Evaluation of a Cationic Surfactant CAE on the Premise of Application to Oral Disinfection

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Introduction

Agents for oral diseases are commonly used topically in the fields of oral surgery. For example, some chemicals are important to prevent infection after oral operation. Disinfectants for oral use are required to be less irritating and neither smelling worse nor tasting worse than that for skin use. Although more than twenty kinds of disinfectants have been brought to practical use, only four preparations are indicated to be used directly for the purpose of cleansing wounds or operation field of oral mucous membranes including pharynx: dilute iodine tincture, compound iodine glycerin, oxidol, and acrinol1). And some other iodine preparations and surfactants, officially approved disinfectants and allowed as disinfectant on mucous membrane of operation field, are commonly adapted for buccal application. Such agents as povidon–iodine, benzethonium chloride and domiphen bromide are used in the form of gargles and mouth rinse solution as medicines for the purpose of disinfection of oral cavity. On the other hand, there are so many gargles of quasi drugs, but they are not sufficiently effective as drugs. Therefore, among gargles, those can be used for oral disinfection are above three drugs only. Chlorhexidine gluconate had been used for oral mucosa disinfectant, but after the report in 1984 describing that it induced shock and its use has been limited to fields other than oral disinfection.

Thus, excepting iodine preparations, most of oral mucosa disinfectants are classified to the surfactant group. Surfactants have original characteristic properties, such as emulsifying, detergent, dispersing and foaming activities. Additionally, some of them have also bactericidal action and inhibit growth of pathogenic microorganisms and are very useful for disinfection. Benzalkonium chloride (BKC), one of the quaternary ammonium salts and cationic surfactants, is a widely used disinfectant, and effective for many kinds of pathogenic bacteria. However, BKC sometimes makes caustic irritation on skin and mucous membrane because of its principal mechanism of bacteriostatic action due to its adsorption to the exposed surface, even though it had been known to be relatively little irritating2,3). Recently, various types of chemical agents have been brought to use as disinfectants, but the use on mucous membrane, especially on oral mucosa, is limited in kinds and concentration for
practical use.

Then N\textsuperscript{o}–cocooy L–arginine ethyl ester DL–pyrrolidone carbonate (CAE), has attracted the attention; it is a condensation product of amino acid with natural fatty acids and neutralized with DL–pyrrolidone carbonic acid, and it has desirably good bio–resolvability. Though CAE has high surface activating effect, it is expected to cause lower irritation to skin than usual cationic surfactants considering it's chemical structure and has detergent and broad bactericidal action. It has also antivirus activity besides antibacterial action. CAE was found to be a strong inactivator of lipids containing virus, especially hepatitis B virus (HBV). The effect of CAE against hepatitis B surface antigen (HBsAg), a coat component of HBV, involves more than its surface–activating effect. The inactivation of HBsAg is considered to be due to a specific structure of CAE forming stable HBsAg–CAE complex\textsuperscript{6,7}. Although such evidence has been proven since 1980, CAE is used only as a detergent to date, and no study has been made to put CAE to practical use as a disinfectant.

In this paper, the evaluation of CAE as a disinfectant in practical use was made from the aspects of both effectiveness and safety in comparison with BKC. First, antimicrobial efficiency was examined, especially effects within short time contact was observed. At the same time, from the aspect of safety, cytotoxicity, systemic toxicity, local irritation, sensitization, and hemolysis activity were also examined.

**Materials and Methods**

**Test compounds**

N\textsuperscript{o} – cocooy arginine ethylester DL – pyrrolidone carbonate (CAE, Ajinomoto) for all experiments; and benzalkonium chloride (BKC, Nacalai Tesque), a control substance, for cytotoxicity, antimicrobial, irritation, and hemolysis tests are used.

**I. Cytotoxicity test**

1. Cells and cell culture

Mouse L–929 cells (NCTC clone 929) were obtained from the Dainippon Pharmaceutical. Stock cultured cells were grown in plastic tissue culture flasks (25 cm\textsuperscript{2}/75 cm\textsuperscript{2}, Falcon) with Eagle’s minimum essential medium (MEM–1, Nissui Pharmaceutical) supplemented with 3 % fetal bovine serum (FBS, DIFCO) 0.17% tryptose phosphate broth (DIFCO) and 2 % L–glutamine (Wako), and cells were incubated at 37°C in a humidified atmosphere containing 5 % CO\textsubscript{2} in air. Cells to be used in these experiments were brought into suspension from stock cultures by treatment within 0.1% trypsin (DIFCO)–ethyldiamine tetraacetic acid, disodium salt (EDTA, Dojin) for not more than 1 minute.

2. Measurement of inhibition of cell adhesion

The cell suspension was adjusted to 5 × 10\textsuperscript{4} cells/ml in MEM with 3 % FBS for experiment. Solutions containing BKC or CAE prepared in final concentration of 0.5 to 150 \(\mu\)g/ml with dimethyl sulfoxide (DMSO, MERCK) were added to cell suspension distributed into centrifuge tubes.

Two ml of this suspension was scattered into each tissue culture dish (35 mm, Falcon)
incubated for about 24 hours. After staining with 0.1M citric acid and 0.1M crystal violet, inhibition of cell adhesion was measured by Monocelator (Olympus) based on earlier description\textsuperscript{6}.

3. Observations of change in cell morphology

Change in cell structure was observed to investigate the cytotoxicity in more detail by electron microscopy. This experiment was initiated by plating $1 \times 10^5$ cells in 2 ml of cell suspension into each dish. About 20 hours after plating, the medium was removed from the dishes and replaced with test media containing test agents or with fresh complete medium for control. The structure of cultured cell was observed after exposure from 1 to 360 minutes to CAE or BKC.

After being fixed, dehydrated and freeze-dried (Eiko, ID-2), the cell was examined for structure by scanning electron microscopy (SEM, JOEL, JSM T-300), coated with 150Å gold, and by transmission electron microscopy (TEM, JOEL, 1200EX-\textcopyright II), embedded and sliced.

II. Antimicrobial test

1. Test strains

\textit{Staphylococcus aureus} (FDA 209P), \textit{Escherichia coli} (NIHJ JC-2), \textit{Bacillus subtilis} (NIHJ PCI-219), \textit{Pseudomonas aeruginosa} (RIMD, Strain HD), \textit{Streptococcus mutans} (IFO 13955) and \textit{Candida albicans} (NI 7941) were obtained from the Institute for Fermentation Osaka (IFO).

2. Cultivation

For preculture, brain heart infusion (BHI) medium was used as the liquid growth medium for \textit{S. aureus}, \textit{E. coli} and \textit{S. mutans}, and King's B medium\textsuperscript{7} for \textit{P. aeruginosa} as liquid growth medium. These strains were cultured in a shaking incubator (Taitec, XY-80) at 37°C for 16 to 18 hours. General yeast medium, IFO medium no. 108, which includes 0.5\% peptone, 0.3\% yeast extract, 0.3\% malt extract and 1.0\% glucose, and adjusted to pH 6.0, was employed for \textit{C. albicans}. This preculture was incubated at 28°C for 24 hours in a shaking incubator. Only \textit{B. subtilis} was made as spore suspension by preincubating without shaking for 1 week at 37°C.

For test cultures, five strains, \textit{S. aureus}, \textit{E. coli}, \textit{B. subtilis}, \textit{P. aeruginosa} and \textit{S. mutans} were incubated by using BHI agar medium for solid cultures at 37°C for 24 hours (for 48 hours in \textit{S. mutans} only). \textit{C. albicans} was cultured on Sabouraud agar medium (Nissui Pharmaceutical) at 28°C for 24 hours.

3. Procedure

Bacteria precultured overnight were adjusted to the final concentration of $10^8$/ml and distributed in a unit of 9 ml into each centrifuge tube. One ml of surfactants diluted to various concentrations with sterilized isotonic sodium chloride solution (saline) was then added to these centrifuge tubes, and 5 \mu l was collected and inoculated to agar plate containing medium at 0.5, 1, 2, 3, 4, 5, 30 and 60 minutes. The bacteria were incubated 24 or 48 hours. The effects of surfactants were judged on the basis of presence or absence of bacteria growth.
III. Single dose and repeated dose toxicity test
1. Animals and breeding conditions

ICR strain specific pathogen free male mice 4 weeks old obtained from the Charles River Japan, Inc. were used. After 1 week prebreeding, each test was started.

Mice were bred in plastic cages in a barrier sustained room. The animals fed on pellets (CRF-1, Oriental Yeast) sterilized by autoclave and given water added with 5 ppm chloride ad libitum.

2. Methods

In single dose toxicity test, male mice were forcibly given CAE orally in doses of 1.0 and 1.5 g/kg body weight with a sonde made of metal. This test was continued for 14 days after single administration. CAE solutions were diluted with saline.

In repeated dose toxicity test in mice, CAE was daily administered by oral gavage forcibly with a sonde for 7 days at dose levels of 0.5 and 0.1 g/kg/day. Control groups of each test were given saline in dose of 0.1ml/10g body weight instead of CAE.

For all experimental animals mortality and general signs were observed daily, and body weight was measured. Food consumption (F.C.) and water consumption (W.C.) were measured per group once a day through the experiment period. Food efficiency was calculated from the amount of gain in weight divided by amount of F.C. Then, all mice surviving to the end of the observation period were put to death by exsanguination under etherization. All mice were autopsied and gross observations on organs and tissues were performed. Liver and kidneys were excised from all animals for weighing and histopathological examination. When any other organs and tissues showing macroscopic changes at necroscopy were found, histopathological examinations were also performed. Organ weight was measured in absolute value and ratio to body weight, and then organs were prepared by the routine method of histological studies.

IV. Irritation test
1. Animals

White Japanese rabbits (Kyudo) weighing about 2.7kg or 3.1kg were used.

2. Methods

Histamine (Hist. Wako) were used in this test as positive controls. Evans blue was obtained from Chroma-Gesellschaft Schmid GMBH & Co. CAE and BKC were diluted with saline from 0.1% in multiple proportion. A preliminary experiment was conducted to determine some conditions as time course by using Hist. From these results, concentration of Hist. was determined to be 0.01%, and this method can make clearer spots by injecting test solution immediately after dye injection than in the reverse steps. That is, first, 1% Evans blue 6 ml/kg was injected gently by intravenous injection for 2 to 3 minutes, and 0.1ml of test solution was injected next into dorsal skin. And then the animals were sacrificed 20 minutes after injection and dorsal skin was exfoliated. Dye extraction method described in details as experimental procedure by Katayama et al. was carried out. Absorbance of evans blue dye measured was 620 nm.
V. Dermal sensitization test

1. Animals

These experiments were performed on Hartley strain guinea pigs (Kyudo) one month old.

2. Methods

The guinea pigs maximization test was carried out as described by Sato et al. An area of the shoulder region was shaved with a razor. At the first stage of induction, Emulsified Freund's complete adjuvant (E–FCA, DIFCO) was injected intradermally into the nuchal skin, and then a crisscross lattice, like the symbol sharp, of abrasions was made at the site of injection, on the first day. At the same time, 0.1 g of 50% CAE in petrolatum was applied to a lint patch (Torii, Adhesive plaster for patch test, Φ=1.5 cm), and fixed in place with adhesive surgical drape (3M, Steri–Drape®) for 24 hours. Similar abrasion and application of patches were repeated the following two days.

At the second stage of induction, the same area as above was shaved and then 10% sodium lauryl sulfate (Wako) in petrolatum was applied with a glass rod and with no bandage, on the eighth day. Twenty–four hours later, the test substance at the same concentration in the same vehicle as used at the first stage of induction was spread over a patch made of filter paper (Toyo Roshi, NO. 131). The patch was covered by overlapping, impermeable, plastic adhesive tape (3 M, Blenderm®) for leaving in place firmly for 48 hours.

Challenge was carried out on the twenty–second day. Hair was removed from the flank. A test sample was applied with open patch testing technique. Ten µl of CAE in water, in concentrations of 1.0, 2.0, 10.0 and 20.0%, was applied directly to an area of the flank skin 1.5 cm in diameter. Petrolatum for negative control and Sudan I for positive control were applied in the same way. The challenge site was evaluated at 24 and 48 hours after application, in accordance with the scale for scoring of skin reaction.

VI. Hemolysis

1. Animals

Sprague–Dawley male rats (Kyudo) were etherized. Blood samples were obtained from the heart and gently placed in a test tube containing sodium heparin with 1000 units/ml.

2. Methods

Preparation of erythrocytes was carried out as described by Yasuhara et al. Blood was centrifuged at 800 g for 15 minutes at 0 °C. The erythrocytes were washed twice and resuspended with 3 folds isotonic phosphate buffer (10mM, pH 7.4) for 10 minutes, to make a final concentration of 10⁹ cells/ml. The stock erythrocytes suspension was kept at 0 °C and used for the experiment within a week.

Hypotonic hemolysis experiments followed the modification of the method reported by Seeman and Weinstein. The hypotonic phosphate buffer (NaCl in 10mM, pH 7.4) was adjusted to remain constant at 70% hemolysis in the absence of drug, according to the prepared suspension. Aliquots of 0.2 ml from the erythrocytes suspension were pipetted into a test tube containing 4.8 ml of the test solution at 37°C. The test solution was composed of hypotonic buffer, containing the drug at concentrations from 10⁻³ to 10⁻⁴ M. DMSO, as the
solvent for the drug, was similarly added to the test solution as a control in the same volumes. One hour after addition of suspension, the mixture was centrifuged at 800 g for 5 minutes. Absorption of the supernatant fraction was measured at 540 nm in the spectrometer (Bausch Lomb, Spectromic 20A) to determine the hemoglobin concentration.

**Results**

1. Cytotoxicity
   1. Concentration of inhibition to 50% cell adhesion
      CAE at 78 μg/ml and BKC at 4.3 μg/ml inhibited 50% cell adhesion to culture dishes (Fig. 1). The cytotoxicity of CAE shown was one eighteenth as much as that of BKC.
     2. Observation by SEM and TEM
        Many well-developed microvilli appeared in control (Fig. 2). The cytoplasmic ground substance relatively condensed in various cell shapes, such as spindle-shaped, rounded, and angular cells. The nuclei had dark granular chromatin and the shape of nuclei was elliptic but slightly irregular in shape (Fig. 3). On the peripheral of cell, filopodia which is longer and finer than microvilli, extended and attached to the bottom of culture dishes.
        Cells were observed to change only 1 minute after CAE treatment by SEM. Microvilli tended to decrease and several wrinkled lines were shown longitudinally on the surface of

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**Fig. 1** Effects on culture cells adhesion with CAE and BKC.

**Fig. 2** Scanning electron micrograph of L cells exposed to DMSO only. Bar represents 5 μm.

**Fig. 3** Transmission electron micrographs of L cells exposed to DMSO only. Bars represent 2 μm.
spindle-shaped cells. Clearer and deeper wrinkled lines on the surface were shown in 3 minutes (Fig. 4-a). In 5 minutes, microvilli decreased considerably, and in 10 minutes almost disappeared. At the same time, wrinkled lines were much deeper. And then, the fold-like membranes were found (Fig. 4-b). Extensive damages could be seen on the crevice surface, with a few deep grooves and numerous small hollows over 40 minutes treatment (Fig. 4-c).

While in 1 minute treatment some morphological changes on cell surface were found by SEM, in the observation by TEM there were no changes in cytoplasm or organelles which were seen in control. However, 3 minutes treatment with CAE made some nuclei remarkably irregular. Some cells with slightly less dense cytoplasmic ground substance were found (Fig. 4-d). These changes became more marked with time, and 10 minutes treatment resulted in less dense organelles, especially in mitochondria. Small part of the cell membranes ruffled intensely. Some of them had more irregular nuclei than before (Fig. 4-e). Cells in 40 minutes CAE treatment had swollen organelles and numerous vacuoles in cytoplasm, and almost all of cells of both nuclei and cytoplasm were extremely less dense (Fig. 4-f). Many cells had torn cell membranes and leaked out a part of intracell compounds from tears. The
distribution of chromatin in the nuclei was homogeneous. It seemed to result in cell lysis like nuclei membranes broken in CAE treatment for 40 minutes.

On the other hand, BKC have no effect on cell morphology within 5 minutes. But 10 minutes treatment of BKC made wrinkled lines like those observed with CAE treatment (Fig. 5–a). After 60 minutes, microvilli decreased and cell membranes became smooth (Fig. 5–b). On the same time cells with fold–like membranes appeared. The effect of surfactant revealed markedly. In 180 minutes treatment, microvilli almost disappeared and fold–like membranes were found (Fig. 5–c). It could also be observed, furthermore, that the cells had neither fold–like membranes nor microvilli and had numerous small hollows on the surface. A crevice was seen on the extremely damaged surfaces of some cells.

While low density areas studded the cytoplasm within 10 minutes BKC treatment in the observation by TEM (Fig. 5–d), these increased on the whole cytoplasm until 40 minutes. Swollen mitochondria and numerous vacuoles were observed at this time. Small vacuoles were observed prominently in the cytoplasm located near the cell membranes. Sixty minutes treatment resulted in a destruction of cristae of mitochondria (Fig. 5–e). Swelling of organelles became more and more conspicuous, and nuclei were affected by CAE.
Appearance of most cells at 180 minutes CAE treatment was biolysis, because the following changes were obvious: broken cell membranes, homogeneous density of nuclei, less dense cytoplasmic ground (Fig. 5-f).

II. Antimicrobial effect

BKC and CAE when contacted for 0.5 and 1 minutes, the manifestations of germicidal action against *B. subtilis* were 0.7% of either drug (Fig. 6-a). It required 0.7% for CAE antimicrobial action against *B. subtilis* to become manifest even though prolonging the contact time, while in five sevenths concentration of CAE, BKC became effective by prolongation. The effective concentrations of BKC against the strains without *B. subtilis* were not more than 0.1%, which is the concentration generally in use (Figs. 6−b, c, d, e and f). In case of CAE, less than 0.1% was required to become manifest for antimicrobial action against

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*Fig. 6* Influence of CAE and BKC to bactericidal effects by contact time.


○: CAE, ▲: BKC.
S. aureus, S. mutans and P. aeruginosa over 1 minute contact, though within 0.5 minutes it required 2–20 folds higher concentrations than that of BKC (Figs. 6–b, c and d). Besides, against E. coli, CAE was effective in excess of BKC at more than 1 minute contact, although 0.1% of CAE was not effective within 0.5 minutes (Fig. 6–e). Thus, the augmentation with prolonged contact time in case of CAE was more distinguished than in case of BKC. However, the same results cannot be expected for C. albicans. Even though the contact time prolonged to 60 minutes, 0.2% of CAE was needed to kill C. albicans. From 1/4 to 2 times concentrations of CAE were required to be comparable with the action of BKC (Fig. 6–f).

III. Single dose toxicity study

1. Effects on general signs, mortality and growth of body weights

Only in the group on 1.5 g/kg CAE a mouse died within a day after administration. One animal in lower dose group on 1.0 g/kg of CAE showed abnormal conditions such as piloerection until the following day of administration and substantial loss of body weight from the start. The whole group of 1.0 g/kg CAE showed decrease in the mean of body weight at the first day after administration, but recovered gradually from the second day (Fig. 7). The experimental period was divided into 3 parts; from the first day to the seventh day (first week), from the eighth day to the fourteenth day (second week) and from the first day to the fourteenth day (total period). With regards weight gain, the group of 1.0 g/kg CAE at the first week showed significant decrease as compared with the control. Conversely at the second week the group showed significant increase. In case of 1.5 g/kg CAE group, the gain in weight was not so favorable as compared with control group for several days after administration, but the difference between the groups was insignificant when examined by the period. The mean of body weight at the end of the examination was about the same for all 3 groups (Fig. 8).

![Fig. 7 Change in body weight of male mice of in a single oral dose of CAE.](image)

![Fig. 8 Change in body weight of male mice in a single oral dose of CAE at each week and total period. *: p<0.05, **: p<0.01.](image)
2. F.C. and W.C.

F.C. and W.C. were calculated in amount of per day and per body, and were tested with mean value of each period between control and test groups. Animals in group of 1.0 g/kg CAE reduced F.C. significantly as compared with control (p<0.05). However, the animals of high dosage group showed no significant difference from control in F.C. by period (Fig.9).

W.C. is shown in Fig.10. In the group of 1.0 g/kg CAE, W.C. was significant by reduced during the first week (p<0.05). In the group of 1.5 g/kg CAE it showed no significant difference in any periods.

Fig. 9 Change in food consumption of male mice in a single oral dose of CAE at each week and total period.
Statistical difference from control. *; p<0.05.

Fig. 10 Change in water consumption of male mice in a single oral dose of CAE at each week and total period.
Statistical difference from control. *; p<0.05.

Fig. 11 Change in food efficiency of male mice in a single oral dose of CAE at each week and total period.

Fig. 12 Change in body weight of male mice during 7 days period of CAE administration and subsequent 7 days period of observation.
Evaluation of a Cationic Surfactant CAE (Higashi)

Table 1 Absolute and relative organ weights in male mice on single oral administration of CAE

<table>
<thead>
<tr>
<th>Dose (g/kg)</th>
<th>No. of mice</th>
<th>Absolute organ weight (g)</th>
<th>Relative organ weight (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Liver</td>
<td>Right</td>
</tr>
<tr>
<td>1.0</td>
<td>6</td>
<td>1.94±0.12</td>
<td>0.28±0.02</td>
</tr>
<tr>
<td>1.5</td>
<td>5</td>
<td>1.82±0.26</td>
<td>0.27±0.03</td>
</tr>
<tr>
<td>Control (Saline)</td>
<td>5</td>
<td>1.92±0.20</td>
<td>0.27±0.05</td>
</tr>
</tbody>
</table>

Values are mean ± S.D. No significant difference is from control.

Relative organ weight (%) = \( \frac{\text{Absolute organ weight (g)}}{\text{Body weight (g)}} \times 100 \)

Further analyses of food efficiency based on growth and F.C. data showed that these values varied widely from 6.4 to 9.6% between groups and each period, though in the total period the values were about constant from 8.0 to 8.1% (Fig. 11).

3. Organ weight

No significant difference in organ weights was seen between the two CAE groups and control group (Table 1). In case of liver of the group of 1.0 g/kg CAE, both absolute and relative organ weights tended to be slightly heavier than those of 1.5 g/kg CAE group and control.

IV. Repeated dose toxicity study

1. Mortality and change in body weight

No animal died through the experiment. Squeaking and retarded growth rate were observed in both CAE groups (Fig. 12). Gain in weight was observed by period, which was divided into administration period (the first to the seventh days), observation period (the eighth to the fourteenth days) and total period (the first to the fourteenth days).

Mean of body weight in administration period decreased from the start; conversely in observation period, it rather increased, as much as 118% of control's growth in weight. However on the last day of experiment, mean of body weight was only 107% of that on the first day of experiment, which was only 45% of the control gain rate (116%) during the same period. When observed by the total period, gain in weight showed significant decrease (Table 2). The growth of group of 0.1 g/kg CAE was slower than that of control within administration period. At the fifth day body weight of this group decreased markedly, but it showed a tendency to recover thereafter. And at the end of experiment the mean of body weight was 112% of the original weight. No significant difference was seen between this group and the controls.

2. F.C. and W.C.

F.C. and W.C. were measured and tested in the same way as in single dose toxicity study. The decrease of F.C. was significant in both test groups during the periods excluding the
Table 2  Change in body weights of male mice in repeated oral dose of CAE

<table>
<thead>
<tr>
<th></th>
<th>CAE (p. o./day)</th>
<th>Control</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.5g/kg</td>
<td>0.1g/kg</td>
</tr>
<tr>
<td>Number of mice at start</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>Death during experimental period</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Number of mice at termination</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>Body wt. (g±S. D.)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>At start</td>
<td>29.1±1.3</td>
<td>28.4±1.0</td>
</tr>
<tr>
<td>On 7th day</td>
<td>29.9±2.6 [103]</td>
<td>29.5±1.4 [104]</td>
</tr>
<tr>
<td>[Ratio to start (%)]</td>
<td></td>
<td></td>
</tr>
<tr>
<td>At termination</td>
<td>31.2±1.0 [107]</td>
<td>31.7±1.4 [113]</td>
</tr>
<tr>
<td>Gained b. wt. (g±S. D.)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Administration period</td>
<td>-0.5±1.4** [-20]</td>
<td>1.0±1.5* [40]</td>
</tr>
<tr>
<td>Observation period</td>
<td>2.6±0.2* [118]</td>
<td>2.3±0.6 [105]</td>
</tr>
<tr>
<td>[Ratio to control (%)]</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total period</td>
<td>2.1±1.3** [45]</td>
<td>3.3±1.6 [70]</td>
</tr>
</tbody>
</table>

Termination: 14th day after the first administration.
Statistical difference from control. *: p<0.05, **: p<0.01

administration period of CAE 0.5 g/kg (p<0.01). In other words, no significant difference was found during the administration period of higher dose (Fig.13).

In case of W.C., when compared with control by period, the decrease of the group given CAE 0.5 g/kg in observation period was significant (p<0.05). But in any other period, no
significant difference was seen (Fig. 14).

Food efficiency was analyzed from gain in body weight (Table 2) and F.C. (Fig. 13). While it showed minus values within administration period of CAE 0.5 g/kg, during observation period of both CAE groups it was in excess of control value. In total period, food efficiency values were lower than controls (Fig. 15); food efficiency of CAE 0.5 g/kg and 0.1 g/kg was lower than and was 50% and 78% respectively to that of control.

3. Organ weight

The absolute liver weight of CAE 0.5 g/kg group was slightly less than that of control. The relative liver weight to the last of body weight also decreased.

In 0.1 g/kg CAE group no significant differences were found in neither absolute nor relative organ weight (Table 3).

4. Histopathological examination

Livers selected from both CAE groups had neither necrosis nor vacuolar degeneration. In a few mice, there were seen some fat droplet-like vacuolated cells at peri-hepatic lobule, but the frequency of occurrence was not different from controls.

In the test groups, there were no specially noteworthy changes in kidneys.

V. Irritation test

The changes on vascular permeability with CAE and BKC injections are shown in Fig. 16.

Table 3 Absolute and relative organ weights of male mice after a week of recovery from the oral administration of CAE for a week

<table>
<thead>
<tr>
<th>Dose (g/kg/day)</th>
<th>No. of mice</th>
<th>Absolute organ weight (g)</th>
<th>Relative organ weight (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Liver</td>
<td>Kidney</td>
</tr>
<tr>
<td>0.5</td>
<td>5</td>
<td>1.59±0.10*</td>
<td>0.23±0.02</td>
</tr>
<tr>
<td>0.1</td>
<td>5</td>
<td>1.76±0.16</td>
<td>0.24±0.03</td>
</tr>
<tr>
<td>Control (Saline)</td>
<td>5</td>
<td>1.89±0.21</td>
<td>0.28±0.04</td>
</tr>
</tbody>
</table>

Values are mean±S. D. Statistical difference from control. *: p<0.05.

Relative organ weight (%) = \[
\frac{\text{Absolute organ weight (g)}}{\text{Body weight (g)}} \times 100
\]
The higher the concentrations of both surfactants were, the more irritation occurred, at \( 10^{-3}\% \) and above.

VI. Sensitization test

The results of maximization test are shown in Table 4. In any challenge concentrations of CAE, neither edema nor flamma seen in positive control like Sudan I was found.

VII. Hemolysis

Though \( 10^4\text{M} \) of BKC made no change in erythrocytes, BKC within \( 10^6 \) to \( 2.5\times10^5\text{M} \) showed membrane stabilization and onset of hemolysis from \( 5\times10^4\text{M} (1.8\times10^{-4}%) \) BKC. At \( 2.5\times10^5\text{M}, \) BKC made 100% hemolysis. On the other hand, between \( 10^4 \) and \( 10^5\text{M} \) CAE maintained membrane stabilization. Onset of hemolysis was observed at \( 2.5\times10^5\text{M} (1.3\times \)
10^-3% CAE. At this concentration 100% hemolysis was obtained with BKC. One hundred % hemolysis with CAE occurred at about 5 \times 10^{-4} \text{M} (\text{Fig. 17}).

Discussion

I. Antimicrobial efficacy

HBsAg, a coat component of HBV, has been proven to be unusually stable against disinfective treatment. Nowadays only a few germicides have efficacy sufficiently enough to be used for HBV, such as sodium hypochlorite in chloric compounds, or glutaraldehyde, ethyleneoxide gas, formaldehyde gas in non–chlorics\(^\text{10}\). However chloric compounds have corrosive action into metals, and non chlorics like aldehyde and ethyleneoxide gas are limited to be used for non human, for fear of occurrence of protein eroding, hemolytic toxicity and so on. If hands are contaminated with virus, the only choice is to wipe off with absorbent cotton with sodium hypochlorite or to wash in running water at present.

By the way, CAE is noted to be a strong inactivator of virus especially HBV. The effect of CAE against HBsAg involves more than its surface–activating effect. Sugimoto et al. (1981)\(^\text{10}\) revealed previously that it is essential for the inactivation of HBsAg that contains some special compounds such as L–arginine, and a cationic property exhibit. It is found, moreover, that virus containing no lipids is not inactivated by CAE. These pieces of evidence indicate that CAE may tightly bind to lipids of component in HBsAg, followed by formation of stable aggregates of the denatured HBsAg–CAE complex\(^\text{4,5}\). And at more than 0.25% CAE completely inactivates the antigen for 1 hour at room temperature.

In the examination of the antimicrobial effects of detergents within short contact time, CAE showed slower action than BKC. However by prolonging contact time, CAE shows powerful effect by strain. For example by comparing with 3 minutes treatment, CAE shows effectiveness with lower concentration than BKC against E. coli, though BKC is 2 to 8 folds as powerful as CAE against the other strains conversely. The bactericidal activity of CAE may not exceed that of BKC, but the fact proves that it appears to be justified to be used as a disinfectant, if proper contact time is chosen. In view of the fact that BKC has no activity against HBV, CAE is of high utility value, if it is utilized as a disinfectant.

II. Safety

1. Cytotoxicity

The cytotoxicity of BKC at several cell lines but L-929 cell had been investigated. In case of Chang's culture human conjunctival epithelial cells, the concentration of LD50 is calculated to be 0.72\(\mu\)g/mL of BKC and 2.5\(\mu\)g/mL of BKC causes all cells denatured in morphology or separated from culture surface with 24 hours contact\(^\text{13}\). On the other hand, in case of V79 chinese hamster cultured cells, BKC makes high influence on morphology at 10\(\mu\)g/mL, and minimum inhibitory concentration (MIC) of BKC is 30\(\mu\)g/mL\(^\text{14}\). This value of MIC is similar to several another disinfectants, such as chlorhexidine\(^\text{15}\) and glutaraldehyde\(^\text{16}\). It is one–tenths of that of eugenol\(^\text{17}\) and sodium hypochlorite\(^\text{18}\). Apparent damage to V79 cells with phenol is seen at 300\(\mu\)g/mL. MIC requires 1 mg/mL of phenol\(^\text{19}\). In this study, influence to L cells of BKC
was observed 4.3 \mu g/ml LD50 and 35.6 \mu g/ml of MIC. In any cell lines, the level of influential concentration of BKC is within the range of 1 - 40 \mu g/ml. Since the practical use concentration is different in each disinfectants, so we cannot immediately say the toxicity from these values; however, surfactant BKC seems to be relatively high cytotoxicity among the disinfectants.

The cytotoxicity of CAE is about one-eighth as low as that of BKC. As phenol inhibits 50\% L cells adhesion at 65 \mu g/ml\textsuperscript{30} which shows relatively low toxicity to V79 cells, it is suggested that the cytotoxicity to L cells is high in order of BKC, phenol and CAE.

Morphological study was carried out with CAE and BKC at 50\% inhibition of cells adhesion. CAE caused cell lysis at 40 minutes. On the other hand, BKC required 6 hours to cause cell death, inspite of showing some changes on membranes at 10 minutes. In treatment of BKC, it is suspected that the cells adhere to its culture surface before the influence of BKC make progress gradually. On germs, BKC has an immediate effect, while on culture cells CAE shows an immediate effect. The reason for effects in these ways cannot be determined, but it is thought that one of the reasons is the difference in absolute concentrations for observation which are the inhibitory concentration of adhesion respectively.

2. Systemic toxicity

As previously reported, the LD50 to BKC in experiments with using animals is revealed to be 200 - 445 mg/kg (p. o.)\textsuperscript{21-24}, and 16 mg/kg (i. v.)\textsuperscript{21}. It is seen generally that the fatal dose is about 1 - 3 g in human in case of BKC\textsuperscript{25} and quaternary ammonium detergents\textsuperscript{26} ; this value has no reference. And the case of intake of 13 g (200 mg/kg) is reported to have recovered\textsuperscript{27}. The LD50 to human was reported to be 100 - 400 mg/kg orally\textsuperscript{28-30}, and 5 - 15 mg/kg\textsuperscript{28,29} by parenteral routes. When these cationic surfactants are taken, clinical symptoms manifested are vomiting, nausea, esophageal cauterant damage, hypertension, collapse, convolution, coma, and death within 1 - 4 hours\textsuperscript{30}.

In the single toxicity experiment, one mouse in the group of 1.5 g/kg CAE which is the maximum concentration for a dose has died, but surviving animals show no differences in gain in weight, F.C., W.C., food efficiency and organ weight. At the concentration of 1.0 g/kg, it cannot be concluded that CAE is toxic, judging from the followings; F.C. and W.C. show some decreases but no dose-dependence is seen and organ weight shows no change. From these findings, it is supposed that the dose level causing obvious toxic changes is higher than 1.5 g/kg. And the LD50 is far higher than this dose, as the reference data, though not official, for the grouped mice published show 10.75 g/kg by Ajinomoto, a manufacturer. So the acute toxicity of CAE is judged to be slight. The LD50 (p. o.) to BKC in the grouped mice was reported to be 340 mg/kg\textsuperscript{10}. For this experiment, five fold amount of the LD50 to BKC was given, but the value of LD50 to CAE was not found at all. Consequently, CAE is thought to be quite low in acute toxicity.

In the repeated dose study, gain in weight of the groups of CAE was restrained in the administration period, but recovery was seen after administration period. The group of lower doses of CAE recovered to the similar level of control at the end of experiment. The effect
of repeated dosage of CAE appeared as decrease of F.C. markedly, and appetite diminished. However, correlation with dosage was not found. Therefore, its cause cannot be attributed to only the drug treatment.

The decrease of absolute liver weight seen in the group of 0.5 g/kg CAE is regarded to be the change accompanying the decrease in body weight. And the relative liver weight reduction seen in the group of 0.5 g/kg CAE may be caused by the possibility that the nutrients stored in liver was consumed due to starvation by decrease of F.C.. The fact that no histological findings of changes in organ weight are found and the extent of decrease is quite little appears to indicate that the changes are not meaningful toxicity-wise. In total period, not only F.C. but food efficiency decreased, but no abnormal findings were seen histopathologically for liver or kidneys. Although surface active compounds are sometimes used for the purpose of solubilization of insoluble drugs, 0.5–0.1 g/kg CAE seem to reduce absorption of the food taken in case of this experiment.

The general signs of acute and subacute toxicity to BKC on mice or rats are present as decrease of F.C., diarrhea and so on. The findings from dead animals have shown irritation to mucosa of stomach and intestines at autopsy. Chronic oral toxicity test causes such mucosa trouble. Similar mucosa trouble may also appear in the same way at the administration of higher concentrations of CAE. The decrease in food efficiency observed may be the local trouble in digestion and absorption in stomach and intestines, but not systemic action.

Generally speaking, the concentration of 1 %, which is the repeated dose in this experiment, of a cationic surfactant caused the mucosa trouble. Neither weight loss nor gastroenteric trouble appear by the concentrations of 0.25% (0.025 g/kg) and below. If concentrations over 0.03% surfactant get into eyes, some medical treatment is required immediately. Because contact with a concentrated solution results in caustic skin trouble with deep necrosis and cicatrices, instruction is given to wash with water and with soap thoroughly the skin involved. According to tabulation of toxicity classes by Hodge et al., in case of LD50 value of 0.5 to 15 g/kg in single oral dose to rats, the toxicity rating is 'slightly toxic' or 'practical nontoxic'. Probable lethal dose for human is 250 mg to 1 liter.

When calculated in term of human with 50 kg weight, the repeated dose given in this animal experiment is equivalent to the amount of 50–250 folds as someone drinks by mistake 100 ml of gargle, 0.1% CAE solution, for 7 days in succession. However the dose level which does not cause toxic changes appeared to be much less than 0.1 g/kg.

3. Local irritation, sensitization and effect on erythrocytes

Cationic surfactants sometimes cause irritation on skin or allergy by extended contact, but CAE was found to have no sensitivity in this study. In the experiment of irritation study by observing the rise of vascular permeability, CAE affects in about the same degree as BKC. But CAE exceeds BKC in respect of disappearance of irritation at 10⁻³% and below.

For surfactants, generally the potency of the membrane effect is correlated with pharmacological potency, and those showing biphasic actions on erythrocytes may well have
hepatotoxicity\textsuperscript{30}. All the drugs that have both a membrane stabilizing effect on erythrocytes at lower concentrations and a hemolytic effect at higher concentrations induce enzyme leakage from hepatocytes. Furthermore, the concentration at which hemolysis occurs corresponds to the concentration that cause a marked enzyme leakage from hepatocytes. So it is possible to anticipate whether there is any hepatotoxicity by observation of effect to erythrocyte membranes. It is supposed that the membrane stabilization at low concentration is due to expansion of the membrane caused by a fluidizing effect of surfactants on lipid bilayer. The fact that the erythrocytes exposed to CAE show lytic action at the concentration of 13 times greater than that of BKC suggests that the surface active potency of CAE is lower than BKC and the influence on plasma membrane is little depending on the concentration. Furthermore, showing biphasic action with sudden phase transition at that respective concentration suggests that these drugs may reveal hematotoxicity and BKC might show higher hemolytic potency than CAE. The $1 \times 10^{-3}\%$ CAE which may show enzyme lysis from hematocytes is a very thin solution, but taking into consideration that histological pattern of liver excised from the grouped mice given 1\% CAE shows no differences; only less than $1 \times 10^{-3}\%$ of CAE passed into liver, or the change was quite little and reversible in observation period if any damage had occurred by then.

III. For the use in oral cavity

Surface active agents are used frequently on skin and mucosa next to iodine preparation. Iodine preparations are superior disinfectant in respect to a wide range of applications and medium germicidal potency. But they also have some demerits such as coloring the applied region, and allergy as iode eczema. Thus, when iodine preparation cannot be used, the choice of disinfectants in oral cavity is only a few yet. Surfactants are low in germicidal potency but also have characteristic in detergency not found in iodine preparation.

Previous studies about potential plaque antiadherents of surface active agents showed that the ionic type was more effective in reducing \textit{in vitro} plaque adhesiveness than the nonionics\textsuperscript{40}. Moreover, antimicrobial activity of cationics on \textit{S. mutans} or \textit{S. sanguis} among ionics surfactants is superior\textsuperscript{47}. Mouth wash containing cetylpyridinium chloride reduced both the amount of plaque and severity of gingivitis\textsuperscript{47}. It is reported that some surface active agents increased the fluoride penetration in topical application of fluoride to the teeth as a caries-preventive procedure\textsuperscript{44,45}. By use of surfactants, it is possible to increase the effect of fluoride deposition and penetration. Cationic surfactants such as cetyl trimethyl ammonium bromide or BKC were examined to be used as root canal irrigants instead of sodium hypochlorite solution which causes the caustic action to periodontal tissue or oral mucosa. Not only the antibacterial effects but also dissolving activities of these cationic surfactants were strong as well as hypochlorite solution\textsuperscript{43}.

Thus, there will be high utility of a new surfactant with antibacterial activity in several respects. Though CAE is commercially available as only a detergent for the purpose of removing germs at present, its effects on HBsAg exceed BKC in cytotoxicity, systemic toxicity and local irritation, and some antibacterial effect with extended contact time.
Considering both of the utility and safety, the utilization of CAE as a disinfectant is possible and reasonable. Some drug design for lasting contact of CAE with oral mucosa and gingival sulcus is advisable hereafter in order to establish its clinical use of dental treatment.

**Conclusion**

The evaluation of CAE (N\(^{\text{a}}\)-cocoyl arginine ethylester DL-pyrrolidone carbonate) as a new disinfectant for practical use was examined from the aspects of efficacy and safety. The results were as follows;

1) Inhibitory concentration of 50% adhesion of culture L cells with CAE treatment was as about one eighteenth as that with BKC treatment (benzalkonium chloride).

2) At the concentration of 50% inhibitory cell adhesion, influence on the morphology was different between CAE and BKC. Morphological changes on cells by electron microscopies were found within a few minutes after CAE treatment, and cell lysis over 40 minutes treatment was found. In case of BKC, the influence appeared from 10 minutes, and cells were damaged extremely on the surface at 180 minutes treatment.

3) Bactericidal action of CAE was gradual but manifest enough by prolonging contact time by strain.

4) In acute toxicity test in mice, body weight, food consumption and food efficiency decreased in the group given CAE. However histopathological changes in liver and kidneys were not found. Acute toxicity of CAE may be classified between slightly toxic and practically nontoxic considering the amount of administration.

5) CAE was suggested to have no sensitivity by maximization test using the guinea pig.

6) Irritation activity and surface active potency of CAE were lower than those of BKC.

From the above; it is possible that CAE is applied with much safety and available for use as a disinfectant in oral and dental field.

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**References**

Evaluation of a Cationic Surfactant CAE (Higashi)


界面活性剤 CAE の口腔内消毒剤としての評価に関する基礎的研究

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新規の口腔用消毒薬として、化学構造からみても低毒性であることが期待できるカチオン界面活性剤 N*-cocoyl L-arginine ethylester DL-pyrrolidone carbonate（CAE）に着目し、その安全性と有効性について検証した。

安全性の面では、培養株細胞の50%付着阻止率からみたCAEの細胞毒性は、benzalkonium chloride（BKC）の約18分の1と非常に低いことが判明した。この付着阻止濃度で、CAEが細胞形態に及ぼす影響を電子顕微鏡で観察すると、処理後数分で細胞表面に変化が認められ、3分後には細胞内で核周辺の不整や細胞質の電子密度の低下などが生じた。処理後40分以降には細胞融解が観察された。これをBKCと比較したところ、形態変化の経緯は同じであったが、変化の現れるまでの時間がCAEはやや速かった。

マウスを用いた急性毒性試験では、CAE投与群の体重、摂餌量および食餌効率が、コントロール群と比較して低下傾向を示した。しかし、これらのマウスの肝臓や腎臓の病理学的所見では異常は全く認められず、CAEの毒性は軽度で、実用上ほとんど問題ないと判断された。さらに、モルモットを使ったアレルギー性試験ではCAEの感作原性は陰性と判定され、局所刺激性と界面活性力はBKCより明らかに低かった。溶血作用の過程から、CAEの肝毒性の可能性が考えられたが、その毒性は現在汎用されているBKCよりもかなり低いと思われた。

有効性の面からは、短時間接触によるCAEの殺菌効果を調べた。CAEの作用は速効性ではBKCにやや劣るものの、接触時間や濃度を考慮することにより、十分な殺菌効果を示すことが判明した。

以上のことから、CAEの安全性は極めて高く、剣形虫の持続化を図ることにより、有用な口腔用消毒剤として応用できると判断された。