% ethanol was added (95% ethanol:residue ratio of 15:1 [v/w]) to the flask. The mixture was heated at 78^{o}C for 2 h, and the extract was evaporated. After adding 99% ethanol, the solution was filtered using a custom-made column (6.5 cm X 60 cm) filled with Diaion HP-20 adsorbent (Mitsubishi Chemical Corporation, Minato-ku, Tokyo, Japan). The final deglycyrrhizined licorice root extracts (DG-LRE) were passed through the column, evaporated, and used for assays. The extracts were suspended in dimethyl sulfoxide (DMSO; Sigma, St Louis, MO, USA) at 50 mg/ml for subsequent assays. Absence of glycyrrhizin was confirmed using high performance liquid chromatography.

Clinical strains. The clinical strains of *S. mutans* (KCOM 1054, KCOM 1111, KCOM 1113, KCOM 1116, KCOM 1126, KCOM 1128, KCOM 1136, KCOM 1197, KCOM 1202, KCOM 1207, KCOM 1217) and *S. sobrinus* (KCOM 1157, KCOM 1196, KCOM 1221) isolated from Koreans and obtained from the Korean Collection for Oral

Microbiology (KCOM, Korea) were used in this study [10]. All strains were cultured on a Todd Hewitt (TH) (Difco, Franklin Lakes, NJ, USA) broth or agar plates in a 37°C incubator in air containing 5% CO_2.

Determining the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC). The MIC and MBC values were determined using a microdilution assay according to the National Committee for Clinical Laboratory Standards [11]. The bacterial strains were cultured in TH broth at 37°C in an incubator for 24 h and added into a 96-well plate to a final concentration of 1×10^6 CFU/ml. The DG-LRE solutions were then added to each well to a final concentration of 32, 16, 8, 4, 2, or 1 μg/ml. The final DMSO concentration in each well was 1%. Ampicillin (100 μg/ml) was used as the positive control, and the culture medium only and culture medium plus 1% DMSO groups were used as the double negative control. After 24 h incubation under suitable conditions, the lowest concentration of DG-LRE to exhibit a final concentration of 1×10^5 CFU/ml was taken as MIC value. To determine MBC values, 10 μl of bacterial culture from each well at the MIC value determined above was diluted 100- through 10,000-fold and plated onto TH agar plates for each bacterial strain. The agar plate was incubated at 37°C for 24 h and the number of colonies was then counted. The concentration that killed 99.9% of the bacteria was considered the MBC value.

Time-kill kinetic assay. A time-kill kinetic assay was performed using the same strains to confirm if the antimicrobial effect of licorice root extracts at different concentrations exhibit the bactericidal or bacteriostatic effect. The time-kill curves were assessed at the following DG-LRE concentrations: 0.5× MIC, 1× MIC, 2× MIC, and 4 × MIC. The control curve was obtained in the culture medium for each strain. The bacteria were inoculated in TH broth and incubated overnight in a 37°C incubator. Liquid media containing licorice at the above mentioned concentrations were inoculated with 1×10^6

CFU/ml of an overnight culture and incubated in a 37℃ incubator. At 0, 3, 6, 9, 12, and 24 h after inoculating with the bacteria, each bacterial culture solution was diluted 100- or 10,000-folds and plated onto a TH agar plate. The agar plate was incubated in a 37℃ incubator for 24 h before the bacterial colonies were counted.

III. Results

Summary statistics about the antimicrobial effects of licorice are shown in Tables Ⅰ and Ⅱ. The MIC values of DG-LGE against the clinical strains of mutans streptococci ranged from 4 to 8 μg/ml (Table 1). The MBC of licorice root extracts against the clinical strains of mutans streptococci ranged from 8 to 16 μg/ml (Table 2). These results suggest the proper concentration of the DG-LRE for developing an oral hygiene product such as a gargling solution to prevent dental caries for Koreans.

Fig 1 showed the results of the time-kill kinetic assay. The results showed the antimicrobial activity of DG-LRE mainly results from either bacteriostatic (Figs 1E, 1G, and 1I) or bactericidal effect (Figs 1A, 1B, 1C, 1D, 1F, 1H, 1J, 1K, 1L, 1M, and 1N). DG-LRE over 8 μg/ml provided complete growth inhibition or bactericidal effects to all the clinical strains, which is in accordance with MIC and MBC results

IV. Discussion

Mutans streptococci have been implicated as a primary causative agent of dental caries, thus inhibition of mutans streptococci growth is one of the important goals for the prevention of dental caries [12,13]. In recent years, some common anti-caries agents, such as CHX have been widely studied for preventing dental caries through the inhibition of growth, adhesion, and/or biofilm formation [14,15]. However, the present studies indicate that the common anti-caries agents CHX used at MICs may cause

unexpected side effects like superficial staining of teeth and tongue and altered taste perception [15]. As a result, there is a considerable interest in the development of new anti-biofilm agents for the control of dental caries. In this study, licorice root extracts were used as a candidate for their potential in the prevention of dental caries.

Mutans streptococci are divided into seven species: *S. mutans, S. sobrinus, S. downei, S. rattus, S. cricetus, S. ferus, and S. macacae* [16]. Of these, *S. mutans and S. sobrinus* are strongly associated with human dental caries [17]. The mutans streptococci have been classified as 6 biotypes (I to VI) according to their ability to ferment 4 carbohydrates (mannitol, sorbitol, raffinose, and melibiose) and to deaminate arginine [18,19]. Among MS, *Streptococcus mutans* and *Streptococcus sobrinus* are commonly found in human oral cavity. In order to prevent dental caries, therefore, it is important to know antimicrobial potential against *S. mutans* and *S. sobrinus*.

Most studies on dental caries had been performed using the strains of mutans streptococci derived from Westerns [20,21]. Because there are differences in the susceptibility between the type strains and the clinical isolates of mutans streptococci [9] and the effectiveness of DG-LRE differed among various strains in this study, it is necessary to evaluate the antimicrobial effects against clinical strains of the mutans streptococci isolated from Korean population for clinical use of DG-LRE as an anti-cariogenic agent in Korea. In this regard, we tested an antimicrobial potential of Korean licorice using the clinical strainss of mutans streptococci isolated from Koreans.

The antimicrobial effects of DG-LRE increased in a concentration- and treatment time-dependent manner (Tables I and II). The MIC values against mutans streptococci ranged from 4 to 8 μg/ml. The MBC values against *S. mutans* and *S. sobrinus* were 8 to 16 μg/ml, which is 1 to 2 times higher than the MIC values. Throughout a time-kill kinetic assay, DG-LRE exhibited both bacteriostatic and bactericidal activities against mutans streptococci in suspension, but the main effects were bactericidal (Fig 1). In addition, significant decreases in the viability of *S. mutans* and *S. sobrinus* occurred after about 9h or 12h, when adding over 8 μg/ml of DG-LRE to the bacterial suspensions.

To prevent dental caries process, inhibition of growth of mutans streptococci is

essential [22]. In this regard, this study showed a possibility that the DG-LRE could be used for an anti-cariogenic through inhibiting the growth of mutans streptococci. Considering that DG-LRE concentrations at 8 μg/ml had little cytotoxic effect on normal human gingival fibroblast cells [10], the concentration of DG-LRE at 8 μg/ml can be used *in vivo* for the prevention of dental caries of Korean and can be useful in the development of oral hygiene products for the prevention of dental caries.

This study has some limitations. The human mouth, with its diverse niches and environmental changes, is well known for its unrestricted formation of natural microbial biofilms. Although mutans streptococci are members of the endogenous oral microflora and major contributors to cariogenic biofilms in the oral cavity, mutans streptococci cells do not occur in monoculture in vivo, but rather in a diverse microbial consortium comprised of several 100 species of bacteria. Further study will be needed to be evaluated in an in vivo model to develop a promising approach for prevention of dental caries.

In summary, the results mentioned above suggest that the concentration of DG-LRE over 8 μg/ml can be used *in vivo* for the prevention of dental caries of Korean and can be useful in the development of oral hygiene products for the prevention of dental caries.

V. Conclusion

Licorice is one of the oldest botanicals in traditional Oriental medicine. This study examined the optimal concentration of licorice root extracts without detectable glycyrrhizin against the clinical isolates from the Korean oral cavity by MIC, MBC, and a time-kill kinetic assay. The results got from a series of experiment suggest that regardless of *S. mutans* or *S. sobrinus*, the concentration of licorice root extracts over 8 μg/ml can be used *in vivo* for the prevention of dental caries in Koreans.

Tables

Table 1. MIC values (μg/ml) of licorice root extracts against the clinical strains of *Streptococcus mutans* and *Streptococcus sobrinus* isolated from Koreans

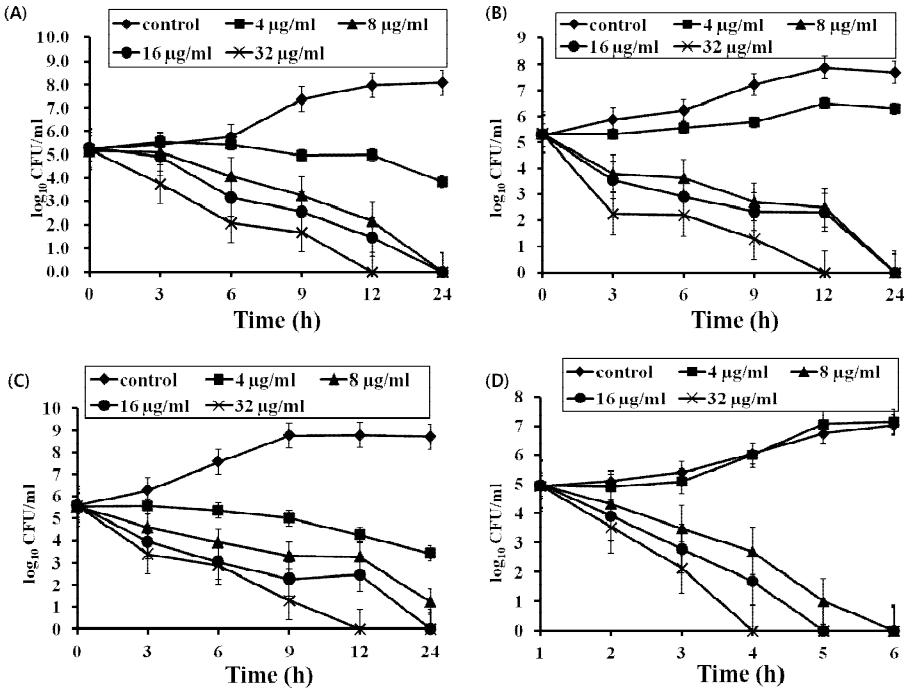
Species and strains	MIC	Species and strains	MIC
S. mutans KCOM 1054	4	*S. sobrinus* KCOM 1157	4
S. mutans KCOM 1111	4	*S. sobrinus* KCOM 1196	4
S. mutans KCOM 1113	4	*S. mutans* KCOM 1197	4
S. mutans KCOM 1116	8	*S. mutans* KCOM 1202	4
S. mutans KCOM 1126	8	*S. mutans* KCOM 1207	8
S. mutans KCOM 1128	4	*S. mutans* KCOM 1217	8
S. mutans KCOM 1136	4	*S. sobrinus* KCOM 1221	4

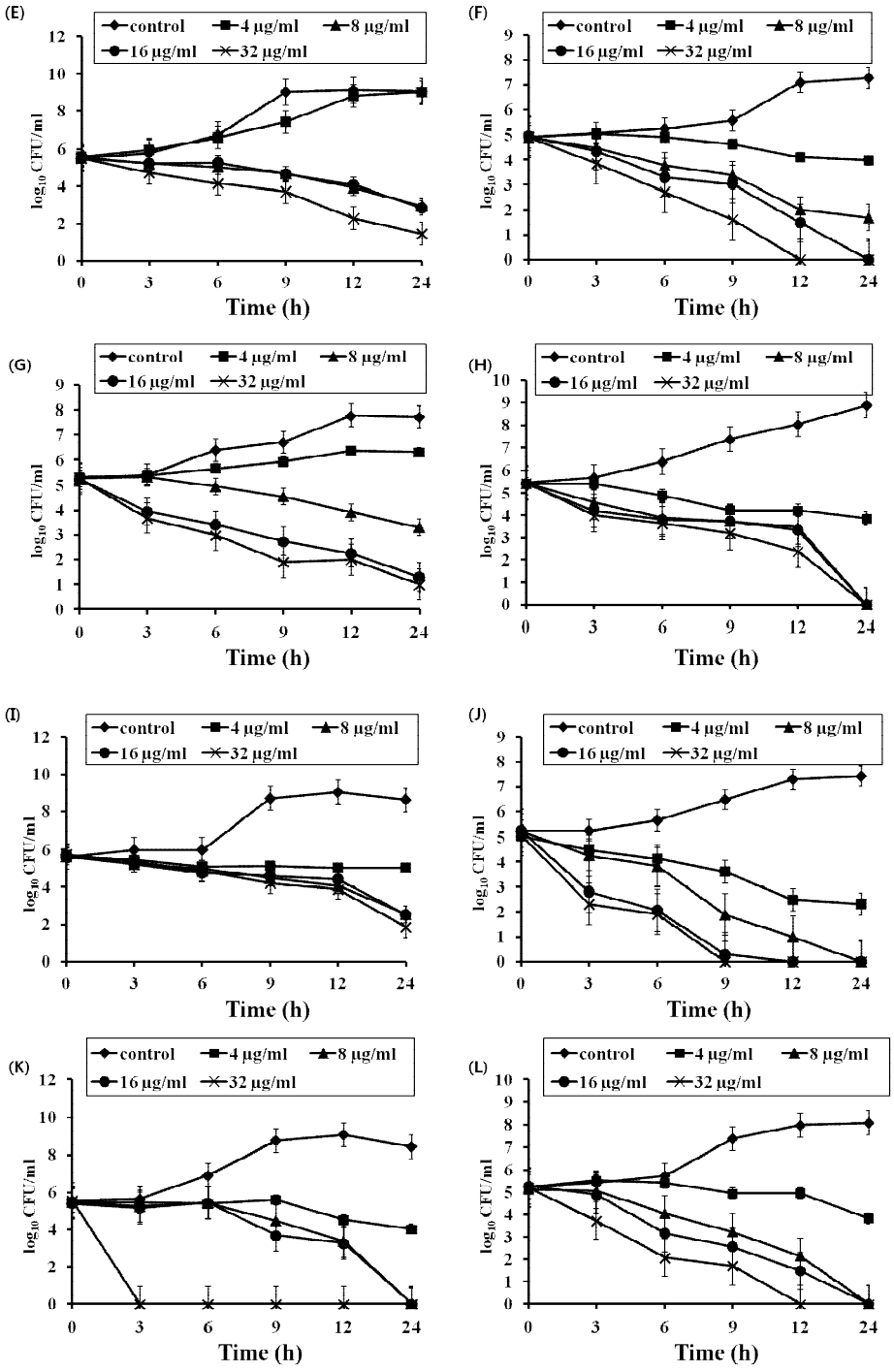
Table 2. MBC values (μg/ml) of licorice root extracts against the clinical strains of *Streptococcus mutans* and *Streptococcus sobrinus* isolated from Koreans

Species and strains	MBC	Species and strains	MBC
S. mutans KCOM 1054	8	*S. sobrinus* KCOM 1157	8
S. mutans KCOM 1111	8	*S. sobrinus* KCOM 1196	8
S. mutans KCOM 1113	8	*S. mutans* KCOM 1197	8
S. mutans KCOM 1116	8	*S. mutans* KCOM 1202	8
S. mutans KCOM 1126	8	*S. mutans* KCOM 1207	16
S. mutans KCOM 1128	16	*S. mutans* KCOM 1217	16
S. mutans KCOM 1136	16	*S. sobrinus* KCOM 1221	8

Figures

Fig.1 Time-kill curves of licorice root extracts against (A) *S. mutans* KCOM 1054, (B) *S. mutans* KCOM 1111, (C) *S. mutans* KCOM 1113, (D) *S. mutans* KCOM 1116, (E) *S. mutans* KCOM 1126, (F) *S. mutans* KCOM 1128, (G) *S. mutans* KCOM 1136, (H) *S. mutans* KCOM 1197, (I) *S. mutans* KCOM 1202, (J) *S. mutans* KCOM 1207, (K) *S. mutans* KCOM 1217, (L) *S. sobrinus* KCOM 1157, (M) *S. sobrinus* KCOM 1196, (N) *S. sobrinus* KCOM 1221 at different licorice concentrations.




(E)
control
4 μg/ml
8 μg/ml
16 μg/ml
32 μg/ml
log10 CFU/ml
Time (h)
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(H)
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(K)
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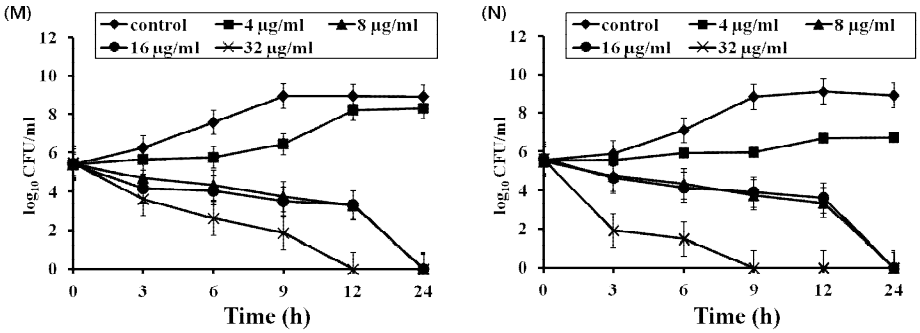

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국문초록

목적: 치아우식증은 *S. mutans* 균과 *S. sobrinus* 균에 의해 유발되는 주요 구강 질병 중 하나이다. 치아우식증을 예방하기 위해 많은 항균 물질들이 개발되어왔고 천연 식물 추출물인 감초도 그 후보군 중 하나이다. 본 실험에서는 고혈압이나 저칼륨혈증을 유발할 수 있는 glycyrrhizin이 감초 농축액에서 검출되지 않도록 레진 필터를 사용하여 정제처리를 하였고 한국인의 치아 우식 이환 억제를 목표로 한국인의 구강에서 추출된 치아우식 유발균을 대상으로, 우식 유발균을 가장 효과적으로 억제할 수 있는 국산 감초 추출물의 적정 농도를 파악하고자 한다.

방법: 한국인의 구강에서 획득한 *S. mutans* 11균주(KCOM 1054, KCOM 1111, KCOM 1113, KCOM 1116, KCOM 1126, KCOM 1128, KCOM 1136, KCOM 1197, KCOM 1202, KCOM 1207, KCOM 1217)와 *S. sobrinus* 3균주(KCOM 1157, KCOM 1196, KCOM 1221), 총 14 종의 표준 임상 균주를 실험 대상으로 하였고 모든 균주는 Todd Hewitt (TH) (Difco, Franklin Lakes, NJ, USA) 액체 배지와 agar 배지 위에서, 그리고 37℃, 5% 이산화탄소 조건의 인큐베이터에서 배양되었다. 국산 감초 추출물의 antimicrobial effect를 보기 위해 MIC(minimum inhibitory concentration), MBC(minimum bactericidal concentrations), time-kill kinetic assay를 진행하였다.

결과: 일련의 실험과정을 통해 얻은 국산 감초 추출물의 MIC 값은 4 혹은 8 μg/ml 이었고, MBC 값은 8 혹은 16 μg/ml 을 나타내었다. Time-kill kinetic assay에서도 bacteriostatic, bactericidal effect 관련하여 각각 MIC, MBC 실험값과 일치하는 결과를 나타내었으며 이는 *S. mutans* 균, *S. sobrinus* 균에 상관없이 감초 추출물의 농도가 8 μg/ml 이상이면 *in vivo*

조건에서 한국인의 치아우식증을 예방하는데 효과적일 것임을 의미한다고 볼 수 있다.