Acanthamoeba is an organism that causes the infection termed Acanthamoeba keratitis (Ishibashi et al., 1988). The infection was first reported in Britain in 1974 (Naginton et al., 1974) and in Japan in 1988 (Ishibashi et al., 1988), and it is estimated that more than hundreds of cases occur each year in contact lens users who are said to represent over 15 million people in Japan so far (Tokoro et al., 2008). In Japan, the number of young people using soft contact lenses has increased recently (Shinoda et al., 2002; Study Group of National Surveillance of Infectious Keratitis in Japan, 2006). Acanthamoeba are protozoans found in soil, freshwater, and seawater (Ishii, 1999). However, non-sterile hands and water can contaminate contact lenses and cause corneal infections (Miyazaki et al., 2007; Yamaura et al., 1993; Zimmerman et al., 2017). Patients with Acanthamoeba keratitis may experience pain presenting with photophobia, ring-like stromal infiltration, epithelial defects, and eyelid edema (Lorenzo-Morales et al., 2015). If Acanthamoeba keratitis is not treated adequately, loss of vision may result.

Currently, cold disinfection with hydrogen peroxide solution or a multipurpose solution (MPS) is commonly used for disinfecting contact lenses (Hiti et al., 2002; Morishige et al., 2012; Atkins, 2006). In particular, an MPS can be used to clean, rinse, and disinfect soft contact lenses with the same liquid. In addition, it serves as a preservative, making it a commonly used solution (Hiti et al., 2002; Atkins, 2006). However, the antiseptic effect of MPS is weaker than that of other disinfection methods, and it is suggested that hypersensitivity may occur (Morishige et al., 2012; Atkins, 2006). Moreover, according to the 2009 National Consumer Center report in Japan, it has been shown that the anti-amoebic activity of commercially available MPSs is very low (National Institute of National Consumer Affairs Center in Japan, 2009; Tanaka et al., 2017). MPS contains polyhexanide.

**Note**

**Anti-Acanthamoeba effect of potassium isostearate for use as a multipurpose solution**

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**Acanthamoeba castellanii** is one of the organisms that cause corneal infection. In this study, attention was focused on potassium isostearate (iso-C18K, a branched chain fatty acid salt) for use in a multipurpose solution (MPS) against Acanthamoeba. An anti-amoebic test against Acanthamoeba castellanii ATCC 30010 (trophozoites type) was conducted. As a result, a growth reduction effect of 4 log units (99.99% suppression) was observed after incubation with 150 mM (5.0 w/v%) iso-C18K for 10 minutes. Furthermore, after the amoeba suspension was mixed with iso-C18K, disruption of cell membranes were observed, and the minimum amoebacidal concentration (MAC) at that time was 9.6 mM (0.31 w/v%). To evaluate the effectiveness as an MPS, assessment by verification tests was conducted using contact lenses. Reducing the concentration of iso-C18K caused a decrease in the number of viable cells, which was confirmed at a MAC of 1.2 mM (0.039 w/v%).

**Key words**: Acanthamoeba castellanii / Anti-amoeba activity / Branched chain fatty acids / Multipurpose solutions / Contact lens.
hydrochloride as the main component (Choy et al., 2012; Santodomingo-Rubido et al., 2006; National Institute of National Consumer Affairs Center in Japan, 2009). However, even after an incubation period of 8 hours, commercially available MPS decreased surviving cell numbers by only ≤ 1/10 cells in four of eight strains (National Institute of National Consumer Affairs Center in Japan, 2009; Tanaka et al., 2017). In addition, MPS is primarily used for eliminating bacteria and fungi, and is hardly considered for preventing eye infections caused by amoebae.

Previous studies regarding anti-amoebic activity have reported antibacterial tests using straight chain fatty acids and their salts (Tanaka et al., 2017) in addition to antibacterial tests against fungi (Era et al., 2015), bacteria (Masuda et al., 2015), and other microbial agents. Fatty acid salts are carboxylic acid salts of chain hydrocarbons and are surfactants formed from fatty acids and alkalis with both hydrophilic and hydrophobic properties. In addition, Shapiro (1996) reported that fatty acids are effective against oral bacteria (Porphyromonas gingivalis, Selenomonas artemidis, and Streptococcus sobrinus). However, an anti-amoebic study using branched chain fatty acids has not been conducted so far. Therefore, in this study, we investigated the anti-amoebic effects of branched chain fatty acid and salt as well as branched chain alcohol against A. castellanii.

First, an anti-amoeba test was conducted to investigate effective samples for amoeba. Isostearic acid (iso-C18), potassium isostearate (iso-C18K) and isoctadecanol (iso-C18-OH) were tested. The samples were obtained from Nissan Chemical Co. (Japan, Tokyo), Ltd. All of the structural formula are shown in Fig. 1. A. castellanii ATCC 30010 was obtained from ATCC (American Type Culture Collection, Manassas, America), and proteose–yeast–glucose (PYG medium, ATCC 712) broth was selected as the growth medium (Tanaka et al., 2017). The number of trophozoites A. castellanii was measured using an optical plastic plankton counter (Matsunami Glass Ind., Ltd., Osaka, Japan). The final amoeba suspension was adjusted to approximately 3.0 × 10⁴ cells mL⁻¹. The adjusted suspension of 1 mL was centrifuged (4°C, 3000 rpm, 10 minutes) to separate the media. 1 mL of 10 % Tween 80 and 1 mL of fatty acid or alcohol were mixed and the pellet was re-suspended contacted, then incubated at 30 °C. The Tween80 has a growth-inhibiting effect on microorganisms such as bacteria (Sawatari et al., 1984), and in the same experiment as Tanaka et al. (2017) one order of decline has been seen at 20 % Tween80. Therefore, 10% concentration of Tween 80 was used in this experiment. This concentration has no proliferation suppression effect and does not show

| Chemical compounds            | Structural formula |
|-------------------------------|--------------------|
| Isostearic acid (iso-C18)     | ![Structure of Isostearic acid (iso-C18)] |
| Potassium isostearate (iso-C18K) | ![Structure of Potassium isostearate (iso-C18K)] |
| Isooctadecanol (iso-C18-OH)  | ![Structure of Isooctadecanol (iso-C18-OH)] |

**FIG. 1.** Structure of branched chain fatty acid and salt, alcohol.
morphological changes even under a microscope. The samples of 500 μL were obtained at 10, 60, and 180 min, then stained with 100 μL Hank’s Balanced Salt Solution (Invitrogen, America) and 400 μL 0.4% Trypan Blue Stain (Invitrogen) and added to the optical plastic plankton counter. Next, 200 μL of sample was added to 800 μL of distilled water, and added on the optical plastic plankton counter and left for 5 minutes. Due to osmotic pressure, the staining solution penetrate dead cells. Dead cells were stained blue, and unstained viable cells were then counted using an optical microscope (ECLIPSE TS100; Nikon, Japan). All experiments were repeated at least three times.

Fig. 2 shows a time-course of anti-amoebic tests with iso-C18, iso-C18K and iso-C18-OH against A. castellanii. The iso-C18 was 1200 mM (42 v/v%) and resulted in a reduction of 4 log units in the growth of A. castellanii after incubation for 10 minutes. Iso-C18 suppressed 99.99% of amoebic growth and exhibited high anti-amoebic activity, whereas control (10% Tween 80) was ineffective. In contrast, iso-C18K (1400 mM, 43 v/v%) had little effect on the growth of A. castellanii after 180 minutes of incubation, having decreased growth by approximately only 0.5 log units. 150 mM (5.0 w/v%) of iso-C18K reduced the growth of A. castellanii by 4 log units after 10 minutes of incubation, whereas control (pH-adjusted water) was ineffective. Sasaki et al. (2019) examined the anti-amoeba effect using samples of 3,5,5-trimethylhexanoic acid (2800 mM) and potassium 3,5,5-trimethylhexanoate (260 mM). As a result, it was confirmed that 3,5,5-trimethylhexanoic acid decreased by 4 orders in 10 minutes and potassium 3,5,5-trimethylhexanoate decreased by 4 orders in 180 minutes. Although the concentration was different, iso-C18K in this study showed a high anti-amoeba effect. Furthermore, in the case of iso-C18K the amoeba cells could not be counted with a microscope, so we fixed the amoeba on the optical plastic plankton counter and tried to capture the moment when the iso-C18K contacts through the gap in the chamber. As a result, rupture of the amoeba cell membrane was confirmed. Fig. 3 (A) is a raw amoeba cell before contact, and (B) is an amoeba cell after rupture after contact with iso-C18K. When iso-C18K was contacted in real time while observing with a microscope, rupture of amoeba cell was confirmed in about 10 seconds contact time. The density at the time of photographing is 150 mM (5 w/v%).

The minimum amoebacidal concentration (MAC) is required to prevent the growth of microorganisms. Thus, lower MAC values indicate higher drug efficacy. The MACs of branched fatty acid, their salt and potassium 3,5,5-trimethylhexanoate decreased by 4 orders in 10 minutes and potassium 3,5,5-trimethylhexanoate decreased by 4 orders in 180 minutes. Although the concentration was different, iso-C18K in this study showed a high anti-amoeba effect. Furthermore, in the case of iso-C18K the amoeba cells could not be counted with a microscope, so we fixed the amoeba on the optical plastic plankton counter and tried to capture the moment when the iso-C18K contacts through the gap in the chamber. As a result, rupture of the amoeba cell membrane was confirmed. Fig. 3 (A) is a raw amoeba cell before contact, and (B) is an amoeba cell after rupture after contact with iso-C18K. When iso-C18K was contacted in real time while observing with a microscope, rupture of amoeba cell was confirmed in about 10 seconds contact time. The density at the time of photographing is 150 mM (5 w/v%).

The minimum amoebacidal concentration (MAC) is required to prevent the growth of microorganisms. Thus, lower MAC values indicate higher drug efficacy. The MACs of branched fatty acid, their salt and branched chain alcohol were measured to examine their anti-amoebic activity against A. castellanii, and samples with the highest activity were determined. As in the case of the anti-amoeba test, 10% Tween 80 was used as the diluting liquid in the case of fatty acids, and pH-adjusted water was used in the case of the fatty acid salt. The samples were prepared in 2-fold dilution series (Tanaka et al., 2017). All experiments were repeated at least three times. The MAC value of iso-C18K was 9.6 mM (0.31 w/v%). Therefore, iso-C18K was found to be the most effective agent against Acanthamoeba as it exhibited an anti-amoebic effect at the lowest concentration (Table 1).

Verification tests using contact lenses were conducted to evaluate the effectiveness of iso-C18K as an MPS. The experiment was conducted according to the method described by Kilvington et al. (1990). The amoeba suspension (1 mL), adjusted to $3.0 \times 10^5$ cells mL$^{-1}$ was added to 12-well plates, and a soft
A contact lens was immersed and allowed to stand for 2 h. Thereafter, 2 mL of the sample, pH-adjusted water, and iso-C18K were added to other wells, and the soft contact lenses were transferred to these sample wells and subjected to disinfection for 6 hours. Because it is difficult to accurately count amoeba adhering to contact lenses, the soft contact lenses were transferred to other wells containing 1 mL of the PYG medium, and amoeba cells adhering to the soft contact lens in the wells were scraped off. The number of viable cells was measured after 7 days of culture at 30°C. A sufficient anti-amoebic effect was considered to be when the number of viable cells was ≤ 10 cells mL$^{-1}$. All experiments were repeated at least three times.

Fig. 4 shows the results of verification tests using iso-C18K. Based on the MAC value of anti-amoeba test 9.6 mM (0.31 w/v%), a 2-fold dilution test was performed. Data were obtained from at least three independent experiments. However, as the anti-amoebic effect was not observed in control cells (pH-adjusted water, pH = 10.8), it could be considered as the anti-amoebic effect of iso-C18K. In addition, it was found that this concentration was effective at concentrations lower than the MAC.

Tests for contact lens disinfectants adhered to international standard ISO 14729 for evaluating the effectiveness: disinfectants need to conform to these standards to be considered effective. In stand-alone tests, it is necessary to evaluate disinfection effects against bacteria such as *Staphylococcus aureus*, *Pseudomonas aeruginosa*, and *Serratia* and fungi, such as *Candida* and *Fusarium* (Rosenthal et al., 2002). Therefore, the antibacterial and antifungal activities of potassium isostearate were tested against these five organisms (three types of bacteria and two types of fungi). The following test bacteria and fungi were selected: *S. aureus* NBRC 12732, *P. aeruginosa* NBRC 13275, *S. marcescens* NBRC 102204, *C. albicans*...
NBRC 1594, and *F. oxysporum* NBRC 31631. *S. aureus* was cultured on NB medium (Becton Dickinson). *P. aeruginosa* and *S. marcescens* were cultured on NBRC 802 medium (polypeptone, 10 g; Wako Pure Chemical Industries, Ltd.; yeast extract, 2 g; Becton Dickinson; MgSO₄·7H₂O, 1 g; Kanto Chemical Co., Inc; distilled water, 1 L; pH 7.0). *F. oxysporum* was cultured on potato dextrose agar (PDA; Nissui Pharmaceutical Co., Ltd.) medium, and *C. albicans* was cultured on YM medium (glucose, 10 g; Waco Pure Chemical Industries; peptone, 5 g; Becton Dickinson; yeast extract, 3 g; Becton Dickinson; malt extract, 3 g; Becton Dickinson; distilled water, 1 L). Two-fold dilutions of iso-C18K were prepared in pH-adjusted KOH solution, and the final concentration was 10 w/v% in pH-adjusted water. Then, 400 μL each of the prepared sample and microbial suspension as mentioned in preparation of cell suspensions, (bacteria: 5.0 × 10⁷ cells/mL, fungi: 5.0 × 10⁷ spores/mL) was taken in a 1.5 mL tube. The suspensions were stirred using a twin mixer and sampled after 10, 60, and 180 min. Samples of 100 μL were applied on the each other agar media described above. The applied samples were cultured under the following conditions: *S. aureus* for 2 days at 37°C, *P. aeruginosa* and *S. marcescens* for 1 day at 30°C, *C. albicans* and *F. oxysporum* for 2 days at 30°C. Results of viable counts [log colony forming units (CFU) mL⁻¹] were calculated by measuring the number of colonies appearing on the agar medium. All experiments were repeated at least three times.

Fig. 5 shows the results of stand-alone tests for iso-C18K. In panel (A), for *S. aureus*, a decrease of 7 log units was observed at 10 minutes up to a concentration of 9.6 mM (0.31 w/v%). However, at 4.8 mM (0.16 w/v%), the antimicrobial effect decreased to approximately 6 log units, even after contact for 180 minutes, and at the concentration of ≤ 2.4 mM (0.078 w/v%), the antimicrobial effect observed was insufficient. Moreover,
in panel (B), for *P. aeruginosa*, a decrease of 7 log units was observed at 10 minutes at a concentration of 4.8 mM (0.16 w/v%), but at the concentration of ≤ 2.4 mM (0.078 w/v%), the antibacterial effect was insufficient. In panel (C), for *S. marcescens*, a decrease of 7 log units was observed at the concentration of 310 mM (10 w/v%) at 10 minutes, but at the concentration of ≤ 9.6 mM (0.31 w/v%), no antibacterial effect was obtained, even after incubation for 180 minutes. In panel (D), *C. albicans* showed a decrease of approximately 2 log units at 180 minutes up to a concentration of 38 mM (1.3 w/v%); however the high antimicrobial effect was not observed at a concentration of ≤ 19 mM (0.63 w/v%). In panel (E), a concentration of 310 mM (10 w/v%) of *F. oxysporum* showed a decrease of 4 log units at 180 minutes. After 180 minutes, the antibacterial effect decreased with decreasing concentration; no antibacterial effect was observed at 4.8 mM (0.16 w/v%).

The log reduction required to pass the primary criteria of the stand-alone test is 3.0 log reduction for bacteria and 0.7 log reduction for fungi ([Rosenthal et al., 2002](#)). As mentioned above, the effective concentration for all five strains was 9.6 mM (0.31 w/v%) at the lowest concentration.

The results of this study suggested that branched chain fatty acid salts have higher anti-amoebic effect than branched chain fatty acid and branched chain alcohol. A previous study regarding anti-amoebic effects against *A. castellanii* showed a decrease in growth of 4 log units when treated with 175 mM potassium caprylate (C8K), potassium caprate (C10K), and potassium laurate (C12K) for 180 minutes ([Tanaka et al., 2017](#)). In addition, cell membrane disruption was observed after mixing the amoeba suspension with C10K or C12K, and the MAC of C10K and C12K was 2.7 mM. Furthermore, in verification tests, both samples showed an anti-amoebic effect at a concentration of 5.5 mM. In this study, MACs of the anti-amoebic test were as follows: 1200 mM (42 v/v%) for iso-C18, 9.6 mM (0.31 w/v%) for iso-C18K and 1400 mM (43 v/v%). The MAC in the verification tests was 1.2 mM (0.039 w/v%) for iso-C18K. In addition to in this study, we confirmed the rupture of amoeba cell membranes, as mentioned in previous studies ([Tanaka et al., 2017](#)). The verification tests of iso-C18K was found to exert anti-amoebic effects at a concentration of approximately 1/4 that of C10K and C12K, as mentioned in a previous study ([Tanaka et al., 2017](#)). Furthermore, the time of contact in the previous study was 180 minutes, but was only 10 minutes in the present study. From these results, it can be deduced that the anti-amoebic effect of iso-C18K is higher than that of C10K and C12K from the previous study.

According to [Godfrey (1966)](#), a surfactant exerts its antimicrobial mechanism by being adsorbed onto the surface of the fungus, accompanied by destruction of the surface lipid membrane and impairment of the penetration-regulating function of the lipoprotein membrane. Alternatively, Godfrey described that respiration is obstructed by the surfactant film formed on the surface of the fungus. In addition, [Obayashi (1961)](#) reacted *E. coli* with various surfactants (Ribonox LCR, Ribonox NCI, CTAB, CPC, Ribonol AS, Lipon P-106, Lipomin SA, and Lipomin COH) and examined changes by electron microscopy. As observed by Obayashi (1961), antimicrobial action was confirmed, and it was observed that cell membranes were ruptured. Moreover, [Watanabe (1980)](#) described the mechanism of such antibacterial action as follows: The antimicrobial agent is concentrated on the surface cell membrane of the fungus, then moves to the action site, hindering normal membrane functions, such as semipermeable membrane properties. The agent penetrates the membrane, enters inside cells and kills by activating denaturation of nucleic acids and proteins, or destroying parts of the cell membrane.

*Acanthamoeba* is a protozoan that to exercise by lobopodium, called bare amoeba ([Ishii, 1999](#)). Unlike shell amoeba (tested amoeba), bare amoebae do not form shells (test, skeleton, conch) and instep (theca, case, box), and as its name suggests, it does not have a hard coated periclar (pellicle, cuticle), such as a ciliate or a developed skin layer (cortex). However, observing the body surface of *Amoeba proteus* using a high-resolution optical microscope reveals a thick membrane structure corresponding to the cell membrane. Because this structure cannot be considered as a naked cell membrane from the viewpoint of thickness, it had been referred to as plasmalemma for a long time ([Mast, 1923](#)) and not as a cell membrane in amoeba. It was then observed by electron microscopy that a structure corresponding to the glyocalyx exists in contact with the unit membrane outside the cell membrane ([Pappas, 1959](#)). [Ishii (1999)](#) roughly divided the plasmalemma into glyocalyx, glycostyle, and scale. Also, he suggested the following: *Acanthamoeba* has a glyocalyx in close proximity to the outside of the cell membrane, with a thickness of 8-10 nm. In addition to being the simplest non-structural structure, the microstructure of glyocalyx is filamentous, tubular, and hexagonal prism; it is regarded as a characteristic of glyocalyx that more complicated patterns are not so clear as to be recognized as independent unit structures. Glycostyle is a delicate structure that is different from glyocalyx. However, unlike glyocalyx, glycostyle can be distinguished clearly from glyocalyx by electron microscopy, and is considered to be one of the components constituting the glyocalyx. However, it is not an exceptional cell coat that is morphologically completely
independent from glycocalyx and therefore, it was said to be distinguishable from scales (Page et al., 1979). Scales are rigid and stable solid structures arranged on the glycocalyx surface in large numbers (Ishii, 1999). First discovered by Grell et al. (1966), typical scales are found in organisms such as Cochliopodium, Dactylamoeba, and Paramoeba eilhardii. Furthermore, it is said that a protein called concanavalin A, which is a type of lectin, binds to the lipopolysaccharide layer. Furthermore, there is a layer consisting of actin and myosin fibers just below the cell membrane (Sonobe et al., 2004). From the above, iso-C18K seems to exert the properties of destroying the plasmalemma (glycocalyx, glycostyle, and scale) and proteins.

Tanaka et al. (2017) suggested that the effect of the anti-amoebic activity depends on the carbon chain of the fatty acid salt, and that samples with 8-12 carbons in straight chain fatty acid salts have an appropriate balance of hydrophilic and hydrophobic groups that are adsorbed onto the cell membrane of Acanthamoeba. As the number of carbon atoms in the fatty acid salt increases, the hydrophobic group increases and the affinity for lipids increases, whereas decreases in carbon atoms increase water solubility and decrease lipophilicity. In addition, Zheng et al. (2005) used the full-length Fabl (bacterial enoyl–acyl carrier protein reductase) gene amplified from genomic DNA of E. coli or S. aureus ATCC 26669 to demonstrate that linoleic acid is a model for unsaturated fatty acids that selectively inhibit Fabl, suggesting that the antimicrobial effects of long chain unsaturated fatty acids are due to inhibition of fatty acid synthesis. The reason why stearic acid, a saturated fatty acid, does not exhibit antibacterial activity was found to be due to the difference in synthesis of unsaturated and saturated fatty acid and Fabl inhibition.

In summary, iso-C18K used in this study, with well-balanced hydrophilic and hydrophobic groups, was easily adsorbed onto the membrane of Acanthamoeba, then moved to the site of action, impeded normal functioning of the membrane, penetrated the membrane, and entered the cell, thereby inactivating the enzyme system and denaturing or destroying nucleic acids and proteins. It is assumed that these effects resulted in rupture of the outer casing of the amoeba.

Taken together, iso-C18K used in this study showed greater anti-amoebic effects than mentioned in previous studies (Tanaka et al., 2017), suggesting the possibility of it use in an MPS. However, as high antimicrobial activity was not obtained in the stand-alone test using three bacteria and two fungi, it is expected that Acanthamoeba keratitis can be suppressed by adding iso-C18K to conventional MPSs.
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