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Original Article

# Controlled release of canine MSC-derived extracellular vesicles by cationized gelatin hydrogels

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ABSTRACT

*Introduction:* Canine mesenchymal stem cell (MSC)-derived extracellular vesicles (EVs) have emerged as a promising form of regenerative therapy. Therapeutic application of EVs remains difficult due to the short half-life of EVs *in vivo* and their rapid clearance from the body. We have developed cationized gelatin hydrogels that prolong the retention of EVs to overcome this problem.
*Methods:* Canine MSCs were isolated from bone marrow. MSC-derived EVs were isolated from the culture supernatant by ultracentrifugation. Gelatin was mixed with ethylene diamine anhydrate to cationized. Distinct cross-linked cationized gelatin hydrogels were created by thermal dehydration. Hydrogels were implanted into the back subcutis of mice in order to evaluate the degradation profiles. Hydrogels with collagenase were incubated at 37 °C *in vitro* to quantize the release of EVs from hydrogels. Lipopolysaccharide (LPS)-stimulated BV-2 cells were used to evaluate the immunomodulatory effect of EVs after release from the hydrogels.
*Results:* The cationized gelatin hydrogels suppressed EV release in PBS. More than 60% of immobilized EVs are not released from the hydrogels. The cationized hydrogels released EVs in a sustainable manner and prolonged the retention time of EVs depending on the intensity of cross-linking after degradation by collagenase. The expression of IL-1β in LPS-stimulated BV-2 cells was lower in EVs released from the hydrogels than in controls.
*Conclusions:* Our results indicate that the controlled release of EVs can be achieved by cationized gelatin hydrogels. The released EVs experimentally confirmed to be effective in reducing proinflammatory response. The cationized gelatin hydrogels appear to be useful biomaterials for releasing canine MSC-derived EVs for regenerative therapy.


## 1. Introduction

Mesenchymal stem/stromal cells (MSCs) are valuable sources of stem cells for the regeneration of damaged tissues in clinical applications. Several studies of rodent spinal cord injury (SCI) models have found that intravenous or intrathecal injection of MSCs promotes functional recovery [1,2]. There have been several clinical trials of MSCs for SCI in dogs [3–5] and humans [6,7]. Injection of MSCs did not cause any complication, and led to improve motor function after MSC injection.

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The therapeutic effects of MSCs are now known to be mediated by various cytokines, growth factors, and extracellular vesicles (EVs) [8–10]. EVs consist of lipid bilayers produced by various cell types. These are classified into three main classes: exosomes, microvesicles, and apoptotic bodies, according to the mode of biogenesis. EVs encapsulate mRNA, miRNA and proteins, and act in cell–cell communication by shuttling complex messages. EVs are involved in cell growth, pathways and altering cell or tissue metabolism in the body. EVs can also influence tissue response to injury and disease. MSC-derived EVs are promising forms of regenerative therapy and immunomodulation. There have been reports that EVs derived from MSCs reduce immune response and promote tissue repair [11,12]. Human MSC-derived EVs exert an effect against multiple diseases in animal models, including brain injury [13], hind limb ischemia [14], myocardial infarction [15], and SCI [16]. Compared to MSC transplantation, injection of MSC-derived EVs have the advantages of being storable and low risk of aneuploidy. Canine MSC-derived EVs also suppress the function of various immune effector cells, and support tissue repair [17].

Different routes of administration are used to deliver EVs to the target tissue. Intravenous injection is the most widely used route for the delivery of EVs. Intravenous injection of EVs into native mice has been shown, however, to cause rapid clearance from blood circulation [13], while accumulation of EVs in the liver, spleen, lung, and gastrointestinal tract has been found after injection [18,19]. The short half-life of EVs *in vivo* and their rapid clearance from the body after intervention means that the therapeutic application of EVs is still difficult. Local injection of EVs may suffer the same fate due to rapid turnover. Furthermore, the regeneration process may be slow and the viability of EVs may degenerate [20]. The development of biomaterials capable of maintaining EVs and the sustained release of EVs is consequently essential for EV-based therapy.

Gelatin is a biodegradable material that has been used in food and medicine because of its good biocompatibility. Gelatin hydrogels are three-dimensional hydrophilic polymeric networks that are physically or chemically cross-linked, which are capable of adsorption without undergoing dissolution. In regenerative medicine, gelatin hydrogels can act as scaffolds, barriers, drug delivery systems, and cell encapsulation matrices. The biosafety and biocompatibility of gelatin hydrogels have been demonstrated through long clinical applications and a number of tissue engineering studies [21,22]. In general, EVs have a net negative charge. Previous studies suggest that anion exchanger is useful for isolation of MSC-derived EVs [13]. We speculate that controlled release of EVs enhance the regeneration of tissue repair, since EVs can maintain their therapeutic effect in the long term. In this study, we developed cationized gelatin hydrogel so as to enhance the retention of EVs and maintain their anti-inflammatory effect after sustained release *in vitro*.

## 2. Materials & methods

### 2.1. Experimental animals

Three healthy female beagles (aged 2–5 years) were purchased from Oriental Yeast (Tokyo, Japan). Sixty ddy male mice (6 weeks old) were purchased from Japan SLC (Shizuoka, Japan). All procedures were performed according to the guidelines of the experimental animal committee of the university, and the protocols (19–97, 19–183) were approved by the experimental animal committee of the university.

### 2.2. MSC isolation and culture

Canine MSCs were isolated from bone marrow, and were cultured as described previously [17]. The bone marrow perfusate was centrifuged, and the precipitates were suspended in 15 mL Dulbecco phosphate-buffered saline (PBS; Nacalai Tesque, Kyoto, Japan). Mononuclear cells were isolated by density centrifuging with a lymphocyte separation solution (Nacalai Tesque) at 400 × *g* for 30 min at room temperature. The buffy coat at the interface was collected, mixed with 20 mL PBS, and centrifuged at 300 × *g* for 5 min. Precipitated cells were washed with PBS and the number of cells was determined using a hemacytometer. Enriched mononuclear cells were plated in 15 cm tissue culture dishes (Corning, Rochester, NY) at a density of $1.5 \times 10^5$ cells/cm$^2$ in complete culture medium, consisting of Dulbecco modified eagle medium including 1 g/L of glucose (DMEM; Nacalai Tesque) with 10% heat-inactivated fetal bovine serum (FBS; GE Healthcare, Chicago, IL) and 1% antibiotic-antimycotic solution (Nacalai Tesque). Incubating took place at 37 °C in 5% $CO_2$. Nonadherent cells were removed by replacing the medium at 48 h after plating. The culture medium was changed 2–3 times per week. When 70–80% confluence was reached, 0.25% trypsin–EDTA solution (Thermo Fisher Scientific, Waltham, MA) was used to harvest the adherent cells. The collected cells were centrifuged at 300 × *g* for 5 min, washed with PBS, and then cryopreserved in liquid nitrogen.

Frozen MSCs were thawed at 37 °C and plated directly in tissue culture dishes of diameter of 15 cm. After 24 h, MSCs were seeded at $8.0 \times 10^3$ cells per cm$^2$. After these cells reached 70–80% confluency, the culture plates were washed with PBS, and each dish was replaced with 15 mL of serum-free medium (STEMPRO MSC SFM; Thermo Scientific). The culture supernatant was collected after 48 h.

### 2.3. Isolation and characterization of EVs

The culture supernatant was centrifuged at 2,320 × *g* for 15 min to remove cells and debris, and was then ultracentrifuged (Himac CP100WX Ultracentrifuge and P28S Rotor; HITACHI, Tokyo, Japan) at 100,000 × *g* for 90 min at 4 °C. The resulting pellet was resuspended in PBS. The protein content was quantified by the Bradford method (Nacalai Tesque).

The samples were heat blocked in SDS-PAGE sample buffer and denatured at 100 °C for 5 min. The samples were electrophoresed on 10% SDS polyacrylamide gels, and transferred on to 0.45 μm nitrocellulose membrane (Bio-Rad, Hercules, CA). The membrane was then incubated for 2 h in 3% blocking buffer (ECL Prime™ blocking agent; GE Healthcare) and probed with mouse monoclonal anti-human TSG101 antibody (clone: 51/TSG101, BD bioscience, Franklin Lakes, NJ) that cross-reacts against canine TSG101 [17,23] at 1:500 at 4 °C overnight. The membrane was washed and incubated with HRP-conjugated goat anti-mouse antibody (Peroxidase AffiniPure Goat Anti-Mouse IgG; Jackson ImmunoResearch, West Grove, PA) at 1:2000 for 1 h at room temperature. Protein was detected using chemiluminescent substrate (LAS4000; FUJIFILM, Tokyo, Japan).

The particle size of MSC-derived EVs was measured via dynamic light scattering (Zetasizer Nano ZS; Malvern, Worcestershire, UK). The analysis was performed at 25 °C using samples diluted with PBS.

### 2.4. Fabrication of cationized gelatin hydrogel

An aqueous solution of 4 wt% gelatin (isoelectric point 9.0, weight-averaged molecular weight 100,000; Nitta Gelatin, Osaka, Japan) was preheated at 37 °C for 2 h 100 mL of 4 wt% gelatin solution was mixed with 1.85 mL of ethylene diamine anhydrate (Nacalai Tesque) to cationized the gelatin. Then, HCl was added to adjust the pH value to 5.0. The solution was dialyzed using a

cellulose dialysis tube (Sekisui Medical, Tokyo, Japan) for 48 h and was then freeze-dried (Takara, Saitama, Japan).

Cationized gelatin hydrogels were prepared by the dehydrothermal crosslinking of cationized gelatin. An aqueous solution of 50 mg/mL cationized gelatin was preheated at 37 °C for 2 h, and was freeze-dried to make the cationized gelatin hydrogel sheets. The sheets were crosslinked by thermal dehydration for 24, 48 and 96 h using a vacuum drying device (DN-30S; Sato Vacuum, Tokyo, Japan). The different sheets were obtained as low, medium and high cross-linked sheets, respectively.

### 2.5. In vivo study of the cationized gelatin hydrogel degradation

To study the degradation profiles of gelatin hydrogels, we employed *in vivo* implantation of 2 mg of cationized gelatin hydrogels. The cationized hydrogels swelled by PBS were implanted into the back subcutis of mice. The implanted hydrogels were then extracted at 3, 7, 10, 14, and 21 days after implantation, and the weight of the hydrogels was measured (n = 4 at each time point).

### 2.6. In vitro release test of EVs from gelatin hydrogels

To study the release of EVs from cationized gelatin hydrogels, we employed *in vitro* degradation of 2.0 mg of the hydrogels. EVs were labelled using a PKH26 red fluorescent cell linker kit (Sigma–Aldrich, St Louis, MO) according to the manufacturer's instructions. In summary, 100 μg/mL of EVs was impregnated into a gelatin hydrogel sheet (low, middle, and high cross-linked) at a volume of 20 μl per sheet overnight at 4 °C. Hydrogel containing EVs was placed in a 1.5 mL tube, and 600 μl of PBS was added and incubated at 37 °C for 26 h. After addition of collagenase D (Roche Diagnostics, Basel, Switzerland), the gels were incubated at 37 °C. At indicated time points (0, 1, 3, 5, 8, 12 and 24 h), the degradation of hydrogel was determined by BCA protein assay kits (Takara Bio, Shiga, Japan), and the amount of released PKH26-labelled EVs was calculated by Spectra Max i3x (Molecular Devices, San Jose, CA).

### 2.7. In vitro assay of immunomodulatory effect of MSC-derived EVs

To study the immunomodulatory effect of EVs after release from cationized gelatin hydrogels, we employed *in vitro* degradation of 2.0 mg of cationized low-linked gelatin hydrogels. In summary, 100 μg/mL of EVs was impregnated into the gelatin hydrogel sheet at a volume of 20 μl per sheet overnight at 4 °C. The low cross-linked hydrogel containing EVs was placed in a 1.5 mL tube, and 60 μl containing 10 μg/mL of collagenase D was added gently and incubated at 37 °C for 72 h. After complete degradation of hydrogels, the supernatant containing EVs was used. For the controls, the low cross-linked hydrogel without EVs were used.

BV-2 cells were cultured in DMEM with 5% heat-inactivated FBS and 1% antibiotic-antimycotic solution at 37 °C in 5% $CO_2$. After the BV-2 cells reached about 70–80% confluency, they were detached using 0.25% trypsin–EDTA solution and seeded at $5.0 \times 10^5$ cells per well in a 6-well plate. After 24 h of incubation, the BV-2 cells were stimulated by replacing 1 mL of cultured medium alone, or cultured medium containing 1 ng/mL of lipopolysaccharide (LPS) (Sigma–Aldrich) and the supernatant.

Total RNA of the cells was extracted 6 h after stimulation of LPS, using a NucleoSpin RNA Plus (Macherey–Nagel, Düren, Deutschland) according to the manufacturer's instructions. The RNAs were converted to cDNA with reverse transcriptase using oligo (dT) primers to prime ReverTraAce (Toyobo, Osaka, Japan). The cDNA was then amplified by the SYBR Green Realtime PCR Master-Mix-Plus (Toyobo) using a StepOnePlus Real-Time PCR system (Thermo Fisher). The following mouse primers were used in this study: 5′-TCCAGGATGAGGACATGAGCAC-3' (forward) and 5′-GAACGTCACACA CCAGCAGGTTA-3' (reverse) for *IL-1β*; 5′-ACACATGTTCTTCTGGGAAATCG-3' (forward) and 5′-TGAAGGACTCTG GCTTTGTC-3' (reverse) for *IL-6*; 5′-ATGAGCACAGAAAGCATGATC-3' (forward) and 5′- TACAGGCTTGTCACTCGAATT-3' (reverse) for *TNF-α*; and 5′-CACTCACGGCAAATTCAACGGCAC-3' (forward) and 5′-GACTCCACGACATACTCAGCAC-3' (reverse) for *GAPDH*. The optimum conditions for PCR amplification of the cDNA were established according to the manufacturer's instructions. Reactions were carried out as follows: initial denaturation at 95 °C for 30 s, followed by 40 cycles of 5 s at 95 °C and 30 s at 60 °C. The data were analyzed using StepOne software (Thermo Fisher), and the cycle number at the linear amplification threshold (Ct) values for the endogenous control gene (*GAPDH*) and the target gene were recorded. The relative gene expression was calculated using the comparative Ct method ($2^{-\Delta\Delta Ct}$). Levels of gene expression in non-LPS treated BV-2 cells were expressed as 1 U.

### 2.8. Statistical analysis

Data are presented as mean ± SD. Real-time quantitative PCR analysis were performed by the Kruskal–Wallis test followed by Dunn's multiple test using GraphPad prism 9 software (GraphPad Software, San Diego, CA). Values of $p < 0.05$ were taken as significant.

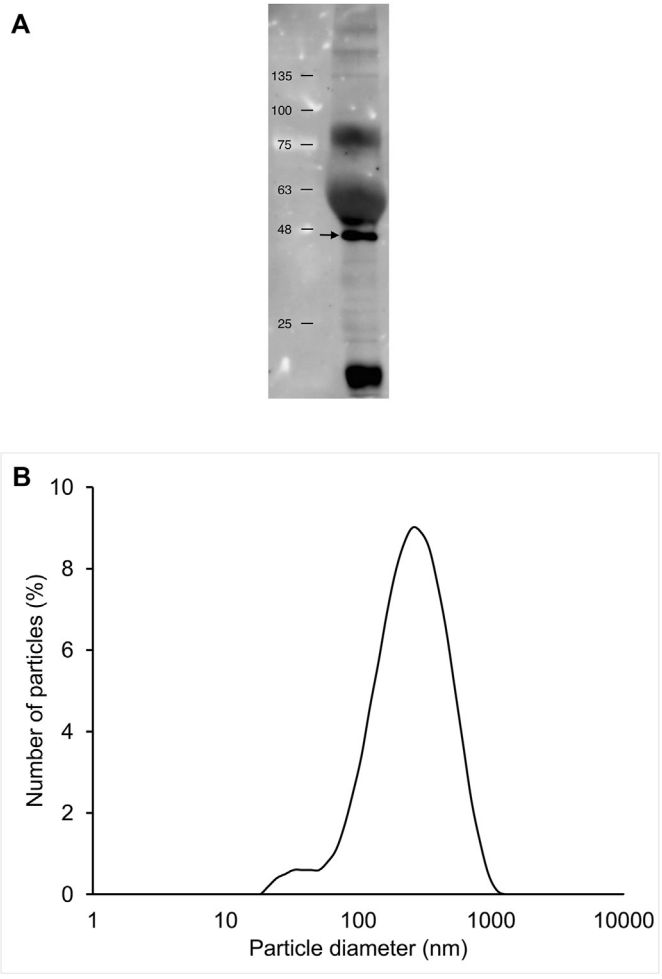


**Fig. 1.** Characteristics of canine MSC-derived EVs. (A) Expression of TSG-101 (arrowhead) in MSC-derived EVs. (B) Diameter of MSC-derived EVs with peak at 299.5 nm.

## 3. Results

### 3.1. Isolation and characterization of EVs

EVs were isolated from the supernatant of canine MSCs by ultracentrifugation. The EVs were characterized by western blotting and a zetasizer. Expression of TSG101 was observed in MSC-derived EVs (Fig. 1). The diameter of EVs derived from canine MSCs has its peak at 299.5 nm.

### 3.2. In vivo study of the cationized gelatin hydrogel degradation

Chemical derivatization modifies the electric charge of gelation. Fig. 2 shows the low, medium, and high cross-linked gelatin cationized gelatin hydrogel sheets. The prepared hydrogel sheets were square (20 mm × 20 mm) and 0.5 mm thick. Fig. 3 shows the time profile of the *in vivo* weight of remaining sheets after implantation of the low, medium, and high cross-linked cationized gelatin hydrogels. The weight of the low cross-linked hydrogels decreased 7−10 days after implantation. The weight of the middle cross-linked hydrogels decreased 14 days after implantation. The high cross-linked hydrogels were detected more than 21 days after implantation.

### 3.3. In vitro release of EVs from cationized gelatin hydrogel

Fig. 4 shows the time profile of degradation and EV release of different cationized cross-linked gelatin hydrogels. *In vitro* degradation of hydrogels showed that 31.0 ± 1.78% were dissolved in the low cross-linked hydrogels at 26 h. In the middle cross-linked hydrogels, 23.7 ± 0.48% were dissolved at 26 h. The high cross-linked gels showed the slowest degradation rate, and 18.5 ± 0.57 (%) were dissolved at 26 h. The release rates of EVs from low, middle, and high cross-linked hydrogels in PBS were respectively 18.3 ± 16.2%, 37.1 ± 2.59%, and 32.0 ± 1.70%. After collagenase was added, all cross-linked hydrogels were dissolved.

*In vitro* release results showed that 100% of EVs were released from the low cross-linked hydrogels at 5 h. In the middle cross-linked hydrogels, 74.4 ± 9.64% were dissolved at 5 h. High cross-linked gel showed slowest release rate, in which 57.3 ± 3.82% of EVs were released for 5 h.

### 3.4. In vitro immunomodulatory effect of released EVs from cationized hydrogels

Based on the results of the *in vivo* study of the cationized gelatin hydrogel degradation and the *in vitro* release of EVs from cationized gelatin hydrogel, we used EVs release from the low cross-linked hydrogels for this study. The expression of IL-1β in LPS-stimulated BV-2 cells was lower in the group treated with EVs released from the low cross-linked hydrogels than the control group (Fig. 5, $p < 0.01$). There was a tendency for the expression of IL-6 in group treated with EVs released from the low cross-linked hydrogels to be lower than in the control group ($p = 0.06$). There was no significant difference between groups in levels of TNF-α ($p = 0.937$).

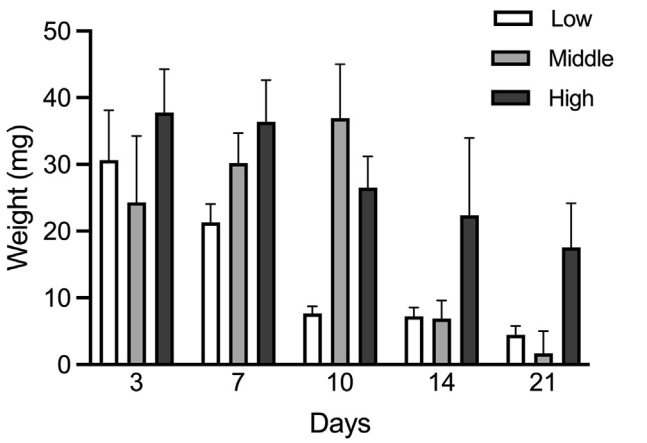


**Fig. 3.** Time profiles of weight remaining after subcutaneous implantation of the low, middle, and high cross-linked gelatin hydrogels.

## 4. Discussion

This study has demonstrated that the controlled release of EVs can be achieved by cationized gelatin hydrogels. The EVs released were confirmed experimentally to be effective in reducing the proinflammatory response. We speculate that cationized gelatin hydrogels can retain EV that have a net negative charge on their surface. *In vitro*, since PBS does not contain any enzymes to degrade the hydrogels, only EVs in free form can be released from the hydrogel. Without the enzymatic degradation of hydrogels to generate water-soluble gelatin fragments, more than 50% of EVs immobilized are not released from the hydrogels. It is possible that EVs interact electrostatically with gelatin hydrogel with positive charge, suppressing EV release by the suspension. The release rates of EVs in the low cross-linked cationized gelatin hydrogels were lower than in the middle and high cross-linked hydrogels. The

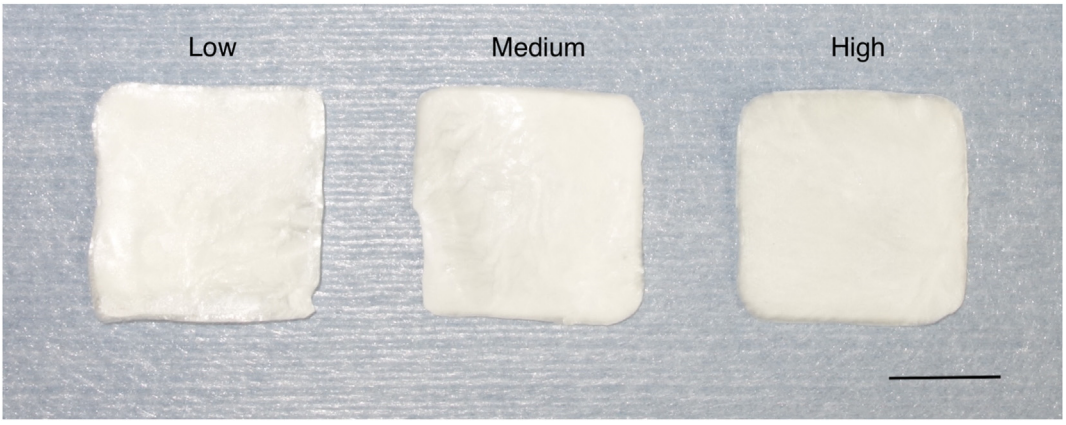


**Fig. 2.** Photograph of cationized gelatin hydrogel sheets. The prepared hydrogel sheets were rectangular (20 mm × 20 mm) and 0.5 mm thick. Left: low, middle: medium, Right: high cross-linked gelatin hydrogel sheets. Bar = 10 mm.

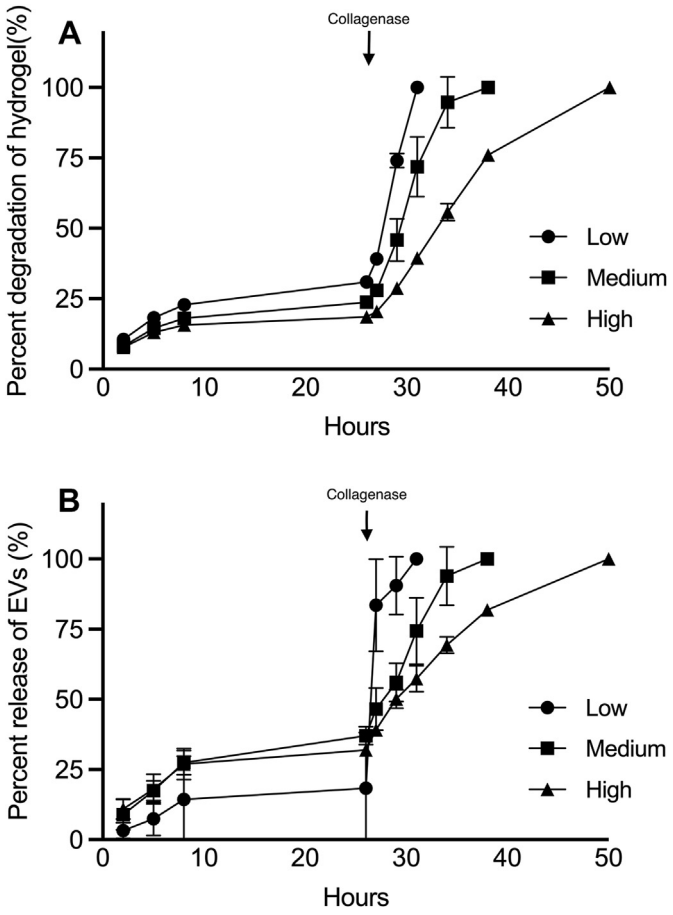


**Fig. 4.** In vitro degradation (A) and release profiles (B) of EVs from the low, middle, and high cross-linked gelatin hydrogels. The sheets were placed in PBS without collagenase for 26 h, followed by PBS containing collagenase for another 24 h.

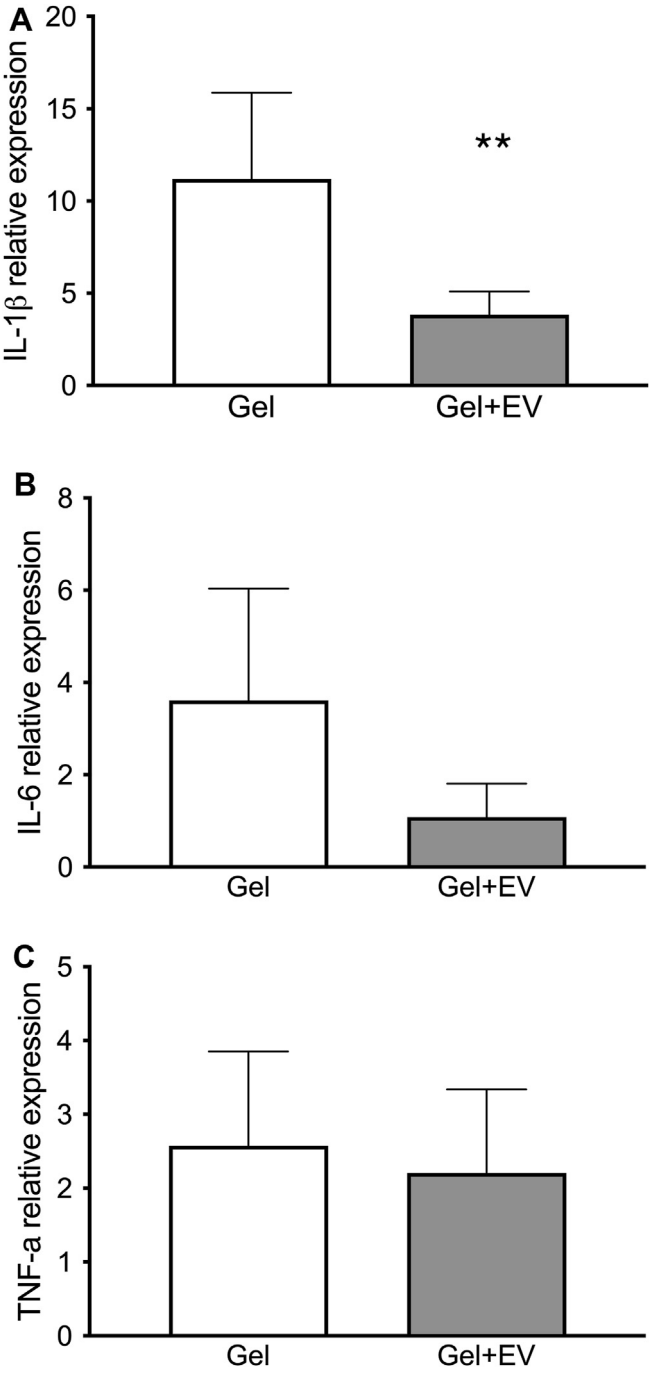


**Fig. 5.** Anti-inflammatory effect of canine MSC-derived EVs released from the low cross-linked gelatin hydrogels on BV-2 cells. qPCR-assays for the expression of IL-1β (A), IL-6 (B), and TNF-α (C) in BV-2 cells cultured with lipopolysaccharide and EVs released from the hydrogels. Levels of gene expression in non-lipopolysaccharide stimulated BV-2 cells are expressed as 1 U.

different crosslinking might affect the retention ability of EVs. To retain EVs, better crosslinking is needed. The good correlation, in the *in vitro* time profile, between hydrogel degradation and EV release indicates that EV release is not governed by a simple diffusion mechanism, but rather by the degradation of cationized gelatin hydrogels. Taken together, these observations confirm that EVs are released by weakening electrostatic interaction following release carrier degradation.

A previous report indicated that canine MSC-EVs cultured under serum-free conditions can reduce the IL-1β in LPS-stimulated BV-2 cells [17]. Our previous report found that human MSC-derived EVs reduce the brain IL-1β in a brain injury mouse model [13]. Consistent with our previous observations, EVs released from the low cross-linked cationized hydrogel reduced the LPS-induced expression of inflammation genes by BV-2. Our results suggest that released canine MSC-derived EVs can retain the immunomodulatory effects of mouse microglial cells, and that the cationized gelatin hydrogels may be useful in clinical application. Further studies are needed to assess whether canine MSC-derived EVs can reduce the immunomodulatory effects of canine cells.

Therapy involving EVs faces a major challenge because of their short half life, which act as a significant limitation on therapeutic effects [18–20]. Biomaterials have been utilized to load EVs. They can prevent loaded EVs from being cleared prematurely, and allow the delivery of a more localized and concentrated dosage. Different polymers have been used to create a sustained delivery system of EVs [24]. The polymers are from natural sources including collagen, gelatin, and chitosan, and synthetic sources, including polyethylene glycol and poly latic-co-glycolic acid [25]. Hydrogels have tunable physical properties that can be exploited to customize the degradation rate. Several studies indicate that biomaterials enhance the retention of EVs and improve the efficacy of EV therapy [26–28]. Several novel hydrogels capable of capturing and delivering EVs have been developed. The hydrogel composed of pluronic F127, oxidative hyaluronic acid and poly-ε-L-lysine can release EVs, and their release rate is more rapid than in acidic pH than in neutral pH

[29]. We have developed a cationized hydrogel for sustaining the release of EVs. Cationized gelatin microspheres that degrade were used to lengthen the period during which plasmid DNA was released [30]. The cationized gelatin hydrogels degraded with time *in vivo*, so EVs were released continuously around the hydrogels until degradation of sheets take place. This system allows controlled biodegradation of the local delivery agent, and protects EVs from rapid degradation.

There are several methods for collecting EVs, including ultracentrifugation, filtration, polymer precipitation, and size-exclusion chromatography [31]. We collected EVs by ultracentrifugal methods, but this method is unsuitable for large scale production of EVs. We have reported that ion exchange chromatography can be used to isolate and enrich EVs in a scalable manner [13]. Isolation methods may influence the yield and the functional characteristics of EVs, however. There is also a lack of good manufacturing practice guidelines for MSC-derived EVs.

This study has several limitations. We did not compare the retention capability of gelatin hydrogels that have negative electric charge and that have no electric charge. Some rodent models are needed to assess the efficacy of sustained release of EVs from cationized gelatin hydrogels. Further studies are needed to determine the efficacy and safety of cationized gelatin hydrogels for retention and release of EVs.

## Declaration of competing interest

The authors declare no conflict of interest.

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