Protection from hemolytic uremic syndrome by eyedrop vaccination with modified Enterohemorrhagic *E.coli* outer membrane vesicles

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ABSTRACT

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(Directed by Professor Kyoung Yul Seo)

Enterohemorrhagic *E. coli* (EHEC) can cause severe diarrhea, hemorrhagic colitis, which is often accompanied by hemolytic anemia, thrombocytopenia, and acute renal failure. But up until now, specific treatments except public health and sanitation are not available. Accordingly, the development of effective vaccines preventing EHEC O157:H7 infection is of prime research interest. Outer membrane vesicles (OMVs) are spherical membrane blebs shed by Gram-negative bacteria and vaccination with OMVs has been reported to induce an immune response and protect vaccinated organisms.

However, an effective and safe OMV vaccine for protection from EHEC O157:H7 infection has not been reported, presumably because OMVs generated from EHEC O157:H7 are intrinsically toxic due to presence of Shiga toxin (STx) and lipopolysaccharide (LPS) endotoxin. Modified OMVs (mOMVs) from cultures of MsbB- and Shiga toxin A subunit (STxA)-deficient EHEC O157:H7 bacteria have less toxic penta-acylated lipid A moiety. Moreover, they do not contain toxic STxA subunit proteins. WaaJ-mOMVs with additional *waaJ* mutation do not have the polymeric O-antigen of O157 LPS.

This study is to explore whether eyedrop vaccination using mOMVs and waaJ-mOMVs is effective for protecting against hemolytic uremic syndrome (HUS) caused by EHEC O157:H7 infection.

Modified OMVs, waaJ-mOMVs, and mOMVs plus polymyxin B (PMB) were administered to BALB/c mice by eyedrop at the onset of experiments. Mice were boosted at 2 weeks, and challenged peritoneally with wild-type OMVs (wtOMVs) at 4 weeks. In order to increase intestinal mucosal immunity, cholera toxins (CT) or polyinosinic:polycytidylic acid (polyI:C) were administered with mOMVs. As parameters for evaluation of the

mOMV-mediated immune protection, serum and mucosal immunoglobulins, body weight change and blood urea nitrogen (BUN)/Creatinin (Cr) were tested, as well as histopathology of renal tissue. Lastly, to confirm the safety of mOMVs for eyedrop use, body weight and ocular histopathological changes were monitored in mice.

The mice group vaccinated with mOMVs elicited greater humoral and mucosal immune responses than did the waaJ-mOMVs and PBS-treated groups. Eyedrop vaccination of mOMVs plus PMB reduced the level of humoral and mucosal immune responses. mOMVs plus CT increase systemic IgG antibody response and intestinal IgA antibody response. After challenge, mice vaccinated with mOMVs were protected from a lethal dose of wtOMVs administered intraperitoneally, conversely mice in the PBS control group were not.

In conclusion, the current study showed that eyedrop vaccination using mOMVs of EHEC O157 bacteria sufficiently induced immunogenicity in mice and intact O157 LPS antigen could be a critical component for enhancing the immunogenicity of the mOMVs. Moreover, eyedrop vaccination with mOMVs was shown to be effective for preventing HUS

pathogenesis in mice against challenge with HUS-causative wtOMVs and thus hold promise for the prevent of EHEC-related pathogenicity.

Key words: outer membrane vesicle, Enterohemorrhagic *E. coli*, eyedrop vaccination

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I. INTRODUCTION

Enterohemorrhagic *E. coli* (EHEC) can cause severe diarrhea, hemorrhagic colitis, which is often accompanied by hemolytic anemia, thrombocytopenia, and acute renal failure, which are the hallmarks of hemolytic uremic syndrome (HUS)[1]. Although typical EHEC strains are classified by the production of Shiga toxin (STx) and the possession of a locus of enterocyte effacement (LEE) in the chromosome, atypical EHEC lacking LEE pathogenicity islands can be associated with HUS, as recently witnessed in a German outbreak of *E. coli* O104:H4[2]. Despite the lethal outbreak of HUS due to *E. coli* O104:H4, EHEC O157:H7 remains the most important

causative strain involved in the manifestation of HUS worldwide[3]. Accordingly, the development of effective vaccines preventing EHEC O157:H7 infection-associated HUS is of prime research interest.

Outer membrane vesicles (OMVs) are spherical membrane blebs shed by Gram-negative bacteria[4]. They carry not only native antigens expressed in the outer membrane, but also exogenous protein epitopes[5] and retain self-adjuvanticity that can be exerted by the inclusion of toll-like receptor agonists (lipopolysaccharide, outer membrane lipoproteins, flagellin, etc.)[6]. Several reports have demonstrated that vaccination with OMVs is sufficient to induce an immune response and protect vaccinated organisms from subsequent pathogen challenge[7-10]. However, up until now, an effective and safe OMV vaccine for protection from EHEC O157:H7 infection and sequelae HUS has not been reported, presumably because OMVs generated from EHEC O157:H7 are intrinsically toxic due to presence of STx exotoxin and LPS endotoxin, which are two major virulence factors that contribute towards the development of HUS. In order to overcome the toxicity of the EHEC O157-OMVs, detoxified OMV (produced from MsbB- and STxA-deficient mutant) was characterized previously for use as a vaccine[5,11]. Moreover, waaJ-

mOMVs lacking the O-antigen side chains were used to test whether the absence of O-antigen in LPS would affect immunogenicity of mOMVs administered via an ocular-mucosal route.

This study is to investigate that eyedrop vaccination with mOMVs of EHEC O157:H7 induce humoral and mucosal immune responses and, without the use of commercial adjuvants, is able to protect immunized mice from further challenge with wtOMVs, which are believed to be produced in the gut of EHEC O157-infected hosts and have been suggested as the causative agent for HUS[12]. Futhermore, current study is to explore whether loss of the O-antigen by truncation of O157-LPS to the core region make waaJ-mOMVs significantly less immunogenic, indicating the LPS O-antigen in the mOMVs plays a significant role in inducing a protective immune response against lethal O157-OMV challenge.

II. MATERIAL AND METHODS

1. Preparation of mOMVs of EHEC O157:H7 strains

Modified OMVs and waaJ-mOMVs were kindly provided by Prof. Sang-Hyun Kim (Korea Research Institute of Bioscience and Biotechnology, Ochang, Korea). MsbB- and STxA-deficient mutants of EHEC O157:H7 (Sakai-DM/*stx1A*/*stx2A*)[11] were used as parental strains for producing mOMVs. The Δ*waaJ*::Cm allele was introduced into the mOMV-producer strain carrying pKD46 by electroporation to create the Δ*waaJ*::Cm mutant of the mOMV-producer strain.

2. Animals

This study was performed in strict accordance with the recommendations in the Guide for the Care and Use Committee of Yonsei University Health System**.** The committee has reviewed and approved the animal study protocol (Approval No: 2011-0261). Specific pathogen-free BALB/c mice, aged 6-10 weeks, were purchased from Charles River Laboratories (Orient Bio, Sungnam, Korea) and were maintained under specific pathogen-free conditions in the experimental facility at Yonsei University Health System

where they received sterilized food and water ad libitum**.** All surgeries were performed after sacrifice by CO_2 narcosis and every effort was made to minimize suffering.

3. Immunization with mOMVs and waaJ-mOMVs

For ocular immunization, five mice were anesthetized and 5 μg mOMVs or waaJ-mOMVs suspended in 5 μl PBS were dropped on the conjunctival sac of each eye by micropipette. Two weeks after vaccination, mice were boosted by the same method. In additional experiments, 5 μg mOMVs plus 5 μg polymyxin B (PMB) suspended in 5 μl PBS were immunized on each eye. For adjuvants, 5 μg mOMVs plus 1 μg CT or 5 μg polyI:C suspended in 5 μl PBS were immunized on each eye.

4. Sample collection

After intraperitoneal injection of mice with pilocarpine (500 mg/kg body weight; Sigma-Aldrich), saliva was obtained. Tear and vaginal wash samples were obtained by lavage with 10 μl or 50 μl PBS. Serum was obtained by tail venipuncture. Fecal extracts were obtained by adding weighed feces to PBS

containing 0.1% sodium azide followed by vortexing. The mixture was then centrifuged and the supernatants were collected.

5. Indirect enzyme-linked immunosorbent assay (ELISA)

ELISA plates (Falcon, Franklin Lakes, NJ) were coated with mOMVs, waaJ-mOMVs, and wtOMVs, respectively, in PBS and incubated overnight at 4℃. Blocking was performed with 1% BSA (Sigma-Aldrich) in PBS, and 2-fold serial sample dilutions were applied to plates. HRP-conjugated goat anti-mouse IgG or IgA (Southern Biotechnology Associates, Birmingham, AL) was added to each well and incubated overnight at 4℃. A tetramethylbenzidine solution (Moss, Pasadena, MD) was used for color development. Plates were measured at 450 nm using an ELISA reader (Molecular Devices, Sunnyvale, CA) after the addition of stopping solution (0.5N HCl).

6. Protection assay against wild-type OMVs

Four weeks after eyedrop immunization with mOMVs, 1.5X LD_{50} of wild-type EHEC O157:H7 OMVs (LD_{50} 0.274 mg/kg)[11] were injected intraperitoneally for the challenge experiment. Body weight changes were

monitored daily for 8 days after injection. For serum sampling by puncturing the retro-orbital area, mice were anesthetized by intraperitoneal injection of ketamine (100 mg/kg body weight) and xylazine hydrochloride (10 mg/kg body weight**)** and serum was obtained daily for 8 days. Blood samples were analyzed for BUN and Cr. For histopathological observation of kidney, a survived mouse from each group was sacrificed on day 4 post challenge by CO_2 narcosis.

7. Histology

Eye tissues including the conjunctiva and eye balls from PBS control and mOMV-treated mice or whole kidney tissues from wtOMV challenged mice were washed with PBS and fixed in 4% formaldehyde for 24 h at 4℃. The tissues were dehydrated by gradual soaking in alcohol and xylene gradients followed by embedding in paraffin. Paraffin-embedded specimens were cut into 5-mm sections and stained with H&E.

8. Safety evaluation

In order to confirm the safety of vaccination, some groups of BALB/c mice were administered with 10 μg of mOMVs or wtOMVs resolved in 10 μl of PBS by eyedrop on both eyes or 10 μg of wtOMVs resolved in 100 μl of PBS intraperitoneally. Body weight changes were monitored on a daily basis for 5 days. Eye tissues were acquired at 24, 48, 72 h after administration of 10 μg of mOMVs for histologic examination.

9. Statistical analyses

All data are expressed as the mean ± SD. Statistical analyses were performed by *t*–tests (Sigma plot).

III. RESULTS

1.Characterization of EHEC O157:H7-derived mOMVs and waaJ-mOMVs

Characteristic molecular patterns of mOMV and waaJ-mOMV was depicted (Fig.1). The lipid A portion of LPS lacked a secondary myristate chain in the mOMV producing strain due to deficiency of MsbB activity (lipid A acyltransferase[13], Fig. 1A). Similarly, loss of WaaJ activity (an α-1,2-glucosyltransferase involved in the LPS core biosynthesis[14]) rendered the waaJ-mOMV producing strain with truncated LPS consisting of lipid A with the core oligosaccharide extended to the galactose residue (Fig. 1A). IB analysis of both mOMV types with monoclonal antibodies (anti-STx2A and anti-STx2B) showed that mOMVs were STxA-deficient but retained STxB subunits (lane 2, Fig. 1B).

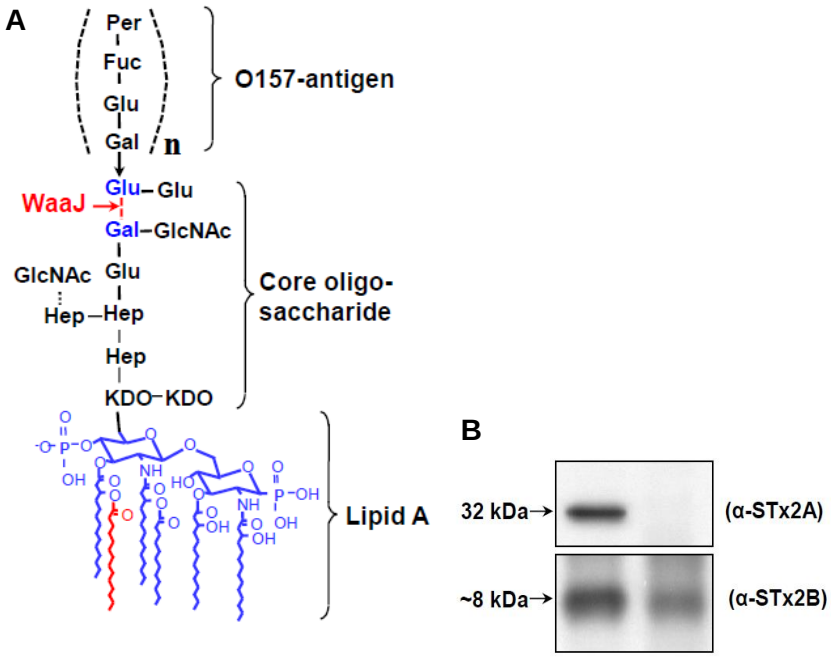

Figure 1 A. Schematic molecular structure of modified outer membrane vesicles from EHEC O157:H7 strain. The lipid A portion of LPS lacks the secondary myristate chain (red-colored) of the mOMV producing strain due to a MsbB deficiency. Lack of WaaJ protein confers the EHEC O157:H7 strain with truncated LPS consisting of lipid A with a core oligosaccharide extended to the galactose residue.

Figure 1 B. IB analysis of OMVs with monoclonal antibodies (anti-STx2A and anti-STx2B). The analysis revealed that the mOMVs (lane 2) were STxA-deficient, but retained STxB subunits compared to the OMVs of parental Sakai-DM strain (lane 1), which produces less endotoxic form of LPS.

2. Systemic and mucosal antibody responses

To assess the immunogenicity of eyedrop mOMVs and waaJ-mOMVs vaccination, groups of mice were administered with mOMVs and waaJ-mOMVs by eyedrop instillation. Serum IgG Ab and mucosal IgA Ab levels in all vaccinated mice were significantly increased than PBS group. Although the levels of both total Ag-specific IgG and IgA Ab of mOMVs vaccinated mice were not significantly higher than that of waaJ-mOMVs vaccinated mice, mOMVs immunization induced slightly higher Ab production levels than waaJ-mOMVs vaccination (Fig. 2A). The immunogenicity continued for 10 weeks (Fig. 2B). Furthermore, induction of significant enhancement of systemic IgG and mucosal IgA antibody production by vaccination of eyedrop mOMVs alone without any addition of adjuvant suggested that mOMV nano-particles could be effective vaccination vehicles by providing major antigens (LPS and outer membrane proteins) in their native form without additive adjuvants.

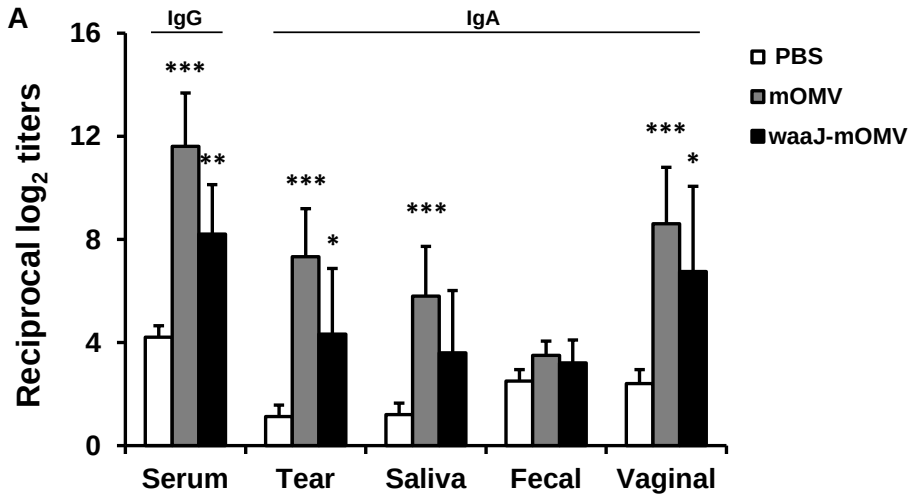

Figure 2 A. Eyedrop vaccination of mOMVs and waaJ-mOMVs resulted in both systemic and mucosal immune responses. Groups of BALB/c mice received 5 μg of the mOMVs or waaJ-mOMVs resolved in 5 μl PBS or PBS alone by eyedrop on each eye twice at a 2-week interval. mOMVs and waaJ-mOMVs-specific antibody titers were measured by ELISA in serum and in various mucosal secretions at 2 weeks after final vaccination. Results are representative of three independent experiments, with five mice in each experimental group. *$p < 0.05$, ** $p < 0.01$, ***$p < 0.001$ compared with the PBS group.

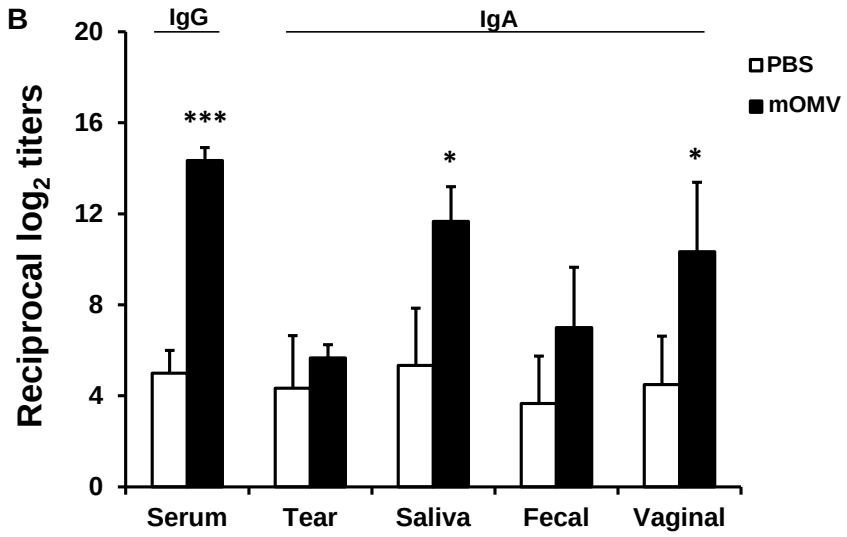

Figure 2 B. Eyedrop vaccination of mOMVs resulted in both systemic and mucosal immune responses for 10 weeks. Groups of BALB/c mice received 5 μg of the mOMVs resolved in 5 μl PBS or PBS alone by eyedrop on each eye twice at a 2-week interval. mOMVs-specific antibody titers were measured by ELISA in serum and in various mucosal secretions at 8 weeks after final vaccination. Results are representative of three independent experiments, with five mice in each experimental group. *$p < 0.05$, ** $p < 0.01$, ***$p < 0.001$ compared with the PBS group.

3. Cross reactivity in systemic and mucosal antibody responses

The formation of serum IgG antibodies cross-reactive to wtOMVs was confirmed in both mOMVs and waaJ-mOMVs vaccinated groups, and there was significantly higher level of mOMVs-specific antibody that cross-reacted to wtOMVs, but not in the waaJ-mOMVs group (Fig. 3).

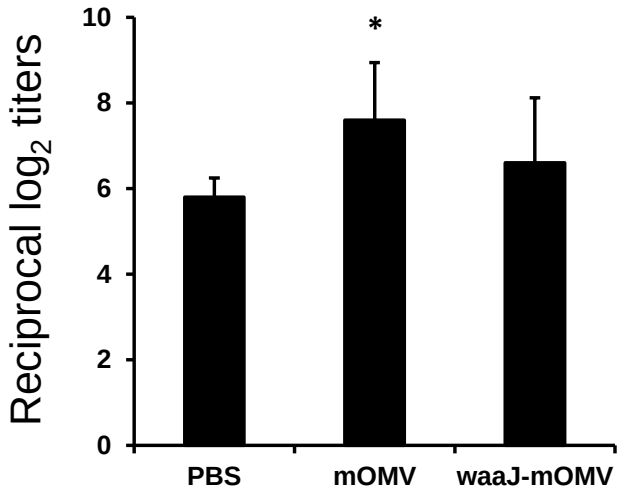

Figure 3. Eyedrop vaccination of mOMVs resulted in wtOMVs cross-reactive Ab production. Groups of BALB/c mice received 5 μg of the mOMVs or waaJ-mOMVs resolved in 5 μl PBS or PBS alone by eyedrop on each eye twice at a 2-week interval. wtOMVs cross-reactive antibody titers were measured by ELISA in serum at 2 weeks after final vaccination. Results are representative of three independent experiments, with five mice in each experimental group. *$p < 0.05$ compared with the PBS group.

4. Eyedrop vaccination of mOMVs plus PMB blocked immune responses in mice

This study evaluated whether premixing mOMVs with polymyxin B (PMB), which is known as an endotoxin neutralizing drug, would decrease the immunogenic potential of mOMVs. PMB treatment significantly down-regulated the levels of serum IgG and mucosal IgA Ab in mice vaccinated with eyedrop mOMVs (Fig. 4A). However, the levels of LPS-specific antibodies were not changed after the addition of PMB (Fig. 4B). These results suggest that the LPS component of mOMVs administered via the eye mucosa can serve not only as a vaccine antigen itself, but also a natural form of strong adjuvant within mOMV vesicle.

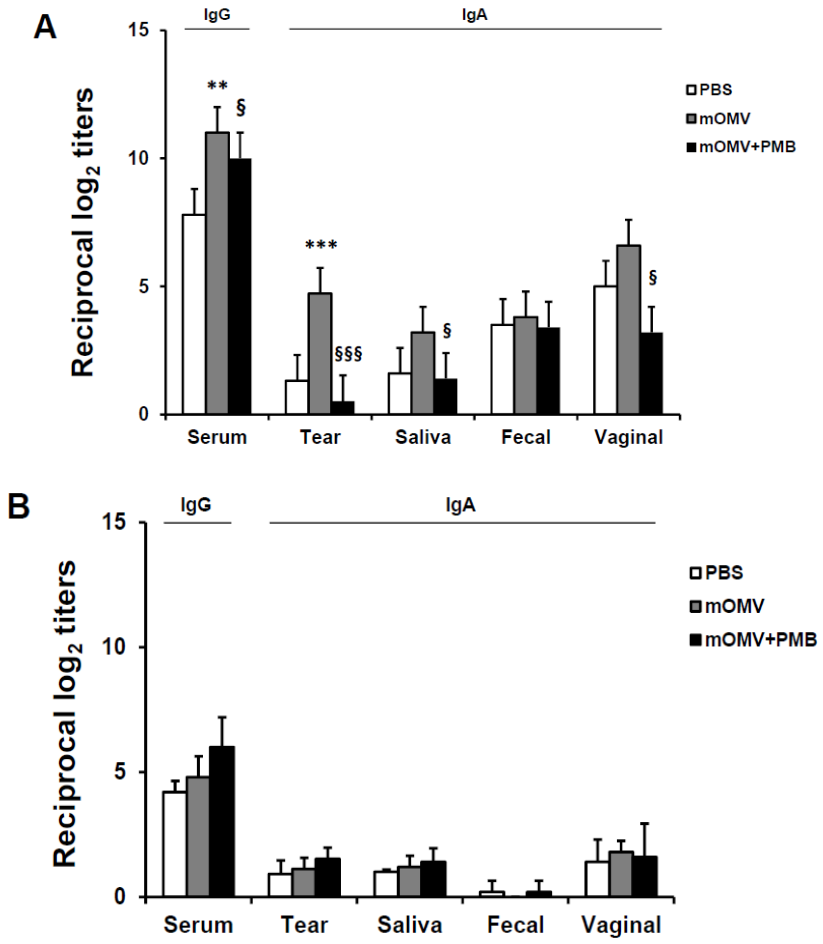

Figure 4 A,B. Eyedrop vaccination of mOMVs plus PMB blocked immune response in mice. Groups of mice given the 5 μg of mOMVs alone or plus 5 μg of PMB resolved in 5 μl of PBS by eyedrop on each eye twice at a 2-week interval. mOMVs- (A) or Anti-LPS_{O157}-specific antibody titers (B) were measured by ELISA in serum or in various mucosal secretions at 2 weeks after final vaccination. ** $p < 0.01$, ***$p < 0.001$ compared with the PBS group; § $p < 0.05$, §§§ $p < 0.001$ compared with mOMV-vaccinated group.

5. Eyedrop vaccination of mOMVs plus adjuvants

To assess the immunogenicity of eyedrop mOMVs with several adjuvants, groups of mice were administered with mOMVs with cholera toxin (CT) or polyI:C eyedrop instillation. Eyedrop vaccination of mOMVs with cholera toxin (CT) sufficiently induced systemic and mucosal antibody response compared to mOMVs alone (Fig. 5A). However, eyedrop vaccination of mOMVs with polyI:C did not sufficiently induce antibody response compared to mOMVs alone (Fig. 5B).

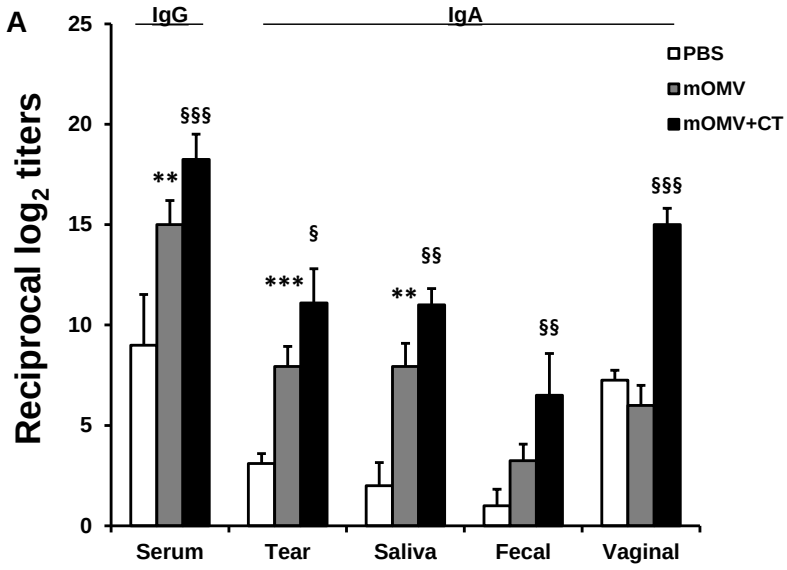

Figure 5 A. Eyedrop vaccination of mOMVs plus CT sufficiently induced antibody response in mice. Groups of BALB/c mice received 5 μg of the mOMVs alone or plus 1 μg of CT resolved in 5 μl PBS by eyedrop on each eye twice at a 2-week interval. mOMVs-specific antibody titers were measured by ELISA in serum and in various mucosal secretions at 2 weeks after final vaccination. Results are representative of three independent experiments, with five mice in each experimental group. *$p < 0.05$, ** $p < 0.01$, ***$p < 0.001$ compared with the PBS group. § $p < 0.05$, §§ $p < 0.01$, §§§ $p < 0.001$ compared with the mOMVs group.

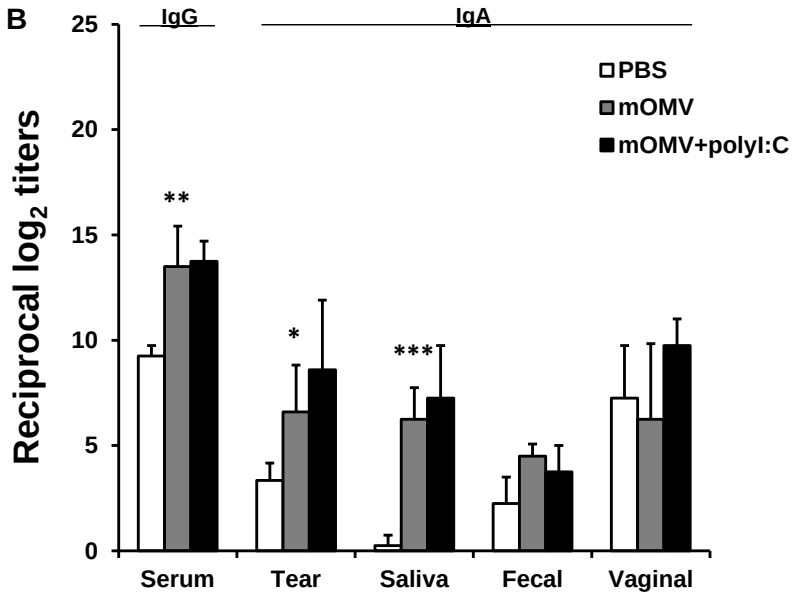

Figure 5 B. Eyedrop vaccination of mOMVs plus polyI:C did not sufficiently induce antibody response in mice. Groups of BALB/c mice received 5 μg of the mOMVs alone or plus 5 μg of polyI:C resolved in 5 μl PBS or PBS by eyedrop on each eye twice at a 2-week interval. mOMVs-specific antibody titers were measured by ELISA in serum and in various mucosal secretions at 2 weeks after final vaccination. Results are representative of three independent experiments, with five mice in each experimental group. *$p < 0.05$, ** $p < 0.01$, ***$p < 0.001$ compared with the PBS group.

6. Protection efficacy assessed for eyedrop vaccination of the mOMVs

To assess the vaccine efficacy of eyedrop immunization of mice with mOMVs, a challenge experiment was performed by intraperitoneal injection of lethal dose (1.5X LD_{50}) of wtOMVs. Mice vaccinated with mOMVs exhibited a slight loss of body weight (Fig. 6A) and were completely protected from challenge with wtOMVs compared to the PBS control group (Fig. 6B). We next analyzed BUN and Cr levels in serum of challenged mice. The serum levels of BUN and Cr in the mOMVs-vaccinated group were not significantly elevated compared with the control group (Fig. 6C,D), and these were consistent with histological observations of the renal tissues of mice vaccinated with mOMVs which showed virtually no sign of renal bleeding compared to that of PBS group (Fig. 6E, right panel). Thus, when results above were considered together, the eyedrop mOMVs-vaccination induced systemic antibodies against the components of mOMVs which binded and neutralized peritoneally injected wtOMVs so that Shiga toxins within wtOMVs were blocked in advance and cleared together with wtOMVs.

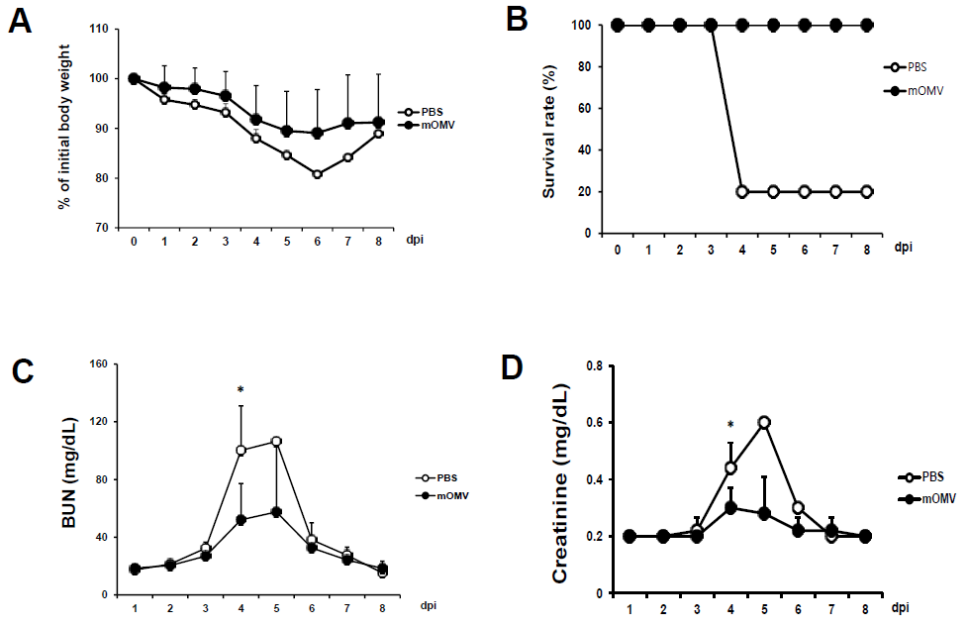

Figure 6 A,B,C,D. Eyedrop vaccination of mOMVs could protect vaccinated mice from wtOMVs challenge. For the protection assay, groups of BALB/c mice vaccinated with 5 μg of the mOMVs resolved in 5 μl PBS or PBS alone by eyedrop on each eye twice at a 2-week interval. At 2 weeks after final vaccination, mice were injected with 1.5X LD_{50} of wild-type EHEC O157:H7 OMVs (LD_{50} 0.274 mg/kg) intraperitoneally. Body weights (A) and survival rates (B) were monitored daily. For the measurement of BUN (C) and creatinine (D) levels as indicators of mouse renal function, serum samples were acquired from all challenged mice daily. Results are representative of three independent experiments, with five mice in each experimental group. *$p < 0.05$ versus PBS group.

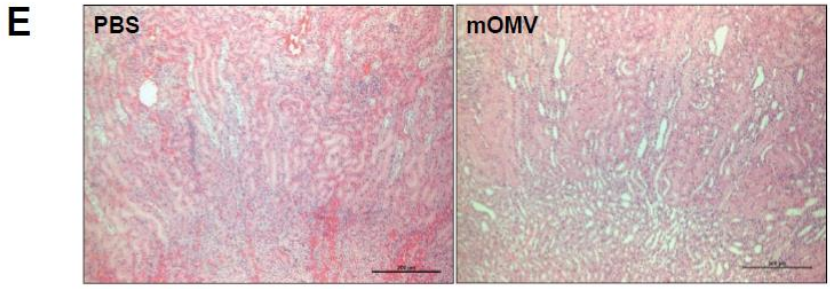

Figure 6 E. Histopathologic evaluation of renal tissue after wtOMVs challenge. For the assessment of renal failure by histological observation, renal tissues were sampled from survived mice of each group at 4 days post challenge and stained with H&E staining (original magnification X 100).

7. Safety of mOMVs administered by eyedrop

Since the LPS components that present in the mOMVs would have a potential to induce an inflammation in administered eyes of mice, the possibility of toxicity of eyedrop mOMVs were assessed. Mice were administered with 10 μg mOMVs by eyedrop and were monitored for several days. As shown in Figure 7, there was no decrease in body weight (Fig. 7A) and no histopathological changes in eye mucosa (Fig. 7B). Surprisingly, the administration of 10 μg wtOMVs by eyedrop induced no significant reduction on mice body weight compared to PBS treated mice, whereas mice that were peritoneally injected with the same amount (10 μg) of wtOMVs exhibited a

severe loss of body weight and eventually died at 4 days post-injection (Fig. 7A). Taken together, these results suggest that vaccination of eyedrop mOMVs has no toxic effect on eyes, and eye mucosa is an efficient and safe vaccine delivery route for inducing protective anti-HUS immunity.

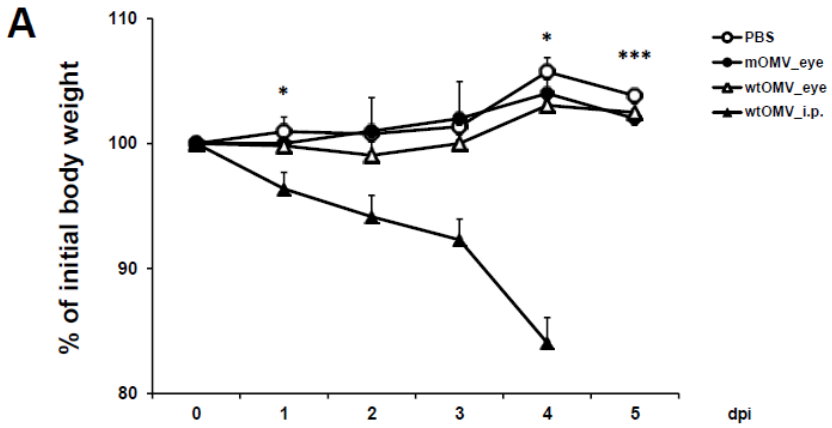

Figure 7 A. Safety evaluation of eyedrop mOMVs in mice. Groups of BALB/c mice were administered with 5 μg of mOMVs or wtOMVs resolved in 5 μl of PBS by eyedrop on each eye or 10 μg of wtOMVs resolved in 100 μl of PBS intraperitoneally. Body weight changes were monitored on a daily basis for 5 days. As a control, PBS was administrated via eyedrop. *$p < 0.05$, ***$p < 0.001$ between ocular wtOMV-treated group and intraperitoneally wtOMV-treated group.

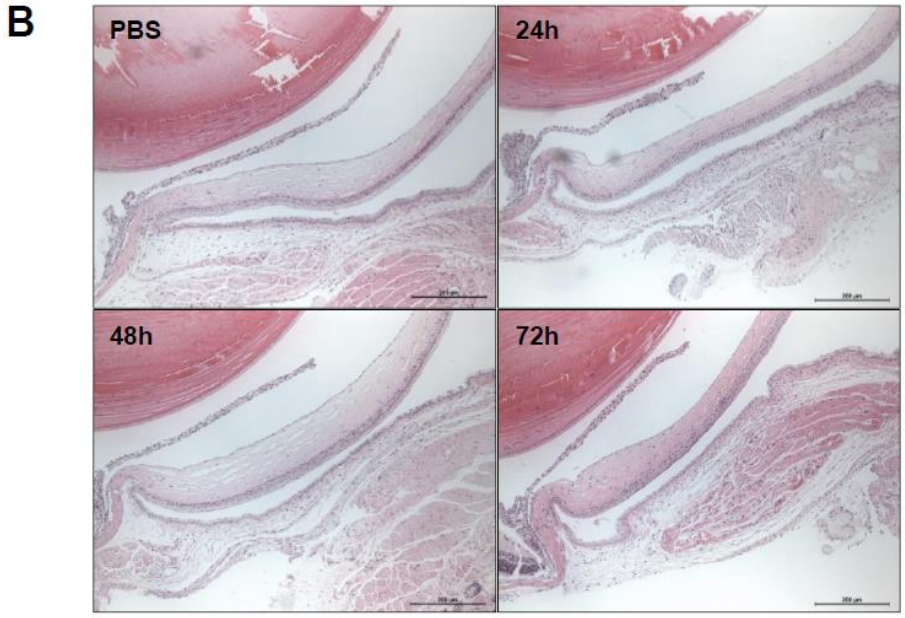

Figure 7 B. Eyedrop mOMVs did not induced ocular inflammation. Eye tissues were acquired at 24, 48 and 72 h after administration of 5 μg of mOMVs for histologic examination (H&E, original magnification X100).

IV. DISCUSSION

Outer membrane vesicles (OMVs) produced from EHEC O157:H7 Sakai strain carried both Shiga toxins and LPS moieties, and caused HUS-like symptoms in a mouse model[12]. Thus, OMVs are now recognized as a natural HUS-causative agent produced within the guts of patients after infection with EHEC O157. Considering that EHEC O157 bacteria are not invasive, it is interesting to note that no mechanism other than the proposed OMV package delivery has been demonstrated for trans-migration of Shiga toxins and the LPS from the gut lumen of HUS patients into the blood stream[12]. This study showed that eyedrop vaccination of mOMVs (engineered to be a safe vaccine) was effective for preventing HUS pathogenesis in mice challenged with HUS-causative wtOMVs. Furthermore, loss of LPS O-antigen in the waaJ-mOMVs rendered the vesicles much less immunogenic, suggesting that the presence of LPS O-antigen in mOMVs is as an important immunogen for inducing effective anti-OMV_{O157} antibodies.

For the development of an effective vaccine against EHEC-related pathogenicity[15,16], STxB subunit-based immunogens that have ability to induce anti-Shiga toxin antibodies have been investigated by several laboratories[17-19]. However, no vaccine able to prevent EHEC infection and/or

STx-mediated HUS for humans and animals has been reported[17,20]. In the preliminary results, sufficiently detectable anti-STxB antibodies by the mOMV vaccination could not raised, although anti-STx antibody plays an important role in the prevention of HUS, presumably by neutralizing the toxins directly. However, the systemic immune responses raised by the mOMVs vaccination could protect the mice from the lethal challenge with wtOMVs given intraperitoneally. This finding supports previous report[12] that STx toxins are mostly enclosed within the OMVs that carry the cargo from the gut where STx is produced to the blood stream (kidney targeting). Therefore, it is likely that anti-mOMV antibodies raised by eyedrop vaccination can block the spread of the toxin by preventing the OMV translocation and/or circulation into the blood stream.

Mucosal vaccination has numerous merits and thus has gained a great deal of attention. Specifically, mucosal vaccination is potentially more convenient and inexpensive compared with parenteral vaccines, in that it does not require specialized skill for administration, and can stimulate secretion of IgA antibodies capable of neutralizing pathogens at sites of entry into the body [21]. Heat-labile toxin (LT) of enterotoxigenic *E.coli* (ETEC) is well known

mucosal vaccine adjuvant; however, intranasal vaccination with LT-adjuvanted vaccine has raised safety concerns regarding the nervous system [22]. Mucosal vaccination using an ocular route[23-25] has been shown to effectively protect host against diverse array of pathogens[26-29]. Since OMVs of ETEC have been reported to induce protective immune responses against colonization of ETEC strains in the murine intestine[30], this study attempted to determine whether eyedrop vaccination of mOMVs from EHEC O157 could effectively prevent HUS after pathogenic *E.coli* attack. But eyedrop vaccination of the mOMVs could not raise enough gut-mucosal immune responses required for lowering the intestinal colonization by the EHEC O157. Instead, it could induce protective immune responses preventing systemic distribution of the STx-containing OMVs. In the context of local gut-associated mucosal antibody response, it is worth attempting to test and compare the effects of mucosal vaccination performed via nasal, sublingual, and oral routes in the further study. Additionally, enhancing the mucosal immunity through the addition of optimal adjuvants like CT, the usability of mOMVs as the effective mucosal vaccine would be more increased.

The experimental eyedrop vaccine model used in this study was intended to test whether the mOMV could induce mucosal and/or systemic immune responses or not, which is enough to protect the vaccinated animal from the lethal HUS-causative agent (wtOMVs), for the first time, via ocular route of immunization. Therefore, the intestinal colonization model was not employed in this study to evaluate the mOMV-mediated mucosal vaccine effectiveness against the EHEC O157 infection in the gut. It will also be interesting to test whether mOMVs derived from the Sakai-O157 strain can act as an effective vaccine against heterologous EHEC infections. Although, the exposure of the R3-core in the LPS lacking the O157 antigen in the waaJ-mOMV can elicit the R3-core-specific anti-LPS antibodies that may cross-react to non-O157 Shiga toxin-producing *E. coli* (STEC) pathogens possessed with R3-type LPS core commonly[31], this study did not test this possibility that would be another theme to be pursued in further experiment. This study has given the prioriy in checking out both the formation of neutralizing antibody against mOMVs and waaJ-OMVs and the protection against wtOMVs.

In addition to mOMVs, which consist of the much-less endotoxic LPS (penta-acylated lipid A) and are devoid of STxA subunits, this study tested the

immunogenicity of waaJ-mOMVs, which are devoid of the O-antigen side chains of O157 LPS in the mOMV backbone. O-antigen truncation of LPS of bacterial pathogens is often associated with decreased immunogenicity compared with full-length LPS[32]. But, sometimes, exposure of the core oligosaccharides with O-antigen truncated LPS is supposed to provide additional immunogenicity[33]. In the case of waaJ-mOMVs, O-antigen truncation rendered the vesicles less immunogenic than the ocular mOMV vaccinatioan model, suggesting that O-antigen in mOMVs may serve as a strong immunogenic component capable of eliciting an antibody response towards the mOMV vaccine. Furthermore, PMB treatment of mOMVs, which was used to block the LPS-mediated adjuvant activity of mOMVs[34], resulted in a significant reduction of anti-OMV_{O157} antibody titers. Interestingly, the ability for inducing anti-LPS_{O157} antibody was not affected by PMB treatment. Since PMB binding to LPS of mOMVs by charge-to-charge interactions can block TLR-4 dependent innate immunity activation pathways[35], it has been suggested that PMB treatment may decrease the potential adjuvant activity of LPS within mOMVs administered to the ocular mucosa. Based on these results, LPS moiety of mOMVs served as an adjuvant by promoting

immunogenicity of vesicle antigens, but did not contribute towards increased production of anti-LPS antibodies.

V. CONCLUSION

The current study showed, for the first time, that eyedrop vaccination using mOMVs of EHEC O157 bacteria was efficient vaccination tool which did not require additional adjuvants. Moreover, eyedrop vaccination with mOMVs was shown to be effective for preventing HUS pathogenesis in mice against challenge with HUS-causative wtOMVs. This study also demonstrated that loss of LPS O-antigen of waaJ-mOMVs rendered vesicles much less immunogenic compared to mOMVs. Thus, taken together, these results suggest that mOMV can be utilized as a safe ocular-mucosal vaccine capable of inducing a protective immune response against HUS-causative OMVs of EHEC O157 bacteria.

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ABSTRACT (in Korean)

장출혈성대장균의 변형외막수포를 이용한 안약백신의 용혈성요독증후군의 보호효과

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최 경 섭

장출혈성대장균은 설사, 출혈성 장염을 일으키며, 용혈성빈혈, 혈소판감소증, 급성 신부전 등 심각한 증상과 동반된다. 아직까지 장출혈성대장균에 대한 치료로 보건 위생학적인 면에서의 조치 외에는 특별한 치료가 없으며, 최근에는 효과적인 백신의 개발이 주된 관심 분야로 대두되고 있다. 외막수포는 그람 음성 세균에서 유래된 구형의 막으로 이를 이용한 백신이 면역반응을 향상시켜 감염된 개체를 보호할 수 있다는 연구들이 있었다. 그러나 장출혈성대장균 O157:H7 에서 유래된 외막수포의 경우 Shiga toxin 같은 외독소 및 리포다당류 같은 내독소가 존재하기에

효과적이며 안전한 백신으로의 사용이 어려웠다. 이에 반해 MsbB- 변이와 STxA- 변이가 이루어진 장출혈성대장균에서 유래된 변형외막수포는 독성이 약화된 penta-acylated 지방 A 구조를 가지고 있으며, 독성 STxA 단백질을 함유하고 있지 않다. 또한 waaJ-변형외막수포는 O157 리포다당류의 O-antigen 중합체가 제거되어 있다.

본 연구는 용혈성요독증후군에 대한 보호효과를 목적으로 장출혈성대장균에서 유래된 변형외막수포를 가지고 안약백신의 효과를 보고자 하였다.

BALB/c 마우스에 변형외막수포와 waaJ-변형외막수포를 안약형태로 접종하였다. 2 주 경과후 2 차 접종을 하였으며, 4 주차에 자연형태 외막수포를 복강내 투여하여 면역 형성 및 보호 효과를 평가하였다. 또한 장내 점막면역의 효과를 증가 시키기 위하여 콜레라 독소를 포함한 여러 보조제를 변형외막수포와 함께 안약형태로 투여하였다. 장출혈성대장균의 변형외막수포 매개 면역반응을 평가하기 위하여 혈청과 점액에서 면역글로불린을 ELISA 기법으로 확인하였으며, 보호 효과 평가를 위해 몸무게, BUN/Cr 수치의 변화, 신장의 조직병리학적 검사를 하였다.

마지막으로 변형외막수포의 안약사용의 안정성을 확인하기 위하여 마우스의 몸무게와 안구의 조직병리학적인 변화를 확인 하였다.

변형외막수포로 접종된 마우스에서 전신적, 점막성 면역반응이 waaJ-변형외막수포 접종군 및 대조군보다 높았다. 변형외막수포에 polymyxin B 를 더한 경우 전신적, 점막성 면역반응이 감소하였다. 변형외막수포에 콜레라 독소를 더하여 안약으로 투여시 전신적 및 장내 항체형성이 증가하였다. 변형외막수포로 안약 점안 시킨 마우스에서는 복강을 통해 접종된 치사량 용량의 자연형태 외막수포로부터 보호 되었으며, 대조군에서는 보호 되지 못하였다.

결론적으로 본 연구는 장출혈성대장균으로부터 유도된 변형외막수포가 마우스에서 충분한 면역성을 유발함을 확인하였고, O157 리포다당류 항원이 외막수포의 면역원성을 향상시키는데 중요함을 보였다. 더욱이 본 연구는 변형외막수포를 안약형태로 투여시 장출혈성대장균 관련 용혈요독증후군 유사 증상을 예방할 수 있었음을 보인 것으로, 향후 장출혈성대장균에 의한 병인의 예방효과에 대한 기대를 보여준다.

핵심되는 말: 변형외막수포, 장출혈성대장균, 안약백신