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Effect of pentosan polysulfate sodium on adhesion molecule expression in canine endothelial cells and neutrophils

Kanittha Darawiroj[1)], Takafumi Sunaga[1,*)], Ryo Owaki[1)], Nomsa Mulenga Handondo[1)] and Masahiro Okumura[1)]

[1)] Laboratory of Veterinary Surgery, Department of Veterinary Clinical Sciences, Graduate School of Veterinary Medicine, Hokkaido University, Sapporo, Hokkaido 060-0818, Japan

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Abstract

Pentosan polysulfate sodium (PPS) is a semi-synthetic sulfated polysaccharide with a heparin-like structure, known for its anticoagulant and anti-inflammatory properties. This study investigated the effect of PPS on adhesion molecules involved in neutrophil–endothelial interactions using an in vitro canine model. Canine aortic endothelial cells (CnAOEC) and neutrophils were pretreated with various concentrations of PPS and stimulated with recombinant canine tumor necrosis factor-alpha (rh-cTNFα). The mRNA expression of E-selectin, intercellular adhesion molecule-1 (ICAM-1), P-selectin, C-X-C motif chemokine receptor 2 (CXCR2), P-selectin glycoprotein ligand-1 (PSGL-1), and L-selectin was measured through real-time polymerase chain reaction (qPCR). C-X-C motif chemokine ligand 1 (CXCL1) secretion was quantified by enzyme-linked immunosorbent assay (ELISA). Lymphocyte function-associated antigen-1 (LFA-1/CD11a) expression was examined using flow cytometry. A static adhesion assay was used to evaluate the effect of PPS on neutrophil adhesion to endothelial cells. In conclusion, PPS displayed no cytotoxicity toward CnAOEC, and endothelial P-selectin expression was significantly reduced. Other adhesion molecules showed no significant differences compared with controls. CXCL1 secretion and neutrophil gene expression (CXCR2, PSGL-1, and L-selectin) varied slightly, although without statistical significance. PPS did not affect CD11a expression or neutrophil adhesion to endothelial cells. These results indicate that PPS is non-cytotoxic and may weakly modulate selectin-mediated endothelial activation without affecting integrin- or chemokine-dependent adhesion.

Key Words: CXCL1, L-selectin, PPS, P-selectin, PSGL-1

Introduction

Inflammation is the initial defensive response to injury and invading pathogens[4)]. It is a vital biological process required for survival[16)]. However, its dysregulation is associated with several chronic diseases[3)], and excessive or prolonged inflammation may lead to physiological disturbance, tissue injury, organ dysfunction, or even death[25)]. Neutrophils are the first immune cells recruited from the bloodstream to inflamed tissue. Besides their pathogen-eliminating

* Corresponding author: Takafumi Sunaga
Laboratory of Veterinary Surgery, Department of Veterinary Clinical Sciences, Graduate School of Veterinary Medicine, Hokkaido University, Sapporo, Hokkaido 060-0818, Japan
TEL: +81-011-706-5228
FAX: +81-011-706-5229
Email: sunaga@vetmed.hokudai.ac.jp
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activity, they release cytokines that modulate other immune cells and contribute to resolving inflammation[23].

The recruitment of neutrophils follows a tightly coordinated multistep cascade, including capturing and rolling, firm adhesion, crawling, and endothelial cell transmigration. Each step is regulated through interactions between adhesion molecules on neutrophils and endothelial cells[15]. During the initial capture and rolling phase, endothelial E-selectin and P-selectin interact with P-selectin glycoprotein ligand-1 (PSGL-1) on neutrophils. This slows their movement along the endothelial cell. L-selectin on neutrophils further facilitates secondary capture by binding to PSGL-1 on adjacent rolling neutrophils, promoting slow rolling. Additionally, endothelial cells secrete C-X-C motif chemokine ligand 1(CXCL1), which in turn binds to its receptor C-X-C chemokine receptor type 2 (CXCR2) on neutrophils. These interactions induce a conformational activation of lymphocyte function-associated antigen-1 (LFA-1) on neutrophils, shifting it from a low to high affinity state. Subsequently, activated LFA-1 mediates firm adhesion by binding to intercellular adhesion molecule 1 (ICAM-1) expressed on an endothelial cell. After firm adhesion, neutrophils crawl along the endothelium under the regulation of ICAM-1 and CXCL1, which influences the subsequent endothelial transmigration process through either the paracellular or transcellular route[13,15,17,33].

Pentosan polysulfate sodium (PPS) is a semi-synthetic sulfated polysaccharide derived from beech wood hemicellulose. It is a low-molecular-weight heparin-like compound with anticoagulant and fibrinolytic properties[7,12]. PPS is recognized as a disease-modifying osteoarthritis drug (DMOAD) that is clinically used for the treatment of osteoarthritis and other inflammatory disorders, such as interstitial cystitis, and for the management of thrombotic and atherosclerotic disease in both human and veterinary medicine[5,12]. As a result of its structural and functional similarities to heparin, PPS exhibits considerably lower anticoagulant activity. However, it shows an inhibitory effect on the cell adhesion associated with the selectin-mediated pathway[9]. These pharmacological characteristics suggest that PPS may modulate inflammation and cell recruitment by regulating adhesion molecules.

Nevertheless, information on the effect of PPS on a broader range of adhesion molecules, particularly in canine endothelial cells and neutrophils, remains limited. In addition, data on its dose-dependent responses is currently lacking. Therefore, this study aims to investigate the effect of PPS on the expression of key adhesion molecules involved in neutrophil-endothelial interactions using a canine cell-based in vitro model, and establishing the corresponding dose-dependent effects.

Materials and Methods

Canine aortic endothelial Cell culture

Canine aortic endothelial cells (CnAOEC; Cell Applications, Inc., San Diego, CA, USA; Cat. No. Cn304-05) were obtained as cryopreserved vials at passage 2. For this study, cells from passages 4 to 9 were maintained in the Canine EC Growth Medium (Cell Applications, Inc., Cat. No. Cn211K-500) following the manufacturer's protocol with minor adjustments. In short, CnAOEC were seeded at a density of 1.0×10^4 cells/cm^2 in 100 mm culture dishes (Corning, Lowell, MA, USA) pre-coated with Attachment Factor Solution (AFS; Cell Applications, Inc., Cat. No. 123). The 10 ml of fresh prewarmed at 37 °C of the Canine EC Growth Medium was replaced every other day. At 80–90% confluence, CnAOEC were washed with Hanks' Balanced Salt Solution (HBSS; Thermo Fisher Scientific, Waltham, MA, USA; Cat. No. 14025092) three times, then the cells were detached from the culture plate using Accutase solution (Sigma-Aldrich, St. Louis, MO, USA). The cell suspension was centrifuged at 400 x g for 5 minutes without braking. The cell pellet was resuspended in the Canine EC Growth Medium. Cell count and viability were assessed using the trypan blue exclusion assay (Wako Pure Chemical Industries, Osaka, Japan). Following this assessment, the cells were sub-cultured

according to the same established conditions.

Canine neutrophil isolation

Ten milliliters of fresh whole blood were collected from the cephalic vein of three healthy female beagles into a syringe containing K_2EDTA (Dojindo Laboratories, Kumamoto, Japan). The procedure was approved by the Hokkaido University Ethics Committee (Approval No. 230104) and conducted in accordance with institutional animal care protocols. Neutrophils were isolated from anticoagulated blood by density gradient centrifugation using Polymorphprep (Serumwerk Bernburg, Bernburg, Germany), according to the company's instructions for human neutrophil preparation (500 × g, 30 min, 20 °C). Residual erythrocytes were removed by treating the cell pellet with 1 ml of 1× RBC lysis buffer (Thermo Fisher Scientific) for 8 min at room temperature. The cells were then washed with HBSS and centrifuged at 400 × g for 10 min. The neutrophil pellet was resuspended in the Roswell Park Memorial Institute 1640 medium (RPMI-1640; Thermo Fisher Scientific) supplemented with 10% fetal bovine serum (FBS; Sigma-Aldrich) and 1% penicillin–streptomycin solution (Wako Pure Chemical Industries). Cell count and viability were evaluated using the trypan blue exclusion assay. Freshly isolated neutrophils were immediately subjected to experimental procedures.

Cell viability cell counting kit-8 assay

CnAOEC were seeded into 96-well plates (Corning) pre-coated with AFS at a density of 5×10^3 cells/100 µl per well in six replicates and incubated in a humidified incubator with 5% CO_2 at 37 °C for 24 hours to facilitate cell adhesion. Following incubation, the cells were subjected to treatment with either the Canine EC Basal Medium (Cell Applications, Inc., Cat. No. Cn210-500) as a negative control, pentosan polysulfate sodium (PPS; Oji Pharma Corporation, Ltd., Tokyo, Japan, Lot No: OJI225-01) at concentrations of 1, 10, 50, 80, or 100 µg/ml in the Canine EC Basal Medium, or 10% Dimethyl sulfoxide (DMSO; Wako Pure Chemical Industries) as a positive control. Treatments were applied for 24 hours under identical incubation conditions. Cell viability was determined using the Cell Counting Kit-8 (CCK-8; Dojindo Laboratories) according to the manufacturer's instructions. Briefly, after the treatment period, 10 µl of CCK-8 reagent was added to each well, and this was followed by a 4-hour incubation period. Cell viability was subsequently evaluated by measuring absorbance at 450 nm using a microplate reader (Bio-Rad Laboratories, Inc., Hercules, CA, USA). Viability values were normalized to blank controls. All experiments were conducted in three separate trials.

RNA extraction, reverse transcription, and quantitative real-time polymerase chain reaction (qPCR)

CnAOEC were plated into 6-well plates (Corning) pre-coated with AFS at a density of 2×10^5 cells per well and cultured in Canine Endothelial Growth Medium, which was replaced every other day until the cells reached confluence. Once confluent, the cells were pre-incubated with either the Canine EC Basal Medium alone or supplemented with PPS at concentrations of 1, 10, 50, 80, or 100 µg/ml for 24 hours. After a pre-incubation, the PPS pre-treated groups were exposed to recombinant canine tumor necrosis factor-alpha (rh-cTNFα; Kingfisher Biotech, Saint Paul, MN, USA) at 30 ng/ml. This concentration was selected based on preliminary dose-response experiments on CnAOEC demonstrating maximal endothelial activation while maintaining high cell viability. The positive control group was treated with rh-cTNFα alone, whereas the negative control was maintained in the Canine EC Basal Medium without PPS or in rh-cTNFα. All cultures were incubated for 6 hours at 37 °C in a humidified atmosphere containing 5% CO_2. Following incubation, cells were washed three times with HBSS to remove residual medium, and total RNA was subsequently extracted.

Freshly isolated neutrophils (1×10^6 cells/ml) were pre-incubated with either the RPMI-1640 medium supplemented with 10% FBS and 1% penicillin-streptomycin solution, either alone or with PPS at concentrations of 1, 10, or 50 µg/

Table 1. Primer sequences for quantitative real-time polymerase chain reaction

Primer	Primer sequence	Reference
CXCR2	Forward: 5′ AGCTGCCTTAATCCCCTCAT 3′ Reverse: 5′ CTGAACCTGCTGGGAAACTC 3′	14)
E-selectin	Forward: 5′ GAAATTCTTGGCCCGTCCATC 3′ Reverse: 5′ ATGCAGCCAGCACTTAATCTGAAAC 3′	20)
GAPDH	Forward: 5′ TGTCCCCACCCCCAATGTATC 3′ Reverse: 5′ CTCCGATGCCTGCTTCACTACCTT 3′	20)
ICAM-1	Forward: 5′ CAGGGTTGCCAGGTACAGTT 3′ Reverse: 5′ AGTATGGGCTCAGTGGGTTG 3′	20)
L-selectin	Forward: 5′ GATTTCAACAGGACCAGCAGCA 3′ Reverse: 5′ CCGCAGGGTCCACAAGTAAGA 3′	20)
PSGL-1	Forward: 5′ TTCTGGCTGAGATGGCGATG 3′ Reverse: 5′ AGAGTGGCTGGCTCTGGAGTTC 3′	20)
P-selectin	Forward: 5′ TACAACACCAGCTGTCGCTTCC 3′ Reverse: 5′ GTGCTGTCCACTGTCCCAAGTC 3′	20)

CXCR2: C-X-C motif chemokine receptor 2; GAPDH: glyceraldehyde-3 phosphate dehydrogenase; ICAM-1: intercellular adhesion molecule-1; PSGL-1: P-selectin glycoprotein ligand-1

ml for 24 hours. After a pre-incubation, the PPS-treated groups were challenged with rh-cTNFα at 20 ng/ml for 1 hour. The positive control group received only rh-cTNFα, while the negative control was maintained in RPMI-1640 containing 10% FBS and 1% penicillin-streptomycin solution without PPS or rh-cTNFα. Following stimulation, neutrophils were washed once with phosphate-buffered saline (PBS; Thermo Fisher Scientific, Cat. No. 10010023). The cells were then lysed and preserved at –70°C until all samples had been collected for subsequent RNA extraction.

Total RNA was extracted using the NucleoSpin RNA Purification Kit (Macherey-Nagel, Düren, Germany) according to the manufacturer's protocol. RNA concentration and purity were determined using a NanoEX Lite spectrophotometer (Optima Inc., Tokyo, Japan) by measuring absorbance at 260 nm. Subsequently, 500 ng of total RNA from CnAOEC and 50 ng from canine neutrophils were reverse-transcribed into cDNA with the M-MLV Reverse Transcriptase Kit (Invitrogen, Carlsbad, CA, USA), following the manufacturer's instructions. The qPCR was performed using the KAPA SYBR FAST qPCR Kit (KAPA Biosystems, Woburn, MA, USA) in a two-step amplification protocol.

The relative expression levels of target genes were quantified using the $2^{-\Delta\Delta Ct}$ method, with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) serving as the internal reference gene for normalization. Adhesion-related genes, including E-selectin, P-selectin, ICAM-1, CXCR2, PSGL-1, and L-selectin, were analyzed to evaluate the effects of PPS on endothelial and neutrophil adhesion molecules. Primer sequences used in this study are listed in Table 1[14,20]. The qPCR experiments were performed using three independent endothelial cell samples as biological replicates, and each sample was analyzed in duplicate as technical replicates. For neutrophil analysis, blood was collected from each dog on three separate occasions, and each sample was tested twice. This procedure was carried out for three individual dogs.

ELISA

In a separate experiment, CnAOEC were plated in 96-well plates at a density of 1×10^4 cells per well in 200 µl of the Canine EC Growth Medium and incubated for 24 hours to allow cell attachment. The cells were then pre-treated with PPS for 24 hours and subsequently stimulated with rh-cTNFα at 30 ng/ml under the same treatment and control conditions as described in the endothelial cell qPCR assay, with a stimulation period of 12 hours. After stimulation, the culture supernatant was collected to quantify the concentration of secreted CXCL1 protein using the Canine CXCL1 ELISA kit (RayBiotech,

Inc., Norcross, GA, USA) according to the manufacturer's instructions.

Flow cytometry

Freshly isolated neutrophils were subjected to the same pre-incubation and stimulation procedures as those described for the qPCR assay. To evaluate LFA-1 surface expression and verify cell viability under these experimental conditions, cells were gently washed once with PBS and resuspended at 3×10^5 cells in PBS containing 10% goat serum (Thermo Fisher Scientific). The suspension was incubated for 20 minutes at room temperature to minimize nonspecific antibody binding. The cells were then stained with PE-conjugated mouse anti-human CD11a monoclonal antibody (clone HI111, IgG1; BD Biosciences, San Diego, CA, USA) or with a PE-conjugated mouse IgG1 κ isotype control (clone MOPC-21; BD Biosciences) for 10 minutes in the dark. After staining, the cells were washed twice with PBS containing 10% goat serum. Subsequently, 2 µl of 7-Aminoactinomycin D (7-AAD; BD Biosciences) was added to 200 µl of the stained cell suspension in PBS supplemented with 10% goat serum and incubated for 10 minutes to identify non-viable cells. Flow cytometric analysis was carried out using a BD FACSLyric™ Flow Cytometer (BD Biosciences) to determine the percentage of intact membrane cells (7-AAD negative) within the neutrophil population and the mean fluorescence intensity (MFI) of CD11a. For each dog, the experiment was conducted in three separate runs.

Static adhesion assay

To assess the effect of PPS on neutrophil adhesion to endothelial cells, a static adhesion assay was conducted. This assay is a modification from prior human investigation[31]. Briefly, CnAOEC were seeded in 96-well plates at a density of 1×10^4 cells per well and cultured in the canine EC growth medium. The culture medium was replaced every other day until the cells reached 100% confluence. The confluent monolayers were pre-incubated with PPS at doses of 1, 10, 50, 80, or 100 µg/ml for 3 hours. Next, the cells were challenged with rh-TNF-α at a final concentration of 30 ng/ml for 4 hours. The positive and negative controls were treated under the same established conditions. Freshly isolated neutrophils were labeled with Calcein-AM (Dojindo Laboratories) and added to each well at 1×10^5 cells per well for co-incubation with treated CnAOEC for 20 minutes. All incubation procedures were conducted in a humidified incubator at 37 °C containing 5% CO_2. Fluorescence intensity was measured using a GloMax Discover microplate reader (Promega, Madison, WI, USA) at an excitation wavelength of 485 nm and an emission wavelength of 520 nm to quantify the total number of labeled neutrophils before washing. The cells were then gently washed twice with HBSS to remove non-adherent cells. Subsequently, 100 µl of RPMI-1640 was added to each well for fluorescence re-measurement. Neutrophil adhesion was expressed as the percentage of adherent cells, calculated from the fluorescence intensity values obtained before and after washing, and normalized to the mean adherence observed in the positive control group.

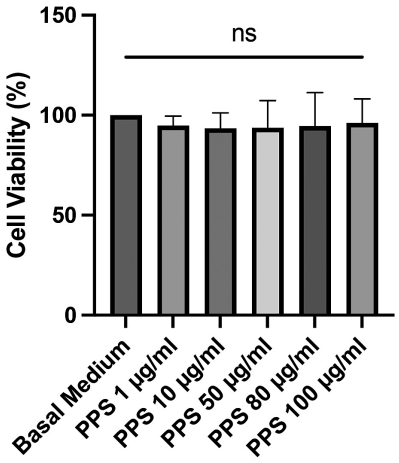

Fig. 1. Effect of PPS on the viability of CnAOEC.
CnAOEC were treated with various concentrations of PPS (1, 10, 50, 80, and 100 µg/ml) for 24 hr, and cell viability was assessed using a CCK-8 assay. Data are presented as the mean ± SD from three independent experiments and were analyzed using RM one-way ANOVA. No statistically significant differences were observed among PPS-treated groups and the control ($P > 0.05$).

Statistical analysis

All data were expressed as the mean ± standard deviation (SD). Statistical analyses

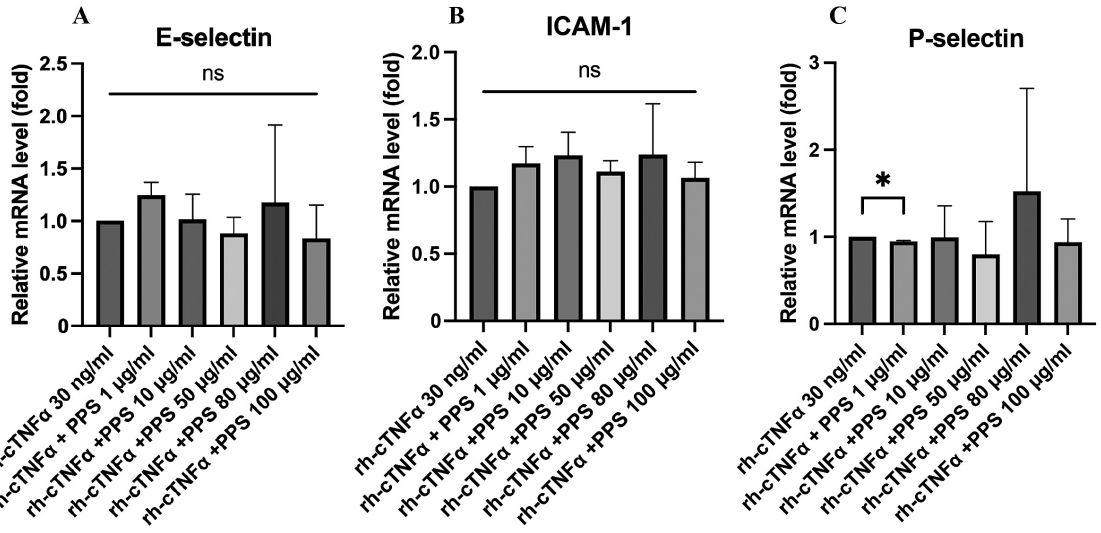

Fig. 2. Effects of PPS on adhesion molecule expression in rh-cTNFα-treated CnAOEC.
CnAOEC were subjected to different concentrations of PPS (1, 10, 50, 80, and 100 µg/ml) for 24 hr and subsequently stimulated with rh-cTNFα for 6 hr. The expression levels of (A) E-selectin, (B) ICAM-1, and (C) P-selectin were quantified by qPCR and normalized to the rh-cTNFα-treated positive control (set at 1.00). Bars represent mean ± SD (n = 3). RM one-way ANOVA revealed a significant decrease in P-selectin expression at 1 µg/ml PPS ($P < 0.05$).

were performed using GraphPad Prism version 10.0 (GraphPad Software, San Diego, CA, USA), with significance set at $P < 0.05$. Data normality was evaluated using the Shapiro–Wilk test. For normally distributed data, a repeated-measures (RM) one-way analysis of variance (ANOVA) followed by Tukey's post-hoc test was applied. When the normality assumption was not met, the Friedman test with Dunn's multiple-comparison test was used.

Results

Effect of PPS on the viability of CnAOEC.

To ensure that the observed effect of PPS on the target adhesion molecules was not confounded by cytotoxicity, CnAOEC viability was evaluated using CCK-8 assay after 24 hours of treatment with various PPS concentrations. The negative control group was normalized to 100% cell viability. Following treatment, CnAOEC viability remained high across all tested concentrations, showing 94.8 ± 4.71, 93.4 ± 7.69, 93.5 ± 13.7, 94.6 ± 16.8, and 96.2 ± 11.9 % cell viability for PPS 1, 10, 50, 80, and 100 µg/ml, respectively, as shown in Figure 1. A one-way ANOVA test revealed no statistically significant difference in cell viability among the control and all PPS-treated groups ($P = 0.974$). These results illustrate that PPS, at the concentrations and exposure duration used in this study, does not compromise the viability of CnAOEC.

Effect of PPS on the expression of endothelial adhesion gene in rh-cTNFα-stimulated CnAOEC

The expression of endothelial adhesion molecules, including E-selectin, ICAM-1, and P-selectin, in CnAOEC stimulated with rh-cTNFα was quantified by qPCR to determine the effect of PPS treatment. All expression levels were normalized to the corresponding positive control. Stimulation with rh-cTNFα induced a marked inflammatory response compared with the negative control, resulting in a 379 ± 95.4-fold increase in E-selectin, a 30.5 ± 4.33-fold increase in ICAM-1, and a 3.30 ± 0.32-fold increase in P-selectin mRNA expression ($P < 0.05$)

The relative mRNA expression of E-selectin was calculated as a fold change relative to the positive control (set at 1.00). Treatment with PPS at 1, 10, and 80 µg/ml resulted in slightly

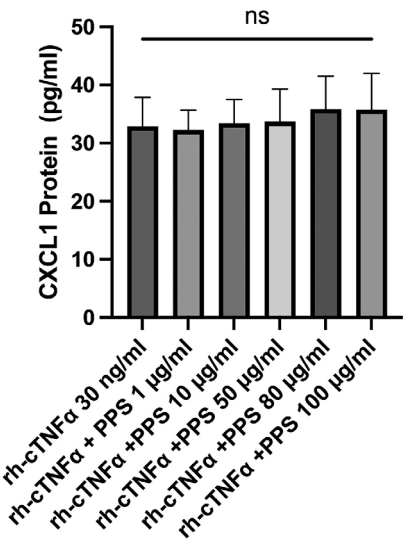

Fig. 3. Effect of PPS on CXCL1 protein expression in rh-cTNFα-stimulated CnAOEC.
CnAOEC were exposed to PPS (1, 10, 50, 80, and 100 µg/ml) for 24 hr, followed by stimulation with rh-cTNFα for 12 hr. The CXCL1 concentration in the culture supernatant was measured using a canine CXCL1 ELISA kit. Data were analyzed using the Friedman test with Dunn's post-hoc correction. Values shown are mean ± SD (n = 3).

increased fold changes of 1.25 ± 0.12, 1.02 ± 0.24, and 1.18 ± 0.74, respectively, whereas 50 µg/ml (0.88 ± 0.16) and 100 µg/ml (0.84 ± 0.32) showed mild downregulation. However, these differences were not statistically significant among the groups ($P = 0.505$) (Figure 2A).

The ICAM-1 mRNA expression was also quantified as a fold change relative to the positive control (set at 1.00). The mean fold changes for PPS at 1, 10, 50, 80, and 100 µg/ml were 1.17 ± 0.13, 1.23 ± 0.17, 1.11 ± 0.08, 1.24 ± 0.38, and 1.06 ± 0.12, respectively (Figure 2B). Despite minor fluctuations across concentrations, no statistically significant differences were observed among the groups ($P = 0.482$).

In contrast, P-selectin expression showed a general decreasing trend in most PPS-treated groups compared with the positive control (set at 1.00). The 1 µg/ml group demonstrated a visible reduction (0.95 ± 0.01), which was statistically significant ($P = 0.044$). Slight decreases were also observed at 10, 50, and 100 µg/ml, with fold changes of 0.99 ± 0.36, 0.80 ± 0.38, and 0.94 ± 0.27, respectively, although these differences were not significant. Whereas treatment with 80 µg/ml PPS resulted in a mild increase (1.52 ± 1.18), but this change was not statistically significant (Figure 2C).

Effect of PPS on CXCL1 protein expression in rh-cTNFα-induced CnAOEC

Following 24 hours of PPS pretreatment and subsequent rh-cTNFα stimulation for 12 hours, the CXCL1 concentration in the culture supernatant was quantified using a canine CXCL1 ELISA kit to evaluate the effect of PPS on CXCL1 protein expression. The mean CXCL1 concentration showed only slight variation among the PPS-treated groups, with values of 32.23 ± 3.45, 33.40 ± 4.13, 33.70 ± 5.57, 35.86 ± 5.65, and 35.78 ± 6.19 pg/ml for 1, 10, 50, 80, and 100 µg/ml, respectively, compared with 32.90 ± 4.96 pg/ml in the rh-cTNFα control group (Figure 3). Note that one group was not normally distributed. Consequently, data were analyzed using the Friedman test followed by Dunn's multiple comparisons test. The Friedman test indicated a statistically significant overall difference among the treatment conditions ($\chi^2 = 11.19$, $P = 0.018$). However, Dunn's post-hoc analysis revealed no significant pairwise differences in CXCL1 secretion ($P \geq 0.132$).

Effect of PPS on adhesion molecule gene expression in cytokine-induced Canine Neutrophils

The effect of PPS on the expression of neutrophil adhesion molecule genes, including CXCR2, PSGL-1, and L-selectin, was investigated using neutrophils isolated from three dogs. Each experimental condition was conducted in triplicate for every donor. Neutrophil viability under the incubation conditions used for the gene expression assays was evaluated by 7-AAD staining in parallel flow cytometry experiments performed under identical protocols. High membrane integrity was maintained across all treatments (90.6–91.6%), with no significant differences between groups ($P \geq 0.094$). Gene expression levels were normalized to the corresponding rh-cTNFα-stimulated control condition, which was set as 1.00 within each dog, and the mean values were used

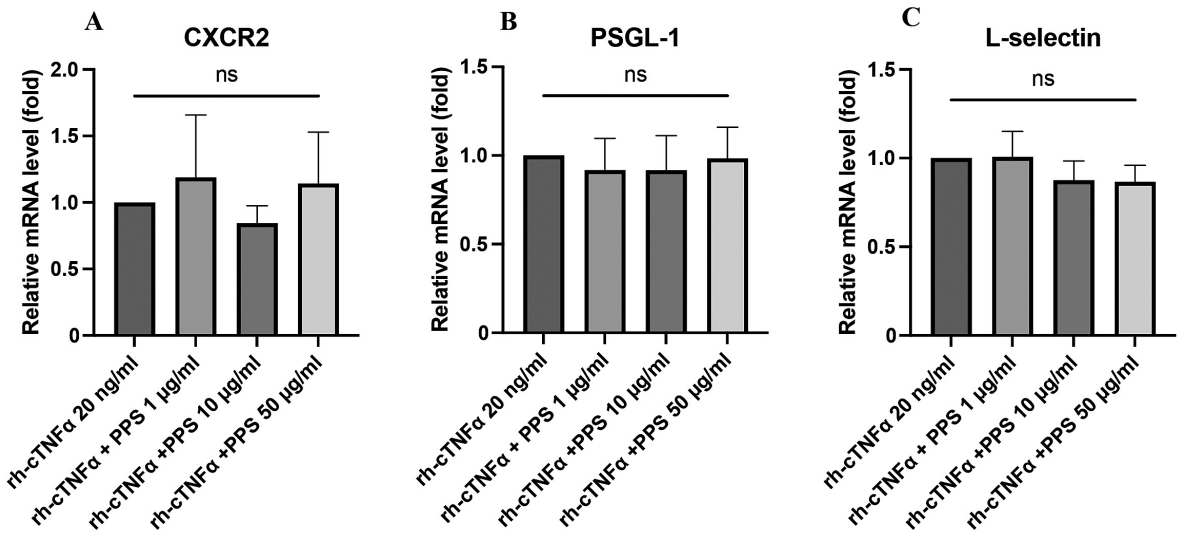

Fig. 4. Effect of PPS on neutrophil adhesion molecule gene expression in rh-cTNFα-stimulated canine neutrophils.

Neutrophils isolated from three clinically healthy dogs were treated with PPS at concentrations of 1, 10, and 50 μg/ml for 24 hr, followed by stimulation with rh-cTNFα for 1 hr. The relative mRNA expression levels of (A) CXCR2, (B) PSGL-1, and (C) L-selectin were quantified by qPCR and normalized to the rh-cTNFα-stimulated control (set as 1.00). Data are presented as mean ± SD (n = 3). Statistical analysis using RM one-way ANOVA showed no significant differences among treatment conditions ($P > 0.05$ for all genes)

for statistical analysis. As shown in Figure 4 A-C, PPS treatment produced only minor variations in the expression of the three genes. CXCR2 mRNA expression slightly increased in fold change at 1 μg/ml (1.19 ± 0.47) and 50 μg/ml (1.14 ± 0.39), whereas a small reduction was observed at 10 μg/ml (0.85 ± 0.13). However, these variations were not statistically significant ($P = 0.427$). For PSGL-1, mRNA expression was modestly lower across all PPS-treated groups, with fold-change values of 0.92 ± 0.18, 0.92 ± 0.19, and 0.98 ± 0.18 for 1, 10, and 50 μg/ml, respectively. These differences were also not statistically significant ($P = 0.605$). Meanwhile, L-selectin mRNA expression showed a gradual decrease with increasing PPS concentration, yielding fold-change values of 1.01 ± 0.14, 0.88 ± 0.11, and 0.87 ± 0.09 for 1, 10, and 50 μg/ml, respectively. Nevertheless, these variations did not reach statistical significance ($P = 0.154$). Collectively, these results indicate that PPS exposure did not significantly influence the expression of CXCR2, PSGL-1, or L-selectin in rh-cTNFα-stimulated canine neutrophils, although a slight tendency toward downregulation was observed.

Effect of PPS on LFA-1 expression on rh-cTNFα-stimulated canine neutrophils.

This study investigated the effect of PPS on the surface expression of CD11a, the α-subunit of LFA-1, in canine neutrophils. Neutrophils were obtained from three dogs, with three independent experiments performed per dog. The mean value for each dog was used for statistical analysis. MFI of CD11a is presented in Figure 5. The rh-cTNFα control group had an MFI of 2,775 ± 911, while the PPS-treated groups exhibited slightly lower values of 2,547 ± 782 at 1 μg/ml, 2,652 ± 867 at 10 μg/ml, and 2,538 ± 804 at 50 μg/ml. Despite this minor reduction, statistical analysis indicated no significant difference in CD11a surface expression among the experimental conditions ($P = 0.283$).

Effect of PPS on static neutrophil adherence to rh-cTNFα-induced CnAOEC

Given the observed downregulation of P-selectin on endothelial cells following treatment with PPS, the potential effect on neutrophil adhesion to rh-cTNFα-stimulated CnAOEC under static adhesion conditions was further investigated. CnAOEC at passages 4-9 were

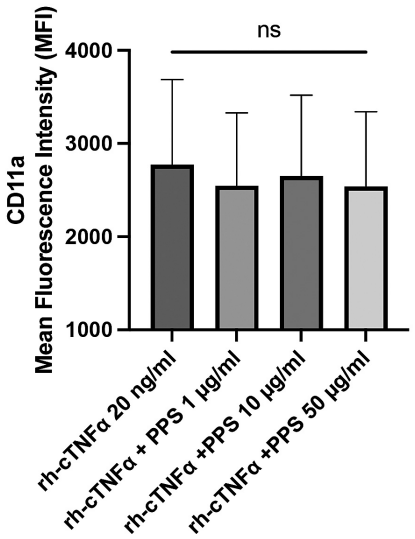

Fig. 5. Effect of PPS on LFA-1 expression on rh-cTNFα-stimulated canine neutrophils.

Canine neutrophils were pretreated with PPS at concentrations of 1, 10, and 50 μg/ml for 24 hr, subsequently stimulated with rh-cTNFα (20 ng/ml) for 1 hr. The expression of CD11a, the α-subunit of LFA-1, was evaluated by flow cytometry. Data are shown as mean ± SD (n = 3). Statistical analysis by RM one-way ANOVA found no significant difference among treatment groups ($P > 0.05$).

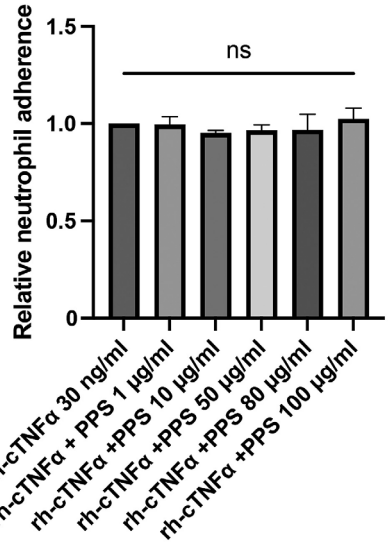

Fig. 6. Effect of PPS on static neutrophil adhesion to rh-cTNFα-stimulated CnAOEC.

CnAOEC were pretreated with PPS (1, 10, 50, 80, and 100 μg/ml) for 3 hr, followed by stimulation with rh-cTNFα (30 ng/ml) for 4 hr. Calcein-AM–labeled neutrophils (1×10^5 cells/well) were added and allowed to adhere for 20 min. Neutrophil adhesion was quantified as relative fluorescence intensity before and after washing. Data represent the mean ± SD from three dogs, each tested in three independent experiments, and the mean per dog was used for statistical analysis. Statistical analysis using the Friedman test with Dunn's post hoc correction showed no significant differences among treatment groups ($P > 0.05$).

used for this experiment. Calcein-AM–labeled neutrophils, isolated from three beagles, were applied to the endothelial cell monolayer following treatment. Three independent experiments were performed for each dog, and the mean per dog was used for statistical analysis.

The results indicated a marked increase in neutrophil adhesion to CnAOEC in the rh-cTNFα-challenged positive control groups compared to the negative control, showing a 5.53 ± 1.38-fold increase in adherence ($P < 0.05$). Furthermore, the effect of different concentrations of PPS on neutrophil adhesion was evaluated. Overall, there was no statistically significant difference in adhesion among the treatment groups ($P = 0.276$). The results are visible in Figure 6. The relative neutrophil adhesion mean values were 1.00 ± 0.04 (PPS 1 μg/ml), 0.95 ± 0.01 (PPS 10 μg/ml), 0.97 ± 0.03 (PPS 50 μg/ml), 0.97 ± 0.08 (PPS 80 μg/ml), 1.02 ± 0.06 (PPS 100 μg/ml) relative to the rh-cTNFα group set as 1.00. Data were analyzed using the Friedman test. The statistical analysis revealed no significant difference across all conditions ($P = 0.276$).

Discussion

This study aimed to investigate the effects of PPS on key adhesion molecules, including E-selectin, ICAM-1, P-selectin, CXCL1, CXCR2, PSGL-1, L-selectin, and LFA-1, and to examine dose-dependent responses in a canine in vitro model. The results confirmed that PPS treatment at 1-100 μg/ml for 24 hours was non-cytotoxic to CnAOEC, indicating good cellular tolerance under these treatments. This experimental setup aligns with previous in vitro studies, where PPS has been applied at 1-200 μg/ml. Reported exposure durations typically range from 6 to 72 hours across various cell types to evaluate toxicity, permeability, or anti-inflammatory effects[2,6,29,30]. Furthermore, PPS has been reported to modulate gene and protein expression in mesenchymal precursor cells within 24-48 hours of exposure, further supporting the chosen incubation time. Overall, these comparisons support that the

conditions used in this study are consistent with established experimental parameters known to balance potential biological activity with cellular safety. This aligns with previous findings that PPS generally maintains cell viability at therapeutic concentrations, with cytotoxicity usually associated with significantly higher concentrations, prolonged exposure, and elevated sulfate levels[1,19,30].

PPS is a low molecular weight heparin-like compound, known for its anticoagulant, anti-adhesive and anti-inflammatory properties[26]. Because of its structural similarity to heparin, it can competitively interfere with selectin-mediated cell adhesion by inhibiting the selectin family[26]. Heparin has been reported as an effective P-selectin and L-selectin inhibitor, mediating neutrophil tethering and rolling by PSGL-1, however, it was not effective against E-selectin in vitro and inhibited neutrophil accumulation in acute inflammation in vivo[18]. In addition, heparan sulfate on endothelial cells helps maintain local chemokine gradients, such as CXCL1, that guide neutrophil migration[21]. Considering these structural and functional similarities, this study supports the potential inhibitory effect of PPS on the targeted adhesion molecules.

In this study, PPS reduced endothelial P-selectin mRNA expression across the tested concentrations, with a significant difference observed at 1 μg/ml. This finding matches with previous evidence that PPS is recognized as an oral P-selectin blocking agent used to treat sickle cell disease[12]. PPS has also been reported to reduce myocardial infarction size by limiting neutrophil accumulation through suppression of terminal complement proteins (C5a and C5b-9)[10,11,28]. These are essential for mediating P-selectin expression by promoting rapid P-selectin translocation from the intracellular to the cell surface[22]. Interestingly, the inhibitory effect on P-selectin mRNA was significant at 1 μg /ml but diminished at higher concentrations. This non-linear response has been observed with other sulfated polysaccharides, in which biological activity often depends on specific charge densities and structural conformations rather than concentration alone9). At higher doses, excessive negative charge density may lead to non-specific electrostatic repulsion, interfering with the specific binding interactions required to modulate selectin expression. Thus, 1 μg/ml may represent the optimal therapeutic window for targeting endothelial P-selectin in this model.

E-selectin expression showed a slight, non-significant reduction at higher doses (50 and 100 μg/ml), suggesting a weaker regulatory effect compared with P-selectin. This finding is consistent with an earlier report showing that PPS mainly interferes with P-selectin-mediated cell rolling, while having no effect on E-selectin-dependent rolling[9]. On the other hand, ICAM-1 expression was slightly upregulated across PPS concentrations, indicating that PPS may not suppress the integrin-mediated firm adhesion phase. This observation differs from earlier reports in other cell types, showing that PPS decreased ICAM-1 and CXCL1 mRNA expression in TNFα-induced mice proximal tubular cells[32], and reduced soluble ICAM-1protein level in mice lung[11]. Although CXCL1 protein levels in this study varied only modestly and without statistical significance, the small decrease observed at 1 μg/ml may indicate a weak inhibitory effect of PPS. This observation is supported by a previous investigation demonstrating that PPS reduced CXCL1 cytokine levels in Chikungunya virus-infected mice, which was associated with reduced neutrophil infiltration[24].

In neutrophils, PPS exposure produced no statistically significant changes in the expression of adhesion-related molecules. Nevertheless, PSGL-1, L-selectin, and LFA-1 showed a consistent tendency toward lower expression across all PPS-treated groups. These results imply a mild suppression of molecules involved in the neutrophil tethering and rolling step, as the PSGL-1-L-selectin complex provides an essential signal for neutrophil rolling[27], and the change from low to intermediate affinity of LFA-1 also regulates this process8). In contrast, CXCR2 expression showed only minor variation, indicating that PPS may had little influence on chemokine-receptor–mediated activation. Together, these findings suggest that PPS has limited impact on neutrophil activation but may weakly modulate the selectin-

mediated rolling phase during the early stage of adhesion. This interpretation aligns with previous studies reporting that sulfated polysaccharides, including heparin and PPS, can mimic natural glycosaminoglycans and competitively interfere with selectin–ligand interactions, thereby reducing neutrophil tethering and rolling[9,18]. The lack of significant changes in the static adhesion assay supports this interpretation. This result is reasonable, since static conditions mainly represent the firm adhesion stage dominated by the LFA-1/ICAM-1 interaction, while the selectin-mediated rolling phase cannot be clearly reproduced. Therefore, the effect of PPS on selectin-dependent adhesion may be subtle and could appear more clearly under flow conditions that better reflect physiological situations.

However, several limitations of this study should be noted. All experiments were conducted under static in vitro conditions, which may not fully reflect the dynamic flow and complex cell interactions that occur in vivo. The number of donor dogs and the tested PPS concentrations were limited, which may affected variability and statistical power. Moreover, most analyses focused on gene and surface expression, while post-translational change and intracellular signaling were not examined in detail. In the adhesion assay, PPS exposure was mainly tested in endothelial cells, and the direct response of neutrophils was not separately evaluated. Future studies using a flow-based adhesion system and co-culture models under cytokine stimulation, combined with a larger sample size and protein-level analyses, are needed to confirm these findings and clarify how PPS modulates neutrophil-endothelial cell interaction.

Conflict of Interest

The authors declare that they have no conflicts of interest related to the design, execution, or interpretation of the study. None of the authors has any financial or personal relationships that could inappropriately influence or bias the content of this work.

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References

1) Akaraphutiporn E, Bwalya EC, Kim S, Sunaga T, Echigo R, Okumura M. Effects of pentosan polysulfate on cell proliferation, cell cycle progression and cyclin-dependent kinases expression in canine articular chondrocytes. J Vet Med Sci 82, 1209–1218, 2020. doi: 10.1292/jvms.20-0091.
2) Bwalya EC, Kim S, Fang J, Wijekoon HMS, Hosoya K, Okumura M. Pentosan polysulfate inhibits IL-1β-induced iNOS, c-Jun and HIF-1α upregulation in canine articular chondrocytes. PLoS One 12, e0177144, 2017. doi: 10.1371/journal.pone.0177144.
3) Cappenberg A, Kardell m, Zarbock A. Selectin-mediated signaling-Shedding light on the regulation of integrin activity in neutrophils. Cells 11, 1310, 2022. doi: 10.3390/cells11081310.
4) Chen L, Deng H, Cui H, Fang J, Zuo Z, Deng J, Li Y, Wang X, Zhao L. Inflammatory responses and inflammation-associated diseases in organs. Oncotarget 9, 7204–7218, 2018. doi: 10.18632/oncotarget.23208.
5) Degenhardt M, Ghosh P, Watzig H. Studies on the structural variations of pentosan polysulfate sodium (NaPPS) from different sources by capillary electrophoresis. Arch Pharm (Weinheim) 334, 27–29, 2001. doi: 10.1002/1521-4184(200101)334:1<27::aid-

ardp27>3.0.co;2-x.

6) Deli MA, Abrahám CS, Takahata H, Katamine S, Niwa M. Pentosan polysulfate regulates scavenger receptor-mediated, but not fluid-phase, endocytosis in immortalized cerebral endothelial cells. Cell Mol Neurobiol 20, 731–745, 2000. doi: 10.1023/a:1007007026500.
7) Ghosh P, Wu J, Shimmon S, Zannettino AC, Gronthos S, Itescu S. Pentosan polysulfate promotes proliferation and chondrogenic differentiation of adult human bone marrow-derived mesenchymal precursor cells. Arthritis Res Ther 12, R28, 2010. doi: 10.1186/ar2935.
8) Green CE, Schaff UY, Sarantos MR, Lum AF, Staunton DE, Simon SI. Dynamic shifts in LFA-1 affinity regulate neutrophil rolling, arrest, and transmigration on inflamed endothelium. Blood 107, 2101–2111, 2006. doi: 10.1182/blood-2005-06-2303.
9) Hopfner M, Alban S, Schumacher G, Rothe U, Bendas G. Selectin-blocking semisynthetic sulfated polysaccharides as promising anti-inflammatory agents. J Pharm Pharmacol 55, 697–706, 2003. doi: 10.1211/002235703765344621.
10) Kilgore KS, Naylor KB, Tanhehco EJ, Park JL, Booth EA, Washington RA, Lucchesi BR. The semisynthetic polysaccharide pentosan polysulfate prevents complement-mediated myocardial injury in the rabbit perfused heart. J Pharmacol Exp Ther 285, 987–994, 1998. doi: 10.1124/jpet.98.1.987.
11) Krishnan R, Stapledon CJM, Mostafavi H, Freitas JR, Liu X, Mahalingam S, Zaid A. Anti-inflammatory actions of Pentosan polysulfate sodium in a mouse model of influenza virus A/PR8/34-induced pulmonary inflammation. Front Immunol 14, 1030879, 2023. doi: 10.3389/fimmu.2023.1030879.
12) Kutlar A, Ataga KI, McMahon L, Howard J, Galacteros F, Hagar W, Vichinsky E, Cheung AT, Matsui N, Embury SH. A potent oral P-selectin blocking agent improves microcirculatory blood flow and a marker of endothelial cell injury in patients with sickle cell disease. Am J Hematol 87, 536–539, 2012. doi: 10.1002/ajh.23147.
13) Ley K, Laudanna C, Cybulsky MI, Nourshargh S. Getting to the site of inflammation: the leukocyte adhesion cascade updated. Nat Rev Immunol 7, 678–689, 2007. doi: 10.1038/nri2156.
14) Li Y, Imaizumi T, Matsumiya T, Seya K, Kawaguchi S, Ding J, Ohkuma H. Polyinosinic-polycytidylic acid induces CXCL1 expression in cultured hCMEC/D3 human cerebral microvascular endothelial cells. Neuroimmunomodulation 27, 38–47, 2020. doi: 10.1159/000506482.
15) Margraf A, Lowell CA, Zarbock A. Neutrophils in acute inflammation: current concepts and translational implications. Blood 139, 2130–2144, 2022. doi: 10.1182/blood.2021012295.
16) Medzhitov R. The spectrum of inflammatory responses. Science 374, 1070–1075, 2021. doi: 10.1126/science.abi5200.
17) Mortaz E, Alipoor SD, Adcock IM, Mumby S, Koenderman L. Update on neutrophil function in severe inflammation. Front Immunol 9, 2171, 2018. doi: 10.3389/fimmu.2018.02171.
18) Nelson RM, Cecconi O, Roberts WG, Aruffo A, Linhardt RJ, Bevilacqua MP. Heparin oligosaccharides bind L- and P-selectin and inhibit acute inflammation. Blood 82, 3253–3258, 1993. doi: 10.1182/blood.V82.11.3253.3253.
19) Ntentakis D, Correa V, Narimatsu T, Glavas P, Vavvas D, Efstathiou N. Effects of pentosan polysulfate sodium on retinal cells viability and RPE regeneration. Investigative Ophthalmology and visual science 64, 2996, 2023. doi: 10.1167/iovs.64.9.2996.
20) Okanishi H, Kagawa Y, Watari T. Expression of selectins and P-selectin glycoprotein ligand-1 in dogs with lymphocytic-plasmacytic enteritis. Vet Immunol Immunopathol 161, 42–48, 2014. doi: 10.1016/j.vetimm.2014.06.005.
21) Phillipson M, Kubes P. The neutrophil in vascular inflammation. Nat Med 17, 1381–1390, 2011. doi: 10.1038/nm.2514.
22) Rinder CS, Rinder HM, Smith BR, Fitch JC, Smith MJ, Tracey JB, Matis LA, Squinto

SP, Rollins SA. Blockade of C5a and C5b-9 generation inhibits leukocyte and platelet activation during extracorporeal circulation. J Clin Invest 96, 1564–1572, 1995. doi: 10.1172/JCI118195.

23) Rosales C. Neutrophil: A Cell with Many Roles in Inflammation or Several Cell Types? Front Physiol 9, 113, 2018. doi: 10.3389/fphys.2018.00113.
24) Rudd PA, Lim EXY, Stapledon CJM, Krishnan R, Herrero LJ. Pentosan polysulfate sodium prevents functional decline in chikungunya infected mice by modulating growth factor signalling and lymphocyte activation. PLoS One 16, e0255125, 2021. doi: 10.1371/journal.pone.0255125.
25) Sherwood ER, Toliver-Kinsky T. Mechanisms of the inflammatory response. Best Pract Res Clin Anaesthesiol 18, 385–405, 2004. doi: 10.1016/j.bpa.2003.12.002.
26) Smith MM, Melrose J. Pentosan polysulfate affords pleotropic protection to multiple cells and tissues. Pharmaceuticals (Basel) 16, 437, 2023. doi: 10.3390/ph16030437.
27) Stadtmann A, Germena G, Block H, Boras M, Rossaint J, Sundd P, Lefort C, Fisher CI, Buscher K, Gelschefarth B, Urzainqui A, Gerke V, Ley K, Zarbock A. The PSGL-1-L-selectin signaling complex regulates neutrophil adhesion under flow. J Exp Med 210, 2171–2180, 2013. doi: 10.1084/jem.20130664.
28) Tanhehco EJ, Kilgore KS, Naylor KB, Park JL, Booth EA, Lucchesi BR. Reduction of myocardial infarct size after ischemia and reperfusion by the glycosaminoglycan pentosan polysulfate. J Cardiovasc Pharmacol 34, 153–161, 1999. doi: 10.1097/00005344-199907000-00024.
29) Veszelka S, Pasztoi M, Farkas AE, Krizbai I, Ngo TK, Niwa M, Abraham CS, Deli MA. Pentosan polysulfate protects brain endothelial cells against bacterial lipopolysaccharide-induced damages. Neurochem Int 50, 219–228, 2007. doi: 10.1016/j.neuint.2006.08.006.
30) Wang YL, Mwale C, Akaraphutiporn E, Kim S, Sunaga T, Okumura M. Sulfate level relied effects of pentosan polysulfate sodium on inhibiting the proliferation of canine articular chondrocytes by targeting PI3K/Akt pathway. Japanese Journal of Veterinary Research 71, 35–47, 2023. doi: 10.57494/jjvr.71.2_35.
31) Wilhelmsen K, Farrar K, Hellman J. Quantitative in vitro assay to measure neutrophil adhesion to activated primary human microvascular endothelial cells under static conditions. J Vis Exp, e50677, 2013. doi: 10.3791/50677.
32) Wu J, Guan TJ, Zheng S, Grosjean F, Liu W, Xiong H, Gordon R, Vlassara H, Striker GE, Zheng F. Inhibition of inflammation by pentosan polysulfate impedes the development and progression of severe diabetic nephropathy in aging C57B6 mice. Lab Invest 91, 1459–1471, 2011. doi: 10.1038/labinvest.2011.93.
33) Yang L, Shi F, Cao F, Wang L, She J, He B, Xu X, Kong L, Cai B. Neutrophils in tissue injury and repair: Molecular mechanisms and therapeutic targets. MedComm (2020) 6, e70184, 2025. doi: 10.1002/mco2.70184.