Hydrerdy Human Amniotic Membrane (HD-AM) is Supporting Aciclovir Included Device of Poly-N-p-Vinylbenzyl-D-Lactonamide (PVLA) Sphere for Treatment of HSV-1 Infected Rabbit Keratitis Model

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Abstract

The human amniotic membrane (AM) has been widely used in ophthalmic surgery, including in ocular surface reconstruction. However, whether AM is effective for the release of impregnated drugs is unknown.

Hydrerdy-AM (HD-AM), glutaraldehyde cross-linked HD-AM (Glu HD-AM) and a solvent of poly-N-p-vinylbenzyl-D-lactonamide (PVLA) did not cause irritation to the rabbit eye. Although the concentration of residual glutaraldehyde in Glu HD-AM was approximately 40 ppm (dry-weight basis), no eye irritation was observed.

Acyclovir (ACV) containing PVLA-bearing spheres (ACV/PVLA) was loaded into HD-AM (ACV/PVLA HD-AM), and then the therapeutic efficacy of ACV/PVLA HD-AM was compared with that of ACV/PVLA solution in a herpex simplex virus-1 (HSV-1) eye infection model. The former was more effective against ocular infection, and its efficacy was dose- and volume-dependent. Thus, ACV/PVLA HD-AM sheets were very effective as a curative treatment for eye infection with HSV-1.

HD-AM can be function as drug delivery system. In the future, it may be possible to combine HD-AM and PVLA in a device that can control the release of hydrophobic medicines.

Keywords: Hydrerdy Human Amniotic Membrane (HD-AM); Drug storage; Herpes simplex virus-1 (HSV-1); Rabbit keratitis model; Aciclovir (ACV); Poly-N-p-vinylbenzyl-D-lactonamide (PVLA)-spheres; Drug delivery

Introduction

Herpes simplex virus (HSV) is one of the main infectious causes of corneal blindness worldwide. A variety of clinical manifestations of both infectious and immunologic diseases affects all levels of the cornea in HSV keratitis. Aciclovir (ACV) is the main therapeutic regime for corneal ulcer [1] because HSV-1 is sensitive to ACV [2]. The common regimen for corneal herpes in Japan is administration of ACV eye ointment (ACV-O). However, because ACV-O must be administered 5 times daily and shows poor water solubility and thus low bioavailability, alternative delivery approaches are needed to increase the therapeutic potential of ACV.

Poly-N-p-vinylbenzyl-D-lactonamide (PVLA) is a superior surface-coating material for culturing hepatocytes [3,4]; it adheres to hepatocytes through a highly specific interaction between its galactose moiety and cell surface asialoglycoprotein receptors [5]. PVLA has been shown to form polymeric micelles in water because of the presence of a hydrophobic polystyrene backbone and hydrophilic sugar moieties [6]. A saline solution containing PVLA may be an effective solvent for ACV.

The human amniotic membrane (AM) has been used as a graft and covering material for patients undergoing reconstructive surgery [7] or treatment for ulcerations [8] or burns [9] because of its anti-inflammatory and antimicrobial effects [10-12], anti-fibroblastic activity and anti-angiogenic properties [10] and potential to reduce scarring [13]. The AM can also produce a wide array of growth factors [14], provide a healthy new substrate suitable for re-epithelialization, and promote epithelial healing [15]. In the field of ophthalmology, the AM has been used with varying degrees of success to treat numerous ocular surface disorders [16-21].

Until recently, the AM was cryopreserved by deep-freezing to −80°C, but there was no reliable method for sterilization. To overcome these limitations, we developed a hydrerdy-AM (HD-AM) under vacuum conditions using far-infrared rays and microwaves, then conducted sterilization by gamma-ray (γ) irradiation. The HD-AM is thin, safe

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Materials and Methods

Preparation of HD-AM and Glu HD-AM

AMs were obtained during elective caesarean sections from volunteers who were serologically negative for human immunodeficiency virus, hepatitis virus types B and C, syphilis and human T-lymphotropic virus-1. AMs were washed several times with sterile 0.01 M phosphate-buffered saline (pH 7.2–7.8, D5652-1L; Sigma-Aldrich, St. Louis, MO, USA) or sterile physical saline (pH 6.4, Otsuka Normal Saline; Otsuka Pharmaceutical Factory, Inc., Tokushima, Japan).

Hyperdry AMs (HD-AMs) were prepared as reported previously [22]. AMs were placed on cooking sheets (Toyo Aluminum Ekco Products, Tokyo, Japan), and dried under 1) a vacuum (approximately 0.4 kPa), 2) far-infrared rays using a 0.4 kW heater to keep the chamber at 50°C, 3) 0.1 kW microwave irradiation (after the air pressure was increased from 0.4 to 4.6 kPa) for 1–3 min, and 4) a hyperdrying device (which again decreased the air pressure to 0.4 kPa on the wet sample). After several repetitions of this cycle of air pressure changes, the samples were dried completely but not frozen. Following this drying treatment, the boiling temperature of the AM samples was decreased to approximately 30°C at 4.60 kPa.

According to the method described by Spoerl et al. [38], the AM was cross-linked with 0.1% glutaraldehyde (20% glutaraldehyde solution; Wako Pure Chemical Industries, Ltd., Osaka, Japan) for 30 min. The cross-linked AM was then washed thoroughly with sterile physical saline or water for injection (pH 5.66, Otsuka Water for Injection; Otsuka Pharmaceutical Factory Inc., Tokushima, Japan), and effective new biological material for clinical use that can be stored at 2–8°C [22] and maintains its growth factors and nutrients because of treatment with our patented hyperdry method [23]. In addition, we developed glutaraldehyde cross-linked HD-AM (Glu HD-AM) as an alternative HD-AM product. Glu HD-AM is a useful substrate for corneal perforations and tissue adhesive to conjunctival defects following multiple trabeculectomy, as it is resistant to collagenases [24,25]. The characteristics of the HD-AM and benefits of Glu HD-AM have been described in clinical studies and case reports [24-32]. In addition, the primary constituents of the AM are a stromal matrix composed mainly of collagen and an epithelial layer [33]. In studies of drug delivery systems (DDS), collagen [34], gelatin [35], alginate [36] and polymer matrices such as ethylene-vinyl acetate copolymer [37] were reported to carry an aqueous solution of basic fibroblast growth factor. The HD-AM and Glu HD-AM are new biological materials made from collagen.

In this present study, we combined two different materials, PVLA and HD-AM, as a new DDS material for ACV. We then evaluated the 1) safety of HD-AM and associated products in an eye irritation test; 2) level of residual glutaraldehyde in HD-AM; 3) therapeutic effects of ACV-containing PVLA-bearing spheres (ACV/PVLA) impregnated in HD-AM (ACV/PVLA HD-AM) sheets compared to treatment with ACV eye drops; and 4) dose- and volume-dependent efficacy of treatment with an ACV/PVLA HD-AM device in a rabbit model of ocular infection with HSV-1. The results showed that PVLA and HD-AM was effective and showed advantages as a DDS for ACV treatment (Figure 1).
dried with a hyperdrying device. γ-irradiation (25 kGy) was used to sterilize HD-AM and Glu HD-AM.

**Judgments of eye irritation**

The experimental procedures used to assess eye injury were based on the scoring systems described by Draize et al. [39] and Kay et al. [40] with the following modifications. Injuries of the cornea, conjunctiva and palpebral mucosa, and iris were scored separately. For scoring, 100 µL aliquots of the tested materials (the liquid used to dilute the PVLA sphere solution and extract solutions containing homogenated HD-AM or Glu HD-AM) were applied to the conjunctival sac. The time intervals were 0.5, 1, 1.5, 2, 4, 24, 48 and 72 h (3 days) after instillation of the tested materials into the eye and evaluation was conducted as shown in Tables 1-3. As a control, saline was instilled into the other eye and evaluated in the same manner.

**Assay of glutaraldehyde**

The residual concentration of glutaraldehyde in Glu HD-AM was determined by reversed-phase high performance liquid chromatography (HPLC) after derivatization with 2,4-dinitrophenylhydrazine (DNPH). For this procedure, 0.1 g of Glu HD-AM was soaked in 3 mL of extraction fluid containing 0.1 M glacial acetic acid and NaOH (pH 4.93), and the sample was gently stirred overnight at 4°C following homogenization. After removal of the solid residues by centrifugation, the extract was buffered to pH 3.0 with 3 mL of 1 M citrate buffer and the residual concentration of glutaraldehyde was determined by reversed-phase high performance liquid chromatography (HPLC) after derivatization with 2,4-dinitrophenylhydrazine (DNPH).

| HR  | HD-AM (N=2) | GLU HD-AM (N=1) | SOLVENT OF PVLA (N=2) |
|-----|-------------|-----------------|-----------------------|
| BEFORE INSTILLATION | 0 | 0 | 0 |
| 0.5 | 0 | - | 0 |
| 1 | 0 | 0 | 0 |
| 1.5 | 0 | - | 0 |
| 2 | 0 | - | 0 |
| 4 | 0 | - | 0 |
| 24 | 0 | 0 | 0 |
| 48 | 0 | 0 | 0 |
| 72 | 0 | 0 | 0 |

Table 1: Classification of HD-AM, GLU HD-AM and PVLA solution without aciclovir (solvent of PVLA).

| Glu HD-AM | Sample No. | Concentration (µM) | Concentration (ppm) |
|-----------|------------|--------------------|---------------------|
| 1 | 3.29 | 36.3 |
| 2 | 3.73 | 43.7 |
| Average | 3.51 | 40 |

Table 2: Concentration of residual Glutaraldehyde in the Glu HD-AM.
aqueous solution (0.5 mg/mL in 20 mL) of PVLA was placed in a 30 mL sample tube in an ice bath and stirred with a magnetic stirrer. PLA (10 mg) was dissolved in 1 mL of methylene dichloride and added dropwise to the aqueous solution. The mixture was sonicated by a probe-type sonicator (UD-200; Tomy, Tokyo, Japan) for 10 min at 4°C for 1 min intervals interspersed with 1 min rests, then evaporated to remove the methylene dichloride. The formed spheres were collected by centrifugation at 17,500 x g for 30 min, and then passed through a 0.2 μm filter for sterilization and adjustment of the diameter. To prepare PVLA-PLA spheres, ACV was mixed into the organic phase.

The 0.5% ACV solution was produced by adding ACV to an ACV solution at a final concentration of 0.1% ACV to yield 0.5% ACV with sonication at 40–45°C for 90 min.

Preparation of ACV/PVLA HD-AM sheets
To prepare ACV/PVLA HD-AM sheets, 100 μL of 0.1% or 0.5% ACV/PVLA solution and a single square (400 mm²) from an HD-AM sheet were incubated in a microtube at 37°C for 1 h. Following this treatment, approximately 50 μL of ACV/PVLA solution remained in the microtube.

Culture of HSV-1
The viruses used in this experiment were generated on a background of the HSV-1 strain McKrae as described by Myles et al. [42] with the following modifications. Viral stocks were propagated in culture dishes containing a confluent monolayer of Vero cells (ATCC CCL-81; Sumitomo Dainippon Pharma Co., Ltd., Osaka, Japan) in Dulbecco's Modified Eagle's Medium (GIBCO 11965-092; Life TechnologiesTM, Japan) in Dulbecco's modified Eagle's Medium (GIBCO 11965-092; Life TechnologiesTM, Tokyo, Japan) containing 5% fetal bovine serum (GIBCO 26140-079; Life TechnologiesTM Japan, Tokyo, Japan) at a multiplicity of infection (MOI) of 0.01. Viral titers were generated using Vero cells.

HSV-1-infected rabbit keratitis model
Male New Zealand white (NZW) rabbits (specific pathogen-free) weighing 2 kg were obtained from Kitayama Labesu, Inc. (Nagano, Japan), handled and maintained in accordance with the tenets of Kay and Calandra classified with the drug as “nonirritating”. Glu by the Draize method, while eye irritation evaluated using the criteria of Nauss et al. [43], with some modifications as shown in Table 4.

Statistical analysis
Data were evaluated as the mean values from three rabbits (triplicate assays). A total of three (n=3) data was used in each analysis. Student's t-test for independent samples was performed using SPSS version 22 for Mac (IBM Japan, Ltd., Tokyo, Japan). Comparisons were two-sided with significance levels of 5% (*) and 1% (**).
PVLA eye drops and 0.5% ACV/PVLA HD-AM sheets. The slit lamp examination score of 0.5% ACV/PVLA HD-AM was significantly lower than that of 0.5% ACV/PVLA eye drops (Figure 3a).

Eye drop treatment resulted in geographic ulcer in two rabbits and dendritic ulcer in one rabbit. In three rabbits, dendritic ulcer was observed after ACV/PVLA HD-AM treatment. The diameter (ϕ) or rectangular area of the epithelium deficiency in the group receiving the eye drop treatment was 1.0–2.0 mm. The ϕ or rectangular area of epithelium deficiency in the group receiving ACV/PVLA HD-AM treatment was 0.5–1.0 mm. The area of epithelium deficiency following eye drop treatment was much greater than that after ACV/PVLA HD-AM treatment on day 7 (Figure 3b).

ACV concentration-dependently inhibited the progress of HSV-1 infection in rabbit eyes

Following treatment of one 0.1% ACV/PVLA HD-AM sheet or three 0.5% ACV/PVLA HD-AM sheets for one-time administration, the progress of HSV-1 infection was decreased in a dose-dependent manner (Figure 4a). Treatment with 0.5% ACV/PVLA HD-AM showed significantly strong inhibition on days 6 and 7.

Table 5: Classification of materials based on eye irritation property.

| Tissue Score | Gross lesions | Score |
|--------------|---------------|-------|
| 0            | Normal        |       |
| 0.5          | Superficial punctate keratitis |     |
| 1.0          | Dendrites ulcer |      |
| 2.0          | Small geographic ulcer |     |
| 3.0          | Geographic ulcer (<50% of surface) | |
| 4.0          | Geographic ulcer (>50% of surface) |   |
| 0.5          | Mild edema and inflammation | |
| 1.0          | Moderate edema and inflammation | |
| 2.0          | Severe edema and inflammation |  |
| 4.0          | Total opacification |  |

Table 6: Gross scoring system for HSV-1 infected rabbit eyes.

| Tissue | Score | Gross lesions |
|--------|-------|---------------|
| Cornea |       |               |
| Epithelium |     |               |
| 0      | Normal |                         |
| 0.5    | Superficial punctate keratitis |     |
| 1.0    | Dendrites ulcer |      |
| 2.0    | Small geographic ulcer |     |
| 3.0    | Geographic ulcer (<50% of surface) | |
| 4.0    | Geographic ulcer (>50% of surface) |   |
| Stroma |       |               |
| 0      | Normal |                         |
| 0.5    | Mild edema and inflammation | |
| 1.0    | Moderate edema and inflammation | |
| 2.0    | Severe edema and inflammation |  |
| 4.0    | Total opacification |  |
The number and size of epithelium-deficient regions on the eyes administered 0.1% ACV/PVLA HD-AM were much greater than those following 0.5% ACV/PVLA HD-AM treatment on day 7 (Figure 4b).

**Discussion**

Ocular irritation tests for HD-AM and Glu HD-AM are important steps in ensuring safety in both the medical industry and medical technology. Irritation testing using rabbits has largely remained unchanged for many years. The Draize, and Kay and Calandra eye irritation tests [39,40] are governmentally endorsed methods described in the OECD testing guidelines (TG) 405 (OECD, 2002). Dreize and Kay set the time interval at 96 h to determine if residual injury was present.
while Calandra conducted evaluated at 168 h (7 days). However, in our experiments, there was no score after 72 h, thus we modified the interval to 72 h. HD-AM, Glu HD-AM, and solvent of PVLA showed no eye irritation.

Denaturation or degradation of the implanted collagen over time is an important problem. In the past, these effects have been prevented by intermolecular cross-linkage by processing with glutaraldehyde solution [44]. These materials, made from porcine and bovine pericardium as pericardial bioprosthesis, are processed into the mitral valve, aortic valve, etc. Various aldehydes have been used for this purpose, including glyoxal, formaldehyde [45], dialdehyde starch, and glutaraldehyde [44], because of their reduced thrombogenicity in bioprosthetic heart valves.
Glutaraldehyde is considered to be less toxic than formaldehyde and relatively stable over time, and is also used as a tissue sterilant against microbial contamination [46]. Cross-linking with glutaraldehyde is also known to render tissue substantially non-antigenic so that implanted tissue does not elicit an adverse immune response in the recipient [44]. However, various post-implantation problems, such as inflammation and other adverse reactions in some patients, are thought to be caused by residual aldehyde in implanted bioprosthetic tissue in some cases.

The mean residual glutaraldehyde level in an extract from Gliu HD-AM was 3.51 μM, which corresponds to approximately 40 ppm (dry-weight basis). Although the HD-AM and Gliu HD-AM did not elicit eye irritation or inflammation, an additional process for decreasing residual aldehyde levels may be preferable prior to surgical implantation of the Gliu HD-AM.

ACV [47], valacyclovir, and famciclovir [48,49] are common medical treatments against HSV-1 in Japan. A 3% ACV eye ointment (ACV-O) is often prescribed to patients with herpetic keratitis. An ACV-O medicine has also been developed to exploit the hydrophobic properties of ACV. However, eye ointment treatment can be inconvenient because ACV-O must be administered 5 times per day, and frequently causes blurred vision after administration. Therefore, new drugs like as eye drops etc. for herpetic keratitis are needed.

PVLA-related materials can encapsulate Z-Asp, which dissolve well in DMSO and caspase inhibitor, in its nanospheres and are specifically absorbed by hepatocytes [50]. In this experiment, we incorporated hydrophobic ACV into PVLA (ACV/PVLA), because of its amphipathic properties. We evaluated both an 1) ACV/PVLA solution as an eye drop-administered drug and 2) ACV/PVLA HD-AM sheet that holds the ACV/PVLA. An in vivo stable HSV-1-infected rabbit model was constructed, beginning on the 4th day after administration of HSV-1 (Figure 2). The effect of treatment with ACV/PVLA HD-AM was significantly better than that of ACV/PVLA solvent administered as eye drops (Figure 3a). Evaluation on day 7 showed that all three indices examined, i.e., 1) the state of the gross lesion, 2) the diameter or rectangular area of epithelium deficiency, and 3) the number of epithelium-deficient regions, indicated that ACV/PVLA HD-AM treatment was better than eye drop treatment (Figure 3b). Thus, the drug-impregnated HD-AM was able to control the release capacity of the drug and achieve a therapeutic effect.

Following ACV/PVLA HD-AM administration, the progress of HSV-1 infection was decreased in a dose-dependent manner (Figure 4a). Moreover, all parameters at the 0.5% concentration of ACV were better than those at the 0.1% concentration (Figure 4b). This indicates that ACV/PVLA HD-AM showed a therapeutic effect against HSV-1. The effect of treatment with 0.5% PVLA sphere solution was approximately 80% of effect of treatment with 3% ACV-O (data not shown). Although the concentrations of ACV included in the PVLA spheres solution were lower than that in general ACV-O, PVLA sphere solution may exhibit controlled release of ACV. However, HD-AM may leave PVLA contain ACV to itself, which was effective for releasing PVLA particles (Figure 3). Based on these two effects, ACV may suppress HSV-1 infection grade in a rabbit model. Regulating drug delivery would increase therapeutic efficacy and decrease potential side effects.

Several beneficial effects of HD-AM have been confirmed in clinical evaluations. HD-AM shows good bone surface prevention [27-29,31], greatly decreases pain [30], and decreases injury dehydration [28,29]. HD-AM is useful as a biomaterial substrate to treat glaucoma filtering bleb leak, corneal perforation [26], and recurrent pterygium [22]. Corneal perforation is often caused by HSV-1. HD-AM may be useful for both treatment of corneal perforation resulting from HSV-1 and controlled drug release with ACV, including PVLA.

Final concentrations of 0.1% and 1.0% ACV (ACV/PVLA solutions) were initially prepared, but both caused precipitation during laboratory-to-laboratory transfer. Increasing the temperature to 40–45°C and sonication for 90 min resulted in a clear and fully dissolved 0.1% ACV/PVLA solution. This 0.1% ACV/PVLA solution was stable at room temperature for additional one month. We thus prepared a stable 0.5% ACV/PVLA solution at 37°C with this procedure. However, the 1.0% ACV included PVLA sphere solution did not remain clear.

Generally, 1 g of ACV will dissolve in approximately 800 mL of water (0.12%). Using PVLA, we increased the concentration of ACV up to 4-fold.

Conclusions

In summary, we demonstrated that 1) administration of HD-AM, Gliu HD-AM, or solvent of PVLA resulted in no eye irritation; 2) following Gliu HD-AM administration, 3.51 μM of residual glutaraldehyde was present (approximately 40 ppm on a dry-weight basis), and no eye irritation was observed; 3) the ACV/PVLA HD-AM sheet was much more effective for treatment in the HSV-1 eye infection rabbit model than the eye drop-administration method, as it also controlled PVLA particle release; and 4) the combination of HD-AM and PVLA-bearing spheres was a very useful for drug delivery as a hydrophobic medicine requires continuous administration.

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