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*Original Article*

# Topical treatment of oral cavity and wounded skin with a new disinfection system utilizing photolysis of hydrogen peroxide in rats

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**ABSTRACT** — The present study aimed to evaluate the acute locally injurious property of hydroxyl radical generation system by photolysis of $H_2O_2$, which is a new disinfection system for the treatment of periodontitis developed in our laboratory. Firstly, generation of the hydroxyl radical by a test device utilizing the photolysis of $H_2O_2$ was confirmed by applying an electron spin resonance (ESR)-spin trapping technique. Secondly, the bactericidal effect of the device was examined under a simulant condition in which *Staphylococcus aureus* suspended in 1 M $H_2O_2$ was irradiated with laser light emitted from the test device, resulting in substantial reduction of the colony forming unit of the bacteria within a short time as 2 min. Finally, acute topical effect of the disinfection system on rat oral mucosa and wounded skin was evaluated by histological examination. No abnormal findings were observed in the buccal mucosal region treated three times with 1 M $H_2O_2$ and irradiation. Similarly, no abnormal findings were observed during the healing of skin treated with 1 M $H_2O_2$ and irradiation immediately after wounding. Since topical treatment with the novel disinfection technique utilizing the photolysis of $H_2O_2$ had no detrimental effect on the oral mucosa and the healing of full thickness skin wounds in rats, it is expected that the acute locally injurious property of the disinfection technique is low.

**Key words:** Hydroxyl radical, Photolysis of $H_2O_2$, Safety evaluation, Topical application, Rats

## INTRODUCTION

Applying the oxidative power of hydroxyl radicals to disinfection systems has been attempted by utilizing sonolysis of water and photolysis of hydrogen peroxide ($H_2O_2$) in our laboratory (Kohno *et al*., 2011; Shirato *et al*., 2012; Ikai *et al*., 2010; Iwasawa *et al*., 2009). In the latter system, designed for the treatment of periodontitis, hydroxyl radicals generated by the photolysis of 1 M $H_2O_2$ increased with laser irradiation time and could kill oral pathogenic bacteria in as little as 1 to 3 min. This time-dose relationship shows that the system is very effective. The 1 M $H_2O_2$ corresponds to approximately 3%, a concentration used in the oral cavity as a disinfectant. A subcommittee of the US Food and Drug Administration (FDA, 2003) also concluded that hydrogen peroxide is safe at concentrations of up to 3%. Therefore, the concentration of $H_2O_2$ used in the disinfection system was fixed to be 1 M. Prior to clinical testing, however, the safety of any such system must be evaluated. There have been many studies on oxidative cellular damage such as membrane lipid peroxidation, protein denaturalization, and nucleic acid modification in relation to reactive oxygen species (ROS) including the hydroxyl radical (Darley-Usmar and Halliwell, 1996; Halliwell 1996a, 1996b). Nonetheless, to our knowledge, few studies have examined the direct involvement of the hydroxyl radical in localized damage *in vivo* possibly because of its extremely short-half life, approximately $10^{-9}$ sec (Sies *et al*., 1992; Pryor, 1986). In the present study, acute locally injurious properties of a new disinfection system utilizing the photolysis of hydrogen peroxide, which generates the hydroxyl radical as a microbicidal active ingredient, were assessed as the first step of safety evaluation.

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## MATERIALS AND METHODS

### Test device

Figure 1(a) shows a schematic illustration of the test device in which a continuous-wave laser was incorporated into a conventional ultrasound scaler (Ricoh Optical Industries, Iwate, Japan). $H_2O_2$ is released from the tip of the device to the lesion concomitantly with laser irradiation (at 405 nm with an output of 7 mW) through an optical fiber. One molar $H_2O_2$ corresponds to approximately 3%, a concentration at which $H_2O_2$ is used in the oral cavity as a disinfectant.

### Reagents

Reagents were purchased from the following sources: 5,5-dimethyl-1-pyrroline N-oxide (DMPO) from Labotec (Tokyo, Japan), $H_2O_2$ from Santoku Chemical Industries (Tokyo, Japan), and 4-hydroxy-2,2,6,6-tetramethylpiperidine (TEMPOL) from Sigma Aldrich (St. Louis, MO, USA). All of the other reagents used were of analytical grade.

### ESR analysis of hydroxyl radicals generated by photolysis of $H_2O_2$

The hydroxyl radical generated by the test device was evaluated. Twenty-five milliliters of 1 M $H_2O_2$ containing 300 mM DMPO in a liquid container was circulated for 2 min at a flow rate of 25 ml/min (Fig. 1(b)). The optimal concentration of DMPO, a spin trap agent, needed to sufficiently trap the hydroxyl radical was examined in our previous report (Nakamura *et al*., 2010). Following the operation, an aliquot of the reaction mixture was subjected to an ESR analysis. The mixture was transferred to a quartz cell for ESR spectrometry and the ESR spectrum was recorded on an X-band ESR spectrometer (JES-FA-100; JEOL, Tokyo, Japan). TEMPOL (20 μM) was used as a standard sample to calculate the concentration of DMPO-OH. The measurement conditions were as follows: field sweep, 330.50 to 340.50 mT; field modulation frequency, 100 kHz; field modulation width, 0.1 mT; amplitude, 200; sweep time, 2 min; time constant, 0.03 sec; microwave frequency, 9.420 GHz; microwave power, 4 mW. All experiments were performed at room temperature.

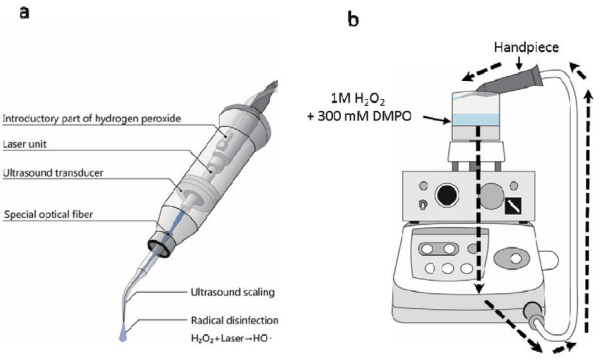


**Fig. 1.** (a) Schematic illustration of the test device for the treatment of infectious oral diseases utilizing photolysis of $H_2O_2$. The system is incorporated into a conventional ultrasound scaler. (b) Schematic illustration showing the experimental procedure in which hydroxyl radicals generated by the test device utilizing photolysis of $H_2O_2$. Twenty-five milliliters of 1 M $H_2O_2$ containing 300 mM DMPO in a liquid container was circulated for 2 min at a flow rate of 25 ml/min. Dashed arrow indicates the flow-circuit of 1 M $H_2O_2$ containing 300 mM DMPO. Following circulation, an aliquot of the reaction mixture was subjected to an ESR analysis.

### Bactericidal assay

A stock culture strain of *Staphylococcus aureus* JCM 2413 was purchased from the Japan Collection of Microorganisms, RIKEN BioResource Center (Saitama, Japan). The *S. aureus* was aerobically cultured on Soybean Casein Digest (SCD) agar (Wako Pure Chemical Industries, Ltd., Osaka, Japan) at 37°C for 1 day. A bacterial suspension of strain JCM 2413 was prepared in sterile physiological saline and the number of cells was adjusted to approximately $5 \times 10^6$ colony forming units (CFU)/ml. Ten microliters of the bacterial suspension was mixed with the same volume of 2M $H_2O_2$ in a 0.2-ml PCR (polymerase chain reaction) tube. Then, the mixture was irradiated with laser light (405 nm with an output of 7 mW) for 1 and 2 min from the test device. The distance between the surface of the mixture and the tip of the hand piece of the device was set at around 7 mm so that the surface of the mixture could be fully irradiated. After irradiation, the sample was mixed with an equal volume of a sterile catalase solution (5,000 U/ml) to terminate the bactericidal effect of the remaining $H_2O_2$. A ten-fold serial dilution of the mixture was then prepared using sterile physiological saline and 10 μl of the dilution was seeded on SCD agar to evaluate the number of viable bacterial cells in the suspension. The agar medium was cultured for 1 to 2 days at 37°C and the number of CFU/ml was determined. The bactericidal effect of the hydroxyl radical generated by the laser irradiation to 1 M $H_2O_2$ (expressed as H(+)L(+)) was compared to the effect of; (1) no treatment, H(-)L(-), (2) laser irradiation alone, H(-)L(+), and (3) 1 M $H_2O_2$ alone, H(+)L(-). For the L(-) condition, the samples were kept in a light shielding box

without the laser irradiation. For the H(-) condition, sterile pure water was added to the reaction system instead of 2 M $H_2O_2$. All tests were performed in duplicate.

### Animals

The experiments reported here were conducted in accordance with the guidelines for animal experiments and animal care adopted by Tohoku University. Five-week old male Wistar rats were purchased from Charles River Laboratories Inc. (Kanagawa, Japan), and used after acclimatization for 1 week. During the acclimatization and experimental periods, animals were given free access to food pellets (Labo MR Stock, Nosan Corp., Tokyo, Japan) and tap water. The rats were housed at 23 ± 3°C under a 12-hr light /12-hr dark cycle.

### Topical treatment of oral cavity

Six animals were allocated to each group, giving a total of twenty-four animals. Under isoflane anesthesia, the left side of the oral cavity (buccal mucosal region) was irrigated once or three times with either : (1) pure water, H(-)L(-), (2) pure water and laser irradiation, H(-)L(+), (3) 1 M $H_2O_2$, H(+)L(-), and (4) 1 M $H_2O_2$ and laser irradiation, H(+)L(+). Each treatment was conducted on a rodent surgery board once a day for 2 min with the corresponding irrigation at a flow rate of 25 ml/min. Immediately following the last treatment, animals were sacrificed by cervical dislocation, and left buccal mucosal tissues and surrounding areas were excised and fixed in 10% neutral formalin.

### Topical treatment of wounded skin

Eighteen animals were allocated to two groups (nine animals per group). Under isoflane anesthesia, the animals were clipped of the fur on their back, and two circular excisions (1 cm in diameter) were made by cutting out the full-thickness skin with scissors as previously reported (Niwano *et al*., 1996a, 1996b). Immediately following the wounding, the peripheral area of the wounded skin was topically treated with either (1) pure water, H(-)L(-), or (2) 1 M $H_2O_2$ and laser irradiation, H(+)L(+). Each wound was subjected to the corresponding treatment for 2 min as in the oral cavity experiment. The wounds were left open, and each rat wore a collar to prevent licking of the wound during the experiment. The wound area (including scab) was traced, and the square size was calculated by using ImageJ provided by the Research Services Branch of the NIH. An average of the two wound areas of each animal was used as a representative value of an individual animal. One or two animals per group were sacrificed by cervical dislocation 3, 5 and 7 days after wounding, and the rest were sacrificed 9 days after wounding. The wounded skin tissue was excised and fixed in 10% neutral formalin.

### Histological examination

Following fixation in 10% neutral formalin, the buccal mucosal tissues and the wounded skin tissues were trimmed, dehydrated by an ethanol series, embedded in paraffin, sectioned, and stained with hematoxylin and eosin for microscopy.

### Statistical analyses

Significant differences in relative wound area between the treatment (1M $H_2O_2$ + laser irradiation) and control (pure water) groups were assessed with Student's *t*-test. A two-way repeated measure ANOVA (analysis of variance) was also applied to all the data for relative wound area.

## RESULTS

### ESR analysis of hydroxyl radicals generated by photolysis of $H_2O_2$

Figure 2 shows representative ESR spectra of the reaction mixtures obtained following the operation of

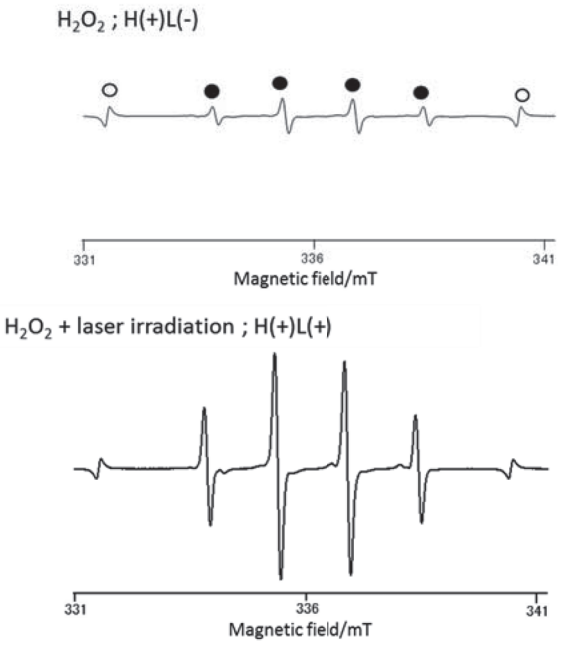


**Fig. 2.** Representative ESR spectra of the reaction mixture obtained following the operation of the device at a flow rate of 25 ml/min for 2 min. The spin adduct DMPO-OH (●) was assigned using hyperfine coupling constants (hfcc): aH=aN=1.49 mT. ○: $Mn^{2+}$ marker.

the device at a flow rate of 25 ml/min for 2 min. Only the signal for DMPO-OH (an adduct of DMPO and the hydroxyl radical) was found. The spin adduct DMPO-OH was assigned using hyperfine coupling constants (hfcc). The hfcc are aH=aN=1.49 mT, which coincide with those of the DMPO-OH adduct reported in a previous paper (Buettner, 1987). When 1 M $H_2O_2$ irradiated with laser light was applied to the test device, a substantial increase in the intensity of the DMPO-OH signal was observed. That is, around 50 μM DMPO-OH as an indicator of the hydroxyl radical was generated in 2 min. Meanwhile, DMPO-OH signal probably derived from the autolysis of $H_2O_2$ was also observed when 1 M $H_2O_2$ was applied to the test device without laser irradiation, but the level was apparently low as compared with that when 1 M $H_2O_2$ was irradiated with laser light.

## Bactericidal assay

Figure 3 summarizes the CFU of *S. aureus* recovered from the specimens. The bacteria were effectively and time-dependently killed by the treatment with 1 M $H_2O_2$ and irradiation (H(+)L(+)), and an approximately 5 logarithmic reduction was obtained within 2 min. Although the treatment with 1M $H_2O_2$ alone (H(+)L(-)) also had a bactericidal effect, the treatment for 2 min killed the bacteria with only a 2 logarithmic reduction. The other conditions (H(-)L(+) and H(-)L(-)) could hardly kill the bacteria even after 2 min.

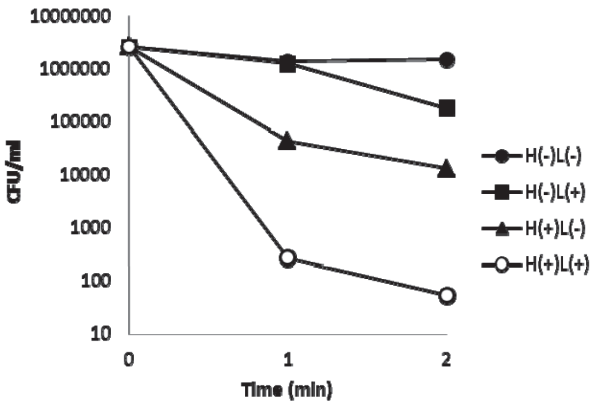


**Fig. 3.** Number of viable *Staphylococcus aureus* recovered from the suspension after each treatment. One molar $H_2O_2$ with laser irradiation could kill the bacteria used in this study with an approximately 5 logarithmic reduction within 2 min. Each symbol represents the mean of duplicate determinations.

## Topical treatment of oral cavity

Figure 4 shows the histological appearance of the buccal mucosal region in the control group (treated three times with pure water; H(-)L(-)) and in the experimental groups (treated three times with pure water and irradiation: H(-)L(+), 1 M $H_2O_2$: H(+)L(-), and $H_2O_2$ and irradiation: H(+)L(+)). The regions in all groups similarly consisted of mucosal stratified epithelium, connective tissue, and muscular layers, with no abnormal findings observed in the experimental groups as compared with the control group.

## Topical treatment of wounded skin

The time course measurement of wound areas is summarized in Fig. 5. Wound areas in both the control (treated with pure water: H(-)L(-)) and the experimental group (treated with 1M $H_2O_2$ + laser irradiation: H(+)L(+)) time-dependently diminished toward wound closure, and the average area became less than 10% of the original size 9 days after wounding. Although no significant differences were found in the wound areas at each time point between the two groups, diminution of the areas in the experimental group tended to be accelerated during the period of day 1 to day 7 as compared with that in the control group. Accordingly, two-way repeated measure ANOVA showed that the treatment with 1 M $H_2O_2$ and irradiation affected

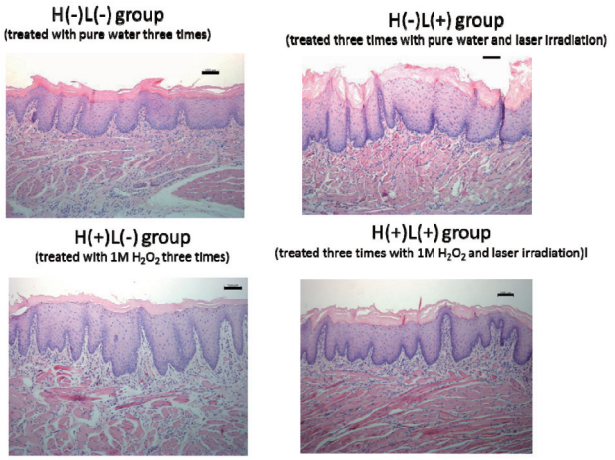


**Fig. 4.** Representative histological appearance of the buccal mucosal regions treated three times with H(-)L(-) (treated with pure water), H(-)L(+); (treated with pure water and laser light irradiation), H(+)L(-); (treated with 1M $H_2O_2$), H(+)L(+); (treated with 1M $H_2O_2$ and laser irradiation). All four regions similarly consisted of a mucosal stratified epithelium, connective tissue, and muscular layers. Hematoxylin-eosin staining. Scale bar: 100 μm.

significantly the time course measurement of wound areas (Table 1).

Figure 6 shows the histological appearance of the marginal region of the wounded skin of rats on day 3 in the two groups. In both groups, immature granulation tissue was formed with numbers of new capillary vessels and infiltrated inflammatory cells under the crusta. Figure 7 shows the wounded skin on day 5 and day 7 in the control group and in the experimental group. In both groups, the granulation tissue tended to be organized into a dense matrix over time, accompanied by re-epithelization partially covering new granulation tissue. Figure 8 shows histological findings of the central regions of the wounded skin on day 9. In both groups, collagen matrix formation and angiogenesis were well accelerated, and epidermis was well elongated with differentiation.

## DISCUSSION

According to the ESR analysis, it was confirmed that the hydroxyl radical expressed as the spin adduct DMPO-OH was substantially generated in 2 min by the disinfection device utilizing photolysis of $H_2O_2$ (Fig. 2). Under a simulant condition in which the bacteria suspended in 1 M $H_2O_2$ was irradiated with laser light emitted from the test device, *S. aureus* was effectively killed with an approximately 5 logarithmic reduction of the CFU within 2 min, indicating that the hydroxyl radical at a level of several dozen μM generated in 2 min by the device effectively contributes to the disinfection process. It is known that reactive oxygen species (ROS), such as singlet oxygen, the superoxide anion, $H_2O_2$, and the hydroxyl radical, cause oxidative damage to tissues or cells if not controlled (Fridovich, 1986; Slater, 1984). ROS are derived from oxygen molecules by electron reduction except for singlet oxygen which is generated as a result of electron transfer within the oxygen molecule (Halliwell and Gutteridge, 1984). Of the ROS, the hydroxyl radical has the highest reactivity (Halliwell and Gutteridge, 1986). Therefore, it was postulated that the hydroxyl radical-based disinfection technique designed for oral infec-

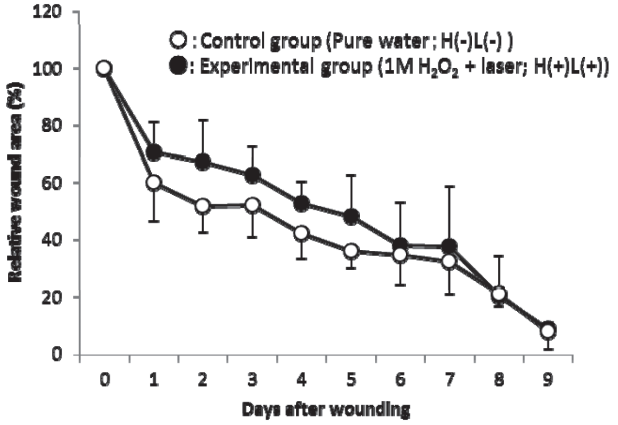


**Fig. 5.** Time course measurement of relative wound areas after wounding. Each value represents the mean and standard deviation (n = 4). An average of two wound areas from each rat was used as a representative value of an individual animal.

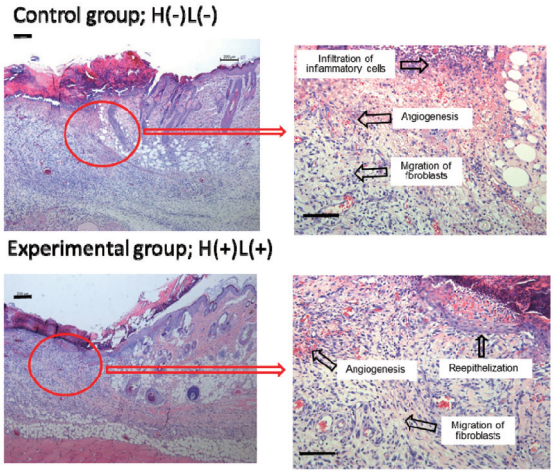


**Fig. 6.** Representative histological appearance of the marginal regions of the wounded skin of rats on day 3 in the control group and in the experimental group (treated with 1 M $H_2O_2$ with laser light irradiation). Immature granulation tissue was formed with numbers of new capillary vessels and infiltrated inflammatory cells under the crusta. Hematoxylin-eosin staining. Scale bar: 200 μm (left), 50 μm (right).

**Table 1.** ANOVA summary table for the time course measurement of wound areas

| | Sum of squares | df | Mean square | F value | P value |
|---|---|---|---|---|---|
| Group | 1530.527 | 1 | 1530.527 | 12.055 | 0.001 |
| Day | 50269.15 | 9 | 5585.461 | 43.995 | < 0.0001 |
| Group x Day | 1526.462 | 9 | 169.607 | 1.336 | 0.2381 |
| Error | 7617.425 | 60 | 126.957 | | |

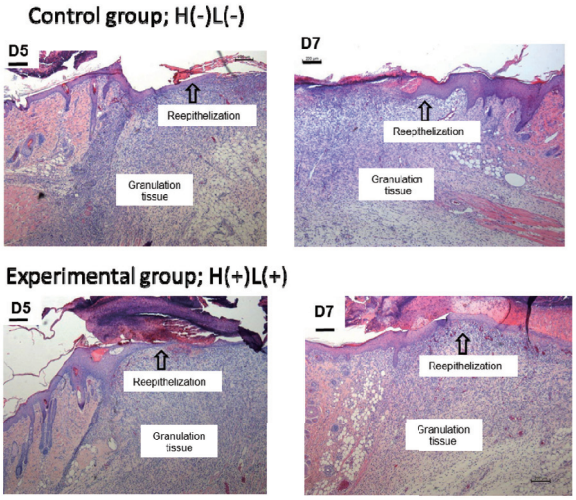


**Fig. 7.** Representative histological appearance of the marginal regions of the wounded skin of rats on day 5 and day 7 in the control group and in the experimental group (treated with 1 M $H_2O_2$ with laser light irradiation). The granulation tissue tended to organize intto a dense matrix over time, accompanied by re-epithelization partially covering new granulation tissue. Hematoxylin-eosin staining. Scale bar: 200 μm.

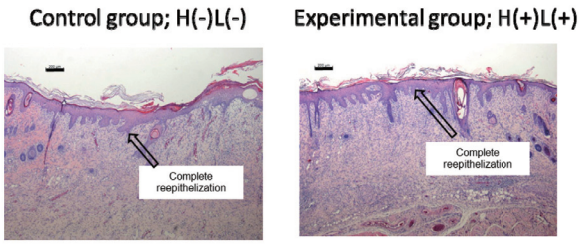


**Fig. 8.** Representative histological findings of the central regions of the wounded skin of rats on day 9 in the control group and the experimental group. In both groups, collagen matrix formation and angiogenesis were well accelerated, and epidermis was well elongated with differentiation. Hematoxylin-eosin staining. Scale bar: 200 μm.

tious diseases such as periodontitis presented in this study might cause acute oxidative damage to the oral mucosa. However, the present study showed that not only single but three treatments with the disinfection technique had no detrimental effect in terms of histological examination. Furthermore, single treatment with the disinfection technique had no detrimental effect on the healing process as examined histologically. In the latter study, the marginal lesion of the wounded area, which completely lacked skin barrier functions provided by the stratum corneum, was exposed to 1 M $H_2O_2$ with laser light irradiation. In addition to the lack of the stratum, some parts of the healing process are initiated from the marginal lesion; fibroblasts migrate, proliferate and produce collagen to form granulation tissue, and epithelial cells proliferate and differentiate on the new granulation tissue (Niwano *et al*., 1996a, 1996b). Since the disinfection technique was applied under the conditions described above, it is concluded that acute locally injurious property of the disinfection technique is considerably low. Meanwhile, the technique would accelerate the wound healing process toward closure as indicated by the time course measurement of wound areas (Fig. 6 and Table 1). Recent *in vitro* and *in vivo* observations suggest that ROS, and mainly $H_2O_2$, interfere with cell signaling acting like a second messenger and inducing adaptive responses, and this is particularly observed in skin wound healing where cells are exposed to $H_2O_2$ following injury (Atrux-Tallau *et al*., 2011; Pan *et al*., 2011; Schreml *et al*., 2011; Eligini *et al*., 2009). Therefore, it is suggested that the disinfection technique does not have injurious effect on the healing process of the wounded skin, and rather have a healing effect likely attributable to $H_2O_2$.

## ACKNOWLEDGMENTS

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