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Storage stability of serum formulations containing ofloxacin for autologous serum eardrop therapy

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Objective: The storage stability of serum formulations containing ofloxacin for autologous serum eardrop therapy was evaluated for microbiological quality and component stability. *Methods*: Sterile serum formulations were prepared by mixing human serum and ofloxacin otic solution (1:1, v/v). To simulate eardrop contamination with external ear surface substances, prepared serum formulations were contaminated with a cotton swab that was rubbed sufficiently on the human external ear. Formulations were stored at 4 °C or room temperature in the dark. Colony forming units (CFUs), ofloxacin, and basic fibroblast growth factor (bFGF) concentrations in the stored serum formulations were determined. *Results*: The growth of microorganisms derived from the external ear was not detected in serum formulations after storage for 14 days, regardless of temperature. However, microbial growth was detected in serum formulations stored without ofloxacin, indicating that this is necessary for storage. In addition, concentrations of ofloxacin and bFGF did not decrease over 14 days, indicating that ofloxacin and bFGF in serum formulations are stable for this time period. *Conclusion*: The present study indicates that the efficacy and safety of serum formulations used as a therapy for perforated eardrums are stable and safe for at least 14 days.

1. Introduction

Autologous serum eardrop therapy (ASET) is a noninvasive therapy for the treatment of perforated eardrums. For this treatment, autologous serum formulations mixed with ofloxacin otic solution at 1:1 (v/v) are used as eardrops (Kakehata et al. 2008; Sasaki et al. 2004). In contrast to surgical treatments, ASET is expected to reduce patient burden.

In the clinical use of external drug formulations, it has recently been reported that microbial contamination from the contact of fingers and affected parts is a major problem (Lagnado et al. 2004). For example, during repeated use of autologous serum eye drop formulations for the treatment of corneal epithelium disorder, *Staphylococcus epidermidis* and *Serratia marcescens* have been reported to contaminate formulations causing infectious inflammation of the cornea (Cho et al. 2013). Numerous gram-positive and gram-negative bacteria are present on the external ear surface (Stroman et al. 2001); thus, repeated use of serum formulation eardrops may lead to contamination and growth of these microorganisms. Cell growth factors in serum formulations, including basic fibroblast growth factor (bFGF), have been associated with the healing of perforated eardrums (Shimada 1993; Mondain and Ryan 1995; Lou et al. 2012). It is conventionally recommended that serum formulations for ASET should be stored under cold temperatures and in the dark (Kakahata et al. 2008; Sasaki et al. 2004). However, the storage stability of serum formulations with regards to microbiological quality and component stability is unknown.

In this study, sterile serum formulations were prepared by mixing human serum and ofloxacin otic solution. The prepared serum formulations were contaminated with human external ear surface substances followed by storage at 4 °C or room temperature in the dark. Colony forming units (CFUs), ofloxacin, and bFGF concentrations in the stored serum formulations were determined to evaluate storage stability.

2. Investigations and results

2.1. Microbiological quality of serum formulations

The effects of storage conditions on microbial growth of serum formulations are shown in Fig. 1. In the presence of ofloxacin, a growth of microorganisms derived from the external ear was not observed after 14 days storage, regardless of storage temperature (Fig. 1A). However, in the absence of ofloxacin, growth of microorganisms was observed in formulations stored at room temperature for longer than four days (Fig. 1A, B).

2.2. Stability of ofloxacin in serum formulations

The effects of storage conditions on the stability of ofloxacin in serum formulations are shown in Fig. 2. Under each condition, the remaining concentration of ofloxacin did not decrease for 14 days.

2.3. Stability of proteins and bFGF in serum formulations

The effects of storage condition on the stability of proteins and bFGF in serum formulations are shown in Fig. 3. Electrophoretic patterns for each storage condition were similar to those just after preparation (Fig. 3A), while concentrations of bFGF for each storage condition were not different from those measured just after preparation (Fig. 3B).

3. Discussion

The findings from this study indicate that the presence of ofloxacin is required in serum formulations in order to avoid microbial contamination from external ear surface substances (Fig. 1). While microbial growth in contaminated serum formulations was not observed when samples were stored at 4 °C in the presence or absence, of ofloxacin, this antibiotic should still be added to serum

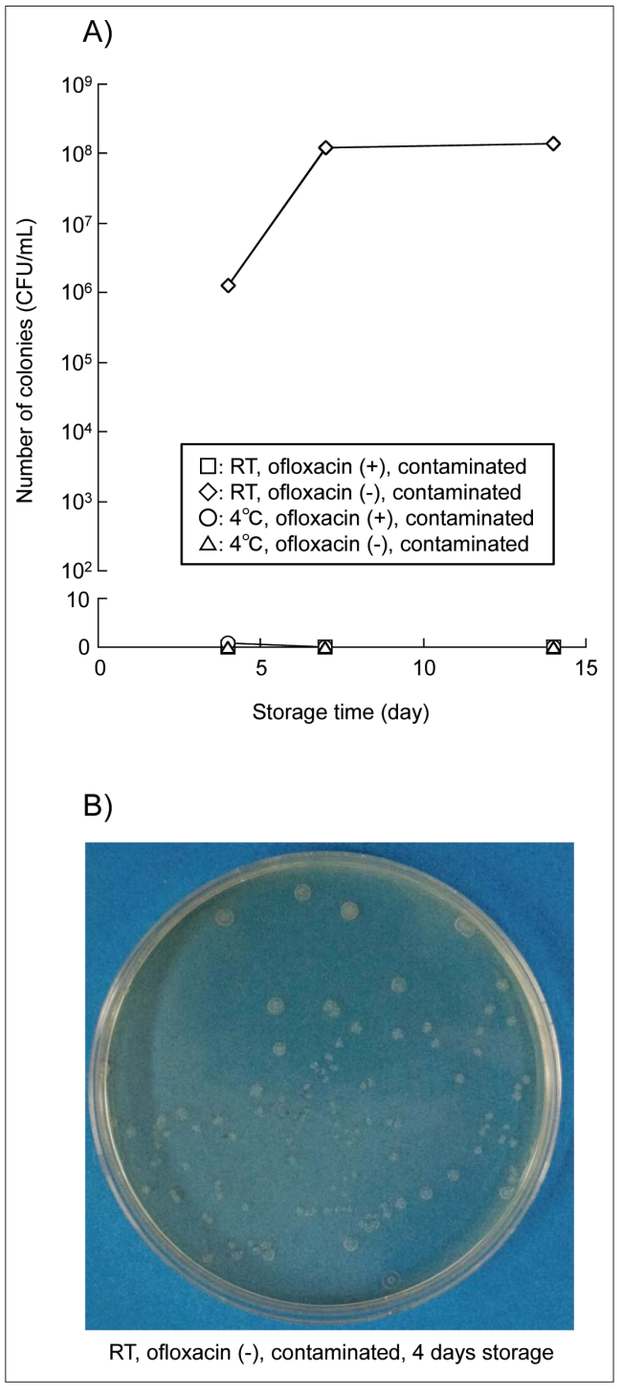

Fig. 1: Effects of storage condition on microbial growth in serum formulations. Serum formulations after four, seven, and 14 days storage were applied to culture media followed by incubation at 37 °C for seven days. After incubation, the number of colonies was obtained. Values are presented as means ± standard deviations (S.D.; n = 5). A) Time profile of the number of colonies; B) Image of bacterial cultures on the medium.

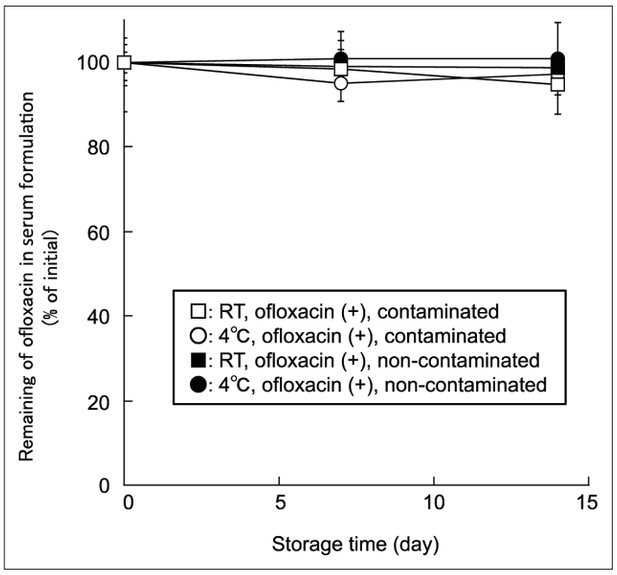

Fig. 2: Effects of storage condition on the stability of ofloxacin in serum formulations. Remaining concentrations of ofloxacin in serum formulations after seven and 14 days storage were determined. Values are presented as means ± S.D. (n = 3-4).

formulations to ensure microbiological quality. In addition, room temperature storage of formulations in the absence of ofloxacin is not recommended because bacterial growth was observed under these conditions. In eardrum perforations of patients with chronic otitis media, microorganisms including *Staphylococcus aureus* and *Pseudomonas aeruginosa* are present in large numbers on external ears (Sakai et al. 2001). Thus, the risk of contamination from these microorganisms into serum formulations is increased. Nevertheless, because ofloxacin has a wide antibacterial spectrum and strong antimicrobial activity, the microbiological quality of serum formulations is thought to improve with the presence of ofloxacin. Although ofloxacin in serum formulations was stable for 14 days (Fig. 2), it is important that formulations containing ofloxacin are not exposed to light for extended time periods because this antibiotic has poor light photostability (Daiichi Sankyo).

Because of the contamination of serum formulations with proteases and microorganisms derived from the external ear, bFGF and other proteins, including cell growth factors, can be inactivated. However, the SDS-PAGE electrophoretic patterns of proteins in contaminated serum formulations did not change after storage for 14 days (Fig. 3A), indicating that those detected by SDS-PAGE were stable over this time period. The most effective serum component for healing perforated eardrums is bFGF (Lagnado et al. 2004; Shimada 1994; Oiki et al. 1989, 1990). Indeed, concentrations of bFGF in contaminated serum formulations did not decrease after storage for 14 days (Fig. 3B), suggesting that contamination of serum formulations by external ear surface substances should not disturb their therapeutic effect. In addition to bFGF, epidermal growth factor (EGF) and transforming growth factor-β (TGF-β) are effective components of serum formulations (Cho et al. 2013). According to previous reports, EGF and TGF-β in serum formulations stored at 4 °C for 14 days, or at -20 °C for three months, were stable (Kakehata et al. 2008; Sasaki et al. 2004; López-Garcia et al. 2014). However, further examinations should be considered as the influence of external ear surface substance contamination on the storage stabilities of EGF and TGF-β in serum formulations has not yet been evaluated. The therapeutic effect on dry eye associated with Sjogren's syndrome depends on serum concentration in autologous serum eye drops (Cho et al. 2013), and in ASET, serum formulations mixed with ofloxacin otic solution at 1:1 (v/v) are generally used. However, the optimal ratio of serum and ofloxacin for maximum therapeutic effect and minimal microbial contamination is unknown. Development of safe and effective serum formulations with an optimal composition is necessary.

In conclusion, the storage stability of serum formulations containing ofloxacin for autologous serum eardrop therapy was evaluated with regards to microbiological quality and component stability. In serum formulations stored for 14 days at 4°C in the dark, growth of microorganisms derived from the external ear was not observed and no inactivation of ofloxacin and bFGF was detected. This study indicates that clinical recommendations for

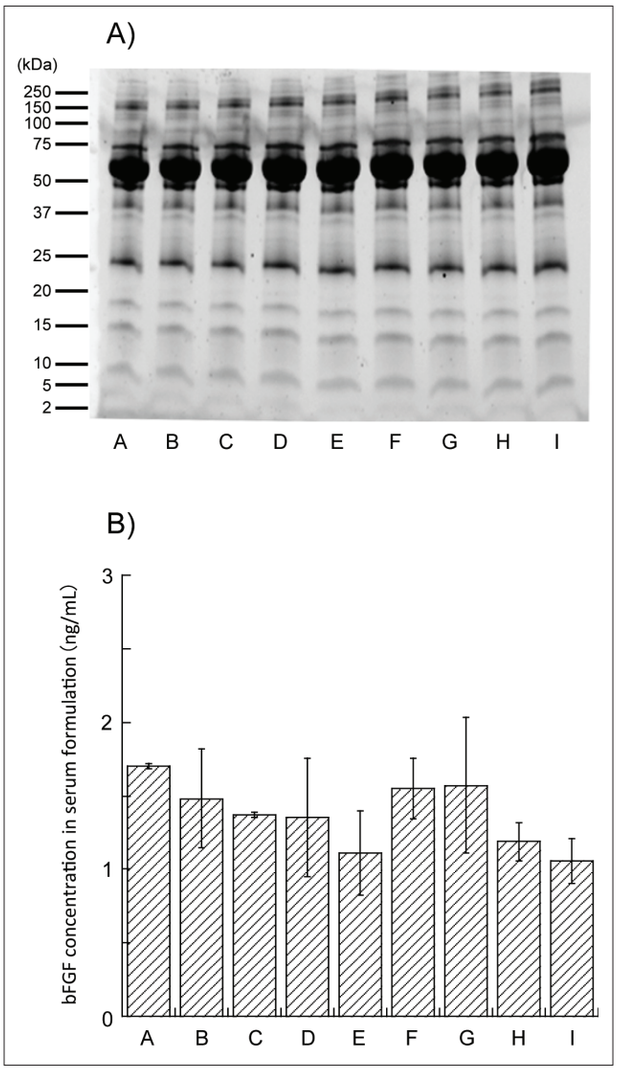

Fig. 3: Effects of storage condition on the stability of proteins and bFGF in serum formulations.
Proteins and bFGF in serum formulations just after preparation and after 14 days storage were detected and determined. Values in panel B are presented as means ± S.D. (n = 3).
A) SDS-PAGE electrophoretic patterns of proteins; B) bFGF concentrations.
Lanes: A) just after preparation, ofloxacin (+), non-contaminated; B) RT, ofloxacin (+), contaminated; C) RT, ofloxacin (−), contaminated; D) 4 ºC, ofloxacin (+), contaminated; E) 4 ºC, ofloxacin (−), contaminated; F) RT, ofloxacin (+), non-contaminated; G) RT, ofloxacin (−), non-contaminated; H) 4 ºC, ofloxacin (+), non-contaminated; I) 4 ºC, ofloxacin (−), non-contaminated.

the storage of serum formulations (ofloxacin presence, under coldness and darkness) are quantitatively suitable and that efficacy and safety are retained for at least 14 days.

4. Experimental

4.1. Materials

Human serum was purchased from Cosmo Bio Co., Ltd. (Tokyo, Japan). Ofloxacin otic solution (Tarivid® otic solution 0.3%) was purchased from Daiichi Sankyo Company, Limited (Tokyo, Japan). Ofloxacin was purchased from Nacalai Tesque, Inc. (Kyoto, Japan). Quinidine sulfate and 2-mercaptoethanol were purchased from Wako Pure Chemicals Co., Ltd. (Osaka, Japan). Nutritional broth and granulated agar were purchased from BD Difco™ (Bedfold, MA, USA). Yeast extract was purchased from Eidia Co., Ltd. (Tokyo, Japan), and all other regents were commercially available and of analytical grade.

4.2. Ethical issues

All procedures involving human participants were performed in accordance with the ethical standards of the institutional and/or national research committee as well as with the 1964 Helsinki Declaration and its later amendments or comparable ethical standards. Informed consent was obtained from all subjects included in the study.

4.3. Preparation, contamination, and storage of serum formulations

Human serum was sterilized through a membrane filter (PVDF Filter, 0.45 µm pore size, Merck Millipore, Billerica, MA) and mixed with ofloxacin otic solution (Kakehata et al. 2998; Sasaki et al. 2004) or sterile phosphate buffered saline (PBS), as a control, at 1:1 (v/v). Serum formulations were either non-contaminated or contaminated with a cotton swab that was sufficiently rubbed on the external ear of subjects. Formulations were stored at 4 °C or room temperature (RT) in the dark.

4.4. Quantitative measurement of contaminating microorganisms in serum formulations

Quantitative measurements were carried out according to the microbial limit test in The Japanese Pharmacopoeia, 16th edition (Japanese Pharmacopoea 2011). For preparation of culture media, nutritional broth (8 g), yeast extract (5 g), and granulated agar (15 g) were dissolved in sterile purified water (1 L) and an 18 mL aliquot was poured into a sterile dish and allowed to solidify. Serum formulations were diluted $10–10^8$ times with sterile saline. Dilutions (100 µL) were applied to culture media using a sterile spreader followed by incubation at 37 °C for seven days. After incubation, the number of colonies was counted macroscopically and the number of colony forming units (CFU) per 1 mL was calculated. Data were taken from dilution factor samples that showed maximal values in the range 0 to 300 CFU/dish.

4.5. Determination of ofloxacin in serum formulations

Ofloxacin concentrations in serum formulations were determined using HPLC. Briefly, 100 µL of serum formulation was diluted 10^3 times with 50 mM phosphate buffer (pH 2.8), mixed with quinidine sulfate (as an internal standard, 40 µL of methanol solution) and acetonitrile (10 µL), and a 20 µL aliquot was subjected to HPLC using a system (Shimadzu Co., Kyoto, Japan) equipped with a 5 µm Mightysil RP-18 GP column (4.6 x 250 mm, Kanto Chemical Co., Inc., Tokyo, Japan). The mobile phase was composed of 50 mM phosphate buffer (pH 2.8)/methanol/acetonitrile (10/4/1, v/v/v) and the flow rate was 0.7 mL/min. The eluate was monitored at Ex. 278 nm and Em. 480 nm and quantified. The amount of ofloxacin remaining in the serum formulations (% of initial) was calculated.

4.6. Detection of proteins and determination of bFGF in serum formulations

Proteins in serum formulations were detected using SDS-PAGE. Briefly, serum formulations were diluted 22.5 times with nuclease-free water. A solution of 4X Laemmli sample buffer (Bio-Rad, Hercules, CA) and 2-mercaptoethanol [9:1 (v/v)] was prepared and mixed with diluted serum formulations at 1:3 (v/v). A 10 µL aliquot was applied to an Any kD Mini PROTEAN TGX Precast Gel (Bio-Rad) and electrophoresed at 200 V. Gels were stained with an oriole fluorescent gel stain (Bio-Rad) and photographed using a chemiluminescence imager (AE-9300 H-CSP Ez-Capture MG, ATTO Co., Tokyo, Japan). The concentration of bFGF in serum formulations was determined using a Human bFGF ELISA Kit (RayBiotech Inc, Norcross, GA).

4.7. Statistical analysis

Differences were identified using the Kruskal-Wallis test with $P < 0.05$ considered significant.

Conflicts of interest: None declared.

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