Veterinary Immunology and Immunopathology 211 (2019) 49–57

Contents lists available at ScienceDirect

Veterinary Immunology and Immunopathology

journal homepage: www.elsevier.com/locate/vetimm

Research paper

Procoagulant activity of bovine neutrophils incubated with conditioned media or extracellular vesicles from *Histophilus somni* stimulated bovine brain endothelial cells

José J. Rivera Rivas*, Charles J. Czuprynski

Department of Pathobiological Sciences, School of Veterinary Medicine, University of Wisconsin-Madison, 2015 Linden Drive, Madison, WI, 53706, USA

ARTICLE INFO

Keywords:
H. somni
Neutrophils
Extracellular vesicles
Pro-coagulant activity
Endotoxin
Endothelial cells
Thrombotic meningoencephalitis

ABSTRACT

Histophilus somni is a Gram negative coccobacillus that causes respiratory, reproductive and central nervous system disease in cattle. The hallmark of *H. somni* infection is diffuse vasculitis and intravascular thrombosis that can lead to an acute central nervous system disease known as thrombotic meningoencephalitis (TME). Because neutrophils are major players in the pathophysiology of septic meningitis, we sought to determine their role in *H. somni*-induced fibrin clot formation *in vitro*. Bovine brain endothelial cells (TBBE cells) were exposed to *H. somni* cells at a 1:25 ratio, respectively. Conditioned media (CM) were collected after a 6 h incubation at 37 °C with 5% CO_2, and then incubated with bovine peripheral blood polymorphonuclear neutrophils (PMNs). Following incubation, fibrin clot formation and tissue factor activity were assessed by a re-calcified plasma clotting assay. We found greater tissue factor activity in cell lysates and CM from *H. somni*-stimulated TBBE cells than unstimulated control TBBE cells. In addition, PMNs exposed to CM or extracellular vesicles from *H. somni*-stimulated TBBE cells expressed von Willenbrand factor, exhibited increased fibrin clot formation, and displayed greater tissue factor activity than PMNs exposed to CM or extracellular vesicles from unstimulated control TBBE cells. These results suggest that bovine PMNs might acquire extracellular vesicles from endothelial cells that leads to thrombus formation in bovine brain microvasculature and contribute to the process that characterizes TME.

1. Introduction

Histophilus somni is a versatile pathogen of cattle and other ruminant species that causes multiple clinical syndromes, including respiratory disease, abortion, arthritis, myocarditis, septicemia and an acute neurological disease known as thrombotic meningoencephalitis (TME) (Corbeil, 2007; Pérez et al., 2010). The latter is characterized by thrombotic vasculitis, fibrinopurulent meningitis and hemorrhage throughout the central nervous system (CNS). Vasculitis and thrombus formation are not confined to small vessels of the CNS; the lungs, gastrointestinal tract and kidneys can also be affected (MacDonald et al., 1973). Interruption of blood supply to the affected organs leads to infarction, necrosis and loss of function, or in the case of TME, a fulminant and rapidly fatal infection.

Vasculitis and thrombus formation are the hallmark of *H. somni* infection (Little, 1986). The mechanisms underlying coagulation dysregulation include tissue factor-mediated thrombin generation and imbalances in the normal physiologic anticoagulant mechanisms, such as antithrombin and protein C. Prior studies demonstrated that *H. somni*-activated bovine brain microvascular endothelial cells exhibit increased expression of pro-inflammatory cytokines, and tissue factor, and reduced expression of anti-coagulation molecules like thrombomodulin and activated protein C (Behling-Kelly et al., 2007). In addition, *H. somni* activated bovine platelets increased their expression of P-selectin and, when incubated with endothelial cells, stimulated the latter's expression of E-selectin and tissue factor (Kuckleburg et al., 2008). Tissue factor, in the presence of calcium and negatively charged phospholipids, forms a complex with factor VIIa that catalyzes sequential activation of factor IX and factor X (Bertina, 2009). The complex of factor Va, calcium and factor Xa, then catalyzes conversion of prothrombin to thrombin, thereby leading to fibrin formation, platelet activation, and ultimately generation of a thrombus.

The possible contributions of bovine neutrophils to *H. somni*-induced vasculitis and thrombus formation have not been investigated. Large number of neutrophils and fibrin thrombi are present in blood vessels of the meninges and brain parenchyma of animals suffering from TME (Little, 1986; MacDonald et al., 1973). Research in other biological systems indicate that neutrophils play a deleterious role in

* Corresponding author.
E-mail address: jjrivera@wisc.edu (J.J. Rivera Rivas).

https://doi.org/10.1016/j.vetimm.2019.03.009
Received 7 January 2018; Received in revised form 6 January 2019; Accepted 23 March 2019

the pathophysiology of deep venous thrombosis, sepsis and stroke (Doring et al., 2013; Fuchs et al., 2012). Peripheral blood polymorphonuclear neutrophils (PMNs) are the first inflammatory leukocytes recruited to sites of infection, and potentiate injury by secreting histones and inflammatory enzymes such as myeloperoxidase, elastase and cathepsin G (Kaplan and Radic, 2012). Proteolytic enzymes can degrade and inactivate anti-coagulant proteins like anti-thrombin, thrombomodulin, protein C and tissue factor pathway inhibitor producing a pro-coagulant microenvironment (Goel, 2003; Higuchi et al., 1992; Massberg et al., 2010; Nogami et al., 2011). Although these events are essential for host defense against microbial dissemination, they can result in thrombus formation, ischemia and tissue damage (Massberg et al., 2010).

In the present study, we examined bovine PMN procoagulant activity in response to endothelial cells that were exposed to *H. somni*. We observed increased pro-coagulant activity (PCA) by neutrophils and bovine brain endothelial cells (TBBE cells) incubated with conditioned medium (CM) from TBBE cells previously incubated with *H. somni*. PMNs exposed to CM from TBBE cells incubated with *H. somni* exhibited increased fibrin clot formation and tissue factor activity compared to PMNs incubated with CM from unstimulated control TBBE cells. In addition, we observed greater tissue factor activity in cell lysates and CM from *H. somni* stimulated TBBE cells than unstimulated control TBBE cells. Our results suggest that bovine PMNs might amplify thrombus formation in bovine brain microvasculature and contribute to the process that characterizes TME.

2. Materials and methods

2.1. Cell culture

Simian virus 40 large T-antigen transformed bovine brain endothelial (TBBE) cells used in this study have been described previously (Behling-Kelly et al., 2007). Cells were cultured in RPMI-1640 (Cellgro; Mediatech, Inc., Herndon, VA) supplemented with 10% heat inactivated fetal bovine serum (FBS) (Atlanta Biologicals, Lawrenceville, GA), 2 mM L-glutamine (Mediatech), 1% penicillin and streptomycin (Cellgro), and passaged by brief enzymatic digestion using 0.1% trypsin EDTA. Cells used in the study were less than 50 passages.

H. somni strain 649, an abortion isolated generously provided by Dr. Lynette Corbeil (Zekarias et al., 2011), was grown as previously described (Behling-Kelly et al., 2007). Briefly, a frozen aliquot of stationary-phase *H. somni* cells was thawed and inoculated into brain-heart infusion (BHI) broth (DifCo; BD Franklin Lakes, NJ) supplemented with 0.5% yeast extract (DifCo; BD Franklin Lakes, NJ) and 0.01% thiamine monophosphate (TMP) (Sigma-Aldrich; St. Louis, MO). The bacteria were cultured without shaking for 16 h at 37 °C with 5% CO_2. Prior to inoculation, bacterial cells were washed 3x with sterile PBS and suspended in phenol red free RPMI-1640 with 10% heat inactivated FBS. Optical density (600 nm) of the bacterial suspension was measured and the number of bacterial cells extrapolated from growth curves performed in our laboratory. The number of viable *H. somni* was confirmed in each experiment by dilution and plating on tryptic soy agar supplemented with 5% sheep red blood cells (RBCs; Becton Dickson).

2.2. Isolation of bovine PMNs

Adult cows from the University of Wisconsin-Madison Dairy Cattle Center were used as used as blood donors as approved by the University of Wisconsin-Madison Institutional Animal Care and Use Committee. At least eight adult cows were available as blood donors any single time. PMNs were isolated from healthy donor Holstein cows by coccygeal venipuncture as described previously (Rivera-Rivas et al., 2009). Peripheral blood was collected in syringes containing 0.36% (vol/vol final concentration) sodium citrate as anticoagulant. Whole blood was centrifuged at 2000 x *g* for 15 min in 50 ml polypropylene conical tubes (Corning Inc., Corning, NY). The blood plasma and buffy coat were carefully removed by gentle aspiration. The remaining cell mixture was incubated with hypotonic lysis solution (8ug/ml NH_4Cl and 1 µg/ml Tris in dH_2O, pH 7.5) for 15 min at room temperature to eliminate red blood cells. Bovine PMNs were harvested from the mixture by centrifugation at 500 x *g* for 5 min. Cells were washed 5 times with Ca^{2+}, Mg^{2+} and phenol red-free Hanks Balanced Salt Solution (HBSS) (Cellgro; Mediatech, Inc., Herndon, VA) and suspended in RPMI 10% FBS. Cell suspensions consisted of greater than 95% PMNs, as determined by differential cell count, and were greater than 95% viable, as judged by trypan blue dye exclusion.

2.3. TBBE conditioned medium

TBBE cells grown in six-well plates were incubated with *H. somni* 649 at a multiplicity of infection (MOI) of 25:1. Plates were centrifuged at 250 x *g* for 10 min and incubated for 1 h at 37 °C with 5% CO_2. Monolayers were washed with warm Ca^{2+} and Mg^{2+} free HBSS and then overlaid with phenol red-free RPMI-1640 with 10% heat inactivated FBS. After 6 h of incubation, CM from *H. somni* infected TBBE cells and control TBBE cells were harvested, centrifuged at 500 x *g* for 15 min 4 °C, filtered through 0.22 µm, aliquoted and stored at −70 °C

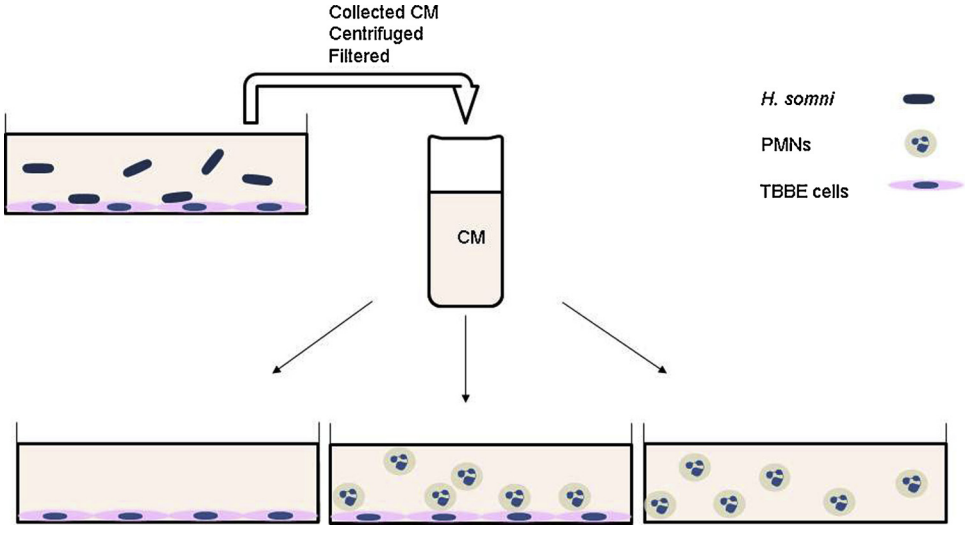

Fig. 1. Experimental design: Conditioned media (CM) collection and treatment of TBBE cells and PMNs. TBBE cells were incubated with or without *H. somni* cells at 37 °C with 5% CO_2. Conditioned media (CM) were collected, centrifuged and filtered to remove cell debris. CM was then added to 96 well plates containing TBBE cells alone, PMNs alone, or TBBE cells and PMNs incubated together. Plates were incubated for 6 h at 37 °C with 30 rpm rotation. Cells were washed 3x with Ca^{2+} and Mg^{2+} free HBSS and suspended in 50 µl phenol red-free Ca^{2+} and Mg^{2+} free HBSS. Cells were lysed with 3x rapid freeze-thaw cycles, and 50 µl of fresh citrated bovine plasma added per well. The plasma was re-calcified with warm $CaCl_2$ to a final concentration of 8.3 mM, and fibrin clot formation determined at 37 °C by measuring absorbance at 595 nm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

(Fig. 1). To confirm removal of viable *H. somni* cells, the conditioned medium was plated on tryptic soy agar supplemented with 5% sheep red blood cells (detection limit < 10 CFU/ml). Alternatively, TBBE monolayers were washed 3x with Ca^{2+} and Mg^{2+} free PBS, and suspended in 50 µl phenol red-free HBSS without Ca^{2+} and Mg^{2+}. Cells were lysed with 3x rapid freeze-thaw cycles and the lysates stored at −80 °C until tissue factor activity assay was performed.

2.4. PMN adhesion to TBBE cells

2.4.1. TBBE cells stimulated with H. somni

Various numbers of *H. somni* cells (0.1–10 multiplicity of infection) were added to TBBE cells in a 96 well plate. Plates were centrifuged at 250 x *g* for 10 min at room temperature to allow bacteria-endothelial cell contact, and then incubated for 60 min at 37 °C with 5% CO_2. Monolayers were washed 3x with HBSS, and then 100 µl of RPMI-10% FBS added and the plates incubated for another 2 h at 37 °C. Bovine PMNs (5×10^5) in a total volume of 200 µl RPMI-10% FBS then were added to the wells and incubated for 30 min at 37 °C with 5% CO_2. The monolayers then were washed 3x with PBS-2% BSA, and lysed in 50 µl of citrate buffer (pH 5.5) with 0.9% Triton X-100.

PMN attachment was estimated by measuring myeloperoxidase activity (MPO) in the cell lysates (Arfors et al., 1987; Cooray, 1994). An equal volume of the MPO substrate 3,30,5,50-tetramethylbenzidine (TMB Substrate; Pierce Rockford, IL) was added to each well and the plate incubated for 35 min at room temperature in the dark. The reaction was stopped by adding 50 µl of 0.18 M H_2SO_4 (Fisher Scientific). Absorbance was measured at 450 nm using a micro-plate reader (DTX 880; Beckman Coulter, Fullerton, CA). MPO activity of the samples was extrapolated from a standard curve generated by serially diluting known amounts of human MPO (EMD Biosciences, Inc., San Diego, CA) as described previously (Arfors et al., 1987; Rivera-Rivas et al., 2009).The percentage of adherent PMNs was calculated as follows: (mU/ml of sample) / (total mU/ml) x 100, where total mU/ml represents the MPO activity for lysates of the total number of PMNs added to the wells.

2.4.2. TBBE cells stimulated with CM

TBBE cells (1×10^5) were cultured in 96-well plates, washed 2x with HBSS and overlaid with 100 µl of conditioned medium (CM) from TBBE cells that had been incubated with *H. somni,* or control TBBE cells. In some experiments, *Salmonella typhosa* lipopolysaccharide (LPS, 1 µg/ml) (Sigma-Aldrich; St. Louis, MO) was used to stimulate TBBE cell monolayers. To diminish the potential effects of *H. somni* endotoxin, polymixin B (10 µg/ml) was pre-incubated for 30 min with TBBE cell CM prior to being added to cell cultures. Bovine PMNs (5×10^5) were added to the wells in a total volume of 200 µl RPMI-10% FBS and incubated for 3 h at 37 °C with 5% CO_2. The monolayers were washed 3x with PBS with 2% BSA (w/v) and lysed by addition of 0.9% Triton X-100 in 50 µl of citrate buffer (pH 5.5). MPO activity in the cell lysates was determined immediately as a marker of neutrophil adherence to TBEE cells as described above.

2.5. Fibrin clot formation by co-cultured TBBE cells and PMNs

TBBE cells (1×10^5) were seeded in flat bottom 96 well plates, with or without bovine PMNs (5×10^5), in 200 µL of phenol red-free RPMI with 10% FBS. Conditioned media from control or *H. somni* exposed TBBE cells were added to the monolayers at 50% final concentration (v \v). Some of the CM received polymixin B at 10 µg/ml before being added to the cells. The TBBE cells then were incubated for 6–24 h at 37 °C while being gently rotated at 30 rpm. Plates were then centrifuged at 250 x *g* for 5 min at room temperature. The supernatants were discarded, the cells washed 3x with Ca^{2+} and Mg^{2+} free PBS, and 50 µl phenol red-free Ca^{2+} and Mg^{2+} free HBSS was added. Cells were then lysed with 3x rapid freeze-thaw cycles and the lysates subjected to analysis by a fibrin deposition assay (Fig. 1).

2.6. Fibrin deposition assay

TBBE cells were washed three times with Ca^{2+} and Mg^{2+} free PBS and resuspended in 50 µL of warm Ca^{2+} and Mg^{2+} free HBSS without phenol red. In some experiments TBBE cells were lysed and 50 µL of cell lysate was used as the sample. In rapid succession, 50 µl of warm platelet poor plasma, followed by 50 µL of warm 25 mM $CaCl_2$ were added to each well. Fibrin clot formation was determined by measuring absorbance at 595 nm every 30 s using a DTX880 plate reader (Beckman Coulter) at 37 °C. The difference of absorbance was expressed as change absorbance or fold change.

2.7. Tissue factor activity assay

Fifty microliters of TBBE cell lysate, TBBE cell conditioned medium or bovine PMNs (5×10^5) in 50 µl of phenol red-free Ca^{2+} and Mg^{2+} free HBSS were added to round bottom wells in a 96 well plate. Platelet poor plasma and 50 µL of 25 mM $CaCl_2$ containing 1 mM of chromogenic factor Xa substrate S2375 (DiaPharma; West Chester, OH) were warmed at 37 °C and added to the wells in rapid succession. The reaction was incubated at 37 °C and the change in absorbance (405 nm) of the para-nitroaniline substrate was determined using a DTX880 plate reader.

2.8. TBBE extracellular vesicle binding to bovine PMNs

TBBE cells (2×10^6) were plated in a six well tissue culture plate and labeled with a red fluorescent cell linker (PKH26; Sigma-Aldrich), according to the manufacturer's instructions. *H. somni* 649 LOS (1 µg/mL), or *H. somni* cells at a multiplicity of infection (MOI) of 25:1, were added to the wells. Plates were centrifuged at 250 x *g* for 5 min at room temperature and then incubated for 1 h at 37 °C with 5% CO_2. Monolayers were washed with warm Ca^{2+} and Mg^{2+} free HBSS and then overlaid with phenol red free RPMI-1640 with 10% heat inactivated fetal bovine serum. After a 6 h incubation, CM from *H. somni* infected TBBE cells and uninfected control TBBE cells were harvested, and centrifuged at 500 x *g* for 5 min to remove cells and debris. Supernatants were collected and centrifuged at 10,000 x *g* for 10 min at 4 °C. The pellets were discarded and the supernatants collected and centrifuged at 21,000 x *g* for 15 min at 4 °C to obtain the extracellular vesicle fractions. In some experiments these were then stained with Annexin V (Invitrogen) following the manufacturer's protocol. Following that the vesicles were washed and stained overnight in the dark with FITC labelled anti-bovine von Willebrand Factor antibody (Serotec) at 4 °C. Microvesicles were washed with FACS buffer (PBS 10% FBS 0.1% Sodium Azide) and suspended in 500 µl of RPMI-10% FBS that was passed through a 0.22 µm filter.

Bovine PMNs (1×10^6) were plated on poly-lysine (Electron Microscopy Science, Hatfield, PA) coated glass coverslips or flat bottom 48 well plates and incubated with TBBE cell extracellular vesicles for 3 h at 37 °C with 5% CO_2. Cells were washed 3x, fixed in the dark for 10 min with 4% paraformaldehyde in ice cold PBS, and washed 2x with PBS. Coverslips were mounted on glass slides with ProLong Gold with DAPI (Thermo Fisher Scientific) and examined by confocal laser scanning microscopy (Leica TCS SP8 microscope; Leica Microsystems Inc., Buffalo Grove, IL). Alternatively, at least 10,000 single cells, defined by forward scatter height vs forward scatter area density plot, were analyzed with an LSR Fortessa flow cytometer (BD Biosciences). Unstained bovine PMNs were used for gating PKH26 positive and negative cells.

2.9. Statistical analysis

Data were analyzed for statistical significance by a one-way analysis of variance, followed by the Tukey post-test, using the Instat software

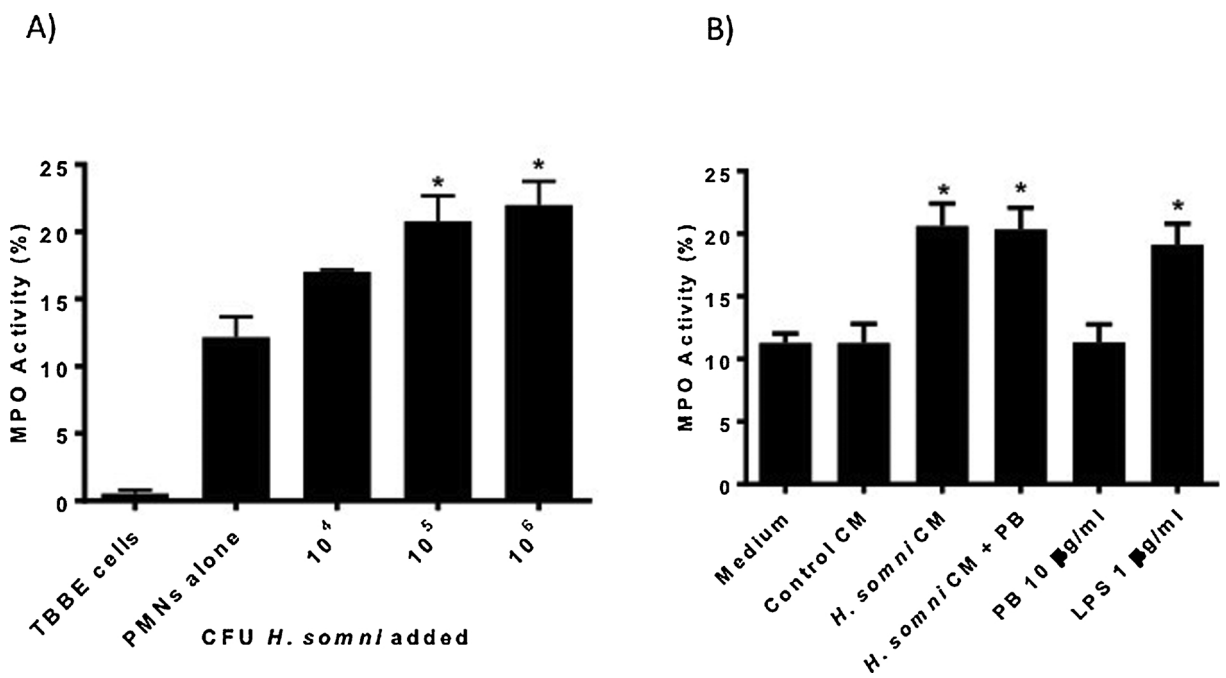

Fig. 2. Bovine PMNs adhere to *H. somni* stimulated TBBE cells. A) TBBE cells (1×10^5) were grown to confluence in 96 well plates, washed with HBSS and various numbers of *H. somni* cells ($10^4 - 10^6$ CFU) added. Plates were centrifuged at 250 x *g* for 5 min to allow bacteria-TBBE cell contact, and incubated for 1 h at 37 °C with 5% CO_2. Monolayers were washed 3x with HBSS, and overlaid with 100 µl of RPMI-10% FBS. After a 2 h incubation at 37 °C, bovine PMNs were added to the TBBE cells at a 5:1 ratio and incubated for 30 min. Monolayers were then washed 3x with HBSS and PMN adhesion estimated by measuring myeloperoxidase (MPO) activity. B) TBBE cell monolayers were prepared in a 96 well plate, washed with HBSS and incubated for 3 h at 37 °C with conditioned medium (CM) from *H. somni* stimulated TBBE cells. Polymixin B (PB) was added to some CM to neutralize LOS. PMNs were added to TBBE cells at a 5:1 ratio and the cells incubated for 30 min. Monolayers were washed 3x with HBSS and PMN adhesion estimated by measuring MPO activity. In both panels % PMN adhesion was estimated by expressing MPO activity as a percentage of total MPO activity for the PMNs added. Data represent the mean ± SEM for three independent experiments, *p < 0.05 compared to PMNs alone (A) or PMNs incubated with control CM (B).

program (GraphPad, San Diego, CA).

3. Results

3.1. Bovine PMNs adhere to H. somni stimulated TBBE cells

Activated endothelial cells transform from a non-thrombogenic to a pro-coagulant surface that promotes leukocyte adhesion, platelet aggregation and clot formation (Harding and Kubes, 2012; Levi, 2010). Because neutrophil infiltration and perivascular cuffing are prominent in brain blood vessels during *H. somni*-induced TME, we first assessed neutrophil adherence to *H. somni* stimulated endothelial cells. TBBE cells were inoculated with *H. somni* at a MOI ranging from 0.1–10, and PMN adhesion evaluated. Exposure of TBBE cells to *H. somni* at a 1:1 or 10:1 multiplicity of infection significantly increased ($P < .05$) bovine PMN adhesion to TBBE cells (Fig. 2A).

We next asked whether increased binding of bPMNs to TBBE cells was dependent on lipooligosaccharide (LOS) in the CM. Conditioned media from TBBE cells exposed to *H. somni* were added to bovine PMNs alone or PMNs co-cultured with TBBE cells. We observed a two-fold increase in adherence of bPMNs to TBBE cells when the co-cultured cells were exposed to CM from *H. somni* stimulated TBBE cells (Fig. 2B). PMN adherence was not significantly reduced when polymixin B was added, suggesting it was not due to *H. somni* LOS present in the CM.

3.2. Conditioned medium (CM) from H. somni stimulated TBBE cells increases fibrin clot formation by co-cultured TBBE cells and PMNs

We previously reported that *H. somni* stimulates pro-inflammatory cytokine release by TBBE cells (Behling-Kelly et al., 2007). We were interested in whether incubation with *H. somni* stimulates TBBE cells to release mediators into the CM that in turn cause pro-coagulant changes in endothelial cells and bovine PMNs. TBBE cells were incubated with *H. somni* for 6 h, and the conditioned media collected, centrifuged and filtered to remove *H. somni* and TBBE cells. Conditioned media from *H. somni* stimulated or control TBBE cells were then added to a new monolayer of TBBE cells, bovine PMNs, or bovine PMNs incubated with TBBE cells (Fig. 1). Fibrin clot formation was assessed by a one-step fibrin deposition assay. As shown in Fig. 3A, CM from *H. somni*-stimulated TBBE cells increased PMN pro-coagulant activity whether or not TBBE cells were present. This effect was unlikely due to *H. somni* lipooligosaccharide (*i.e.* endotoxin) because adding polymixin B to the CM, before the latter was added to the PMNs, did not significantly affect pro-coagulative activity (Fig. 3B). Nor did incubation of PMNs with *S. typhosa* LPS (1 µg/ml) alone result in the same pro-coagulative changes. We next examined whether pro-coagulant activity was dependent on CM concentration. Bovine PMNs were exposed to various concentrations of TBBE cell CM for six hours and pro-coagulant activity quantified by a one-step fibrin clot formation. As shown in Fig. 3C, bPMN pro-coagulant activity was dependent on the CM concentration added.

3.3. H. somni increases tissue factor activity in TBBE cell lysates and in their conditioned medium

We next sought to determine how long TBBE must be incubated with CM to increase pro-coagulant activity. In contrast to the lack of an effect at 6 h incubation (Fig. 3A), TBBE cells incubated for 24 h with CM from *H. somni* stimulated TBBE cells became pro-coagulant as compared to TBBE cells incubated with control CM (Fig. 4A). Once again this response was not likely due to LOS present in the CM, because adding polymixin B had little effect.

Previous work from our laboratory reported that pro-coagulant activity of TBBE cells exposed to *H. somni* was associated with greater tissue factor expression and activity. Tissue factor can be expressed on

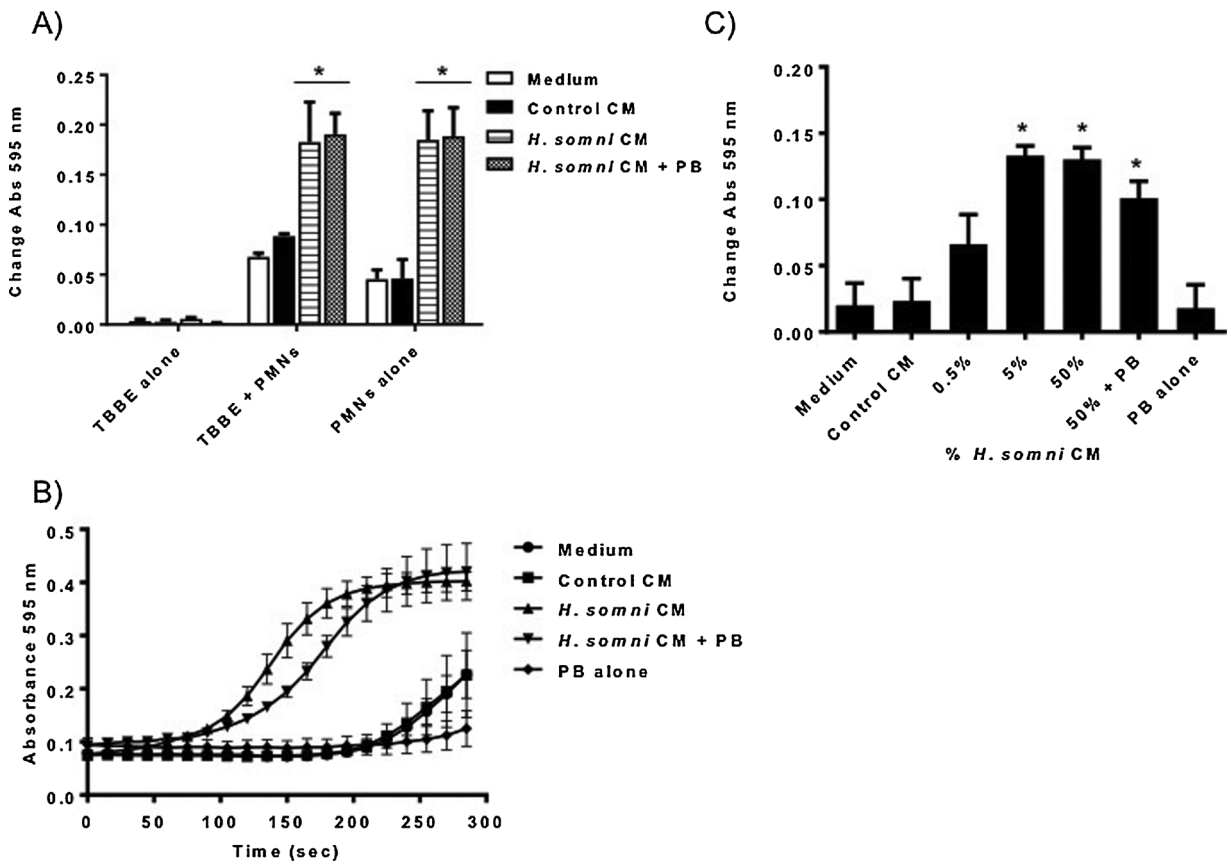

Fig. 3. Fibrin clot formation by TBBE cells and PMNs after exposure to conditioned media (CM) from *H. somni* stimulated TBBE cells. A) TBBE cells were grown to confluence in a 96 well plate, washed 3x with HBSS and overlaid with RPMI-10% FBS. Bovine PMNs were added to TBBE cells at a 5:1 ratio and CM from *H. somni* stimulated or control TBBE cells added at a 50% final concentration (v\v). Polymixin B (PB) (10 μg/mL) was added to some wells to neutralize LOS. The plate was incubated for 6 h at 37 °C with 30 rpm rotation. Cells were washed 3x with Ca^{2+} and Mg^{2+} free PBS, and suspended in 50 μl phenol red-free Ca^{2+} and Mg^{2+} free HBSS. Cells were lysed with 3x rapid freeze-thaw cycles, and 50 μl of fresh citrated bovine plasma added per well. The plasma was re-calcified with warm $CaCl_2$ to a final concentration of 8.3 mM, and fibrin clot formation at 37 °C determined by measuring absorbance at 595 nm. B) Kinetics of fibrin clot formation from one representative experiment (mean ± SD for triplicate wells per sample). Experimental groups tested included: medium (RPMI-10% FBS); control CM (media from unstimulated TBBE cells); *H. somni* CM (medium from *H. somni* stimulated TBBE cells); *H. somni* CM + PB (10 μg/ml) and PB alone. C) One step fibrin clot formation by bovine PMNs after incubation with various concentrations of CM from *H. somni* stimulated TBBE cells. Data represent the mean ± SEM change in absorbance from three independent experiments *$p < 0.05$, compared to control CM. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

the cell surface, or released into the local environment in microparticles (Kagawa et al., 1998; Kushak et al., 2005). We hypothesized that TBBE cells released tissue factor in response to *H. somni*, which resulted in the tissue factor activity observed in TBBE cell CM. To test this hypothesis, we used the factor Xa chromogenic substrate para-nitroaniline to assess CM tissue factor activity. Using this assay, we observed elevated tissue factor activity in TBBE cell CM and TBBE cell lysates after a 6 h incubation with *H. somni* (Fig. 4B).

3.4. Extracellular vesicles from H. somni-stimulated TBBE cells bind to bovine PMNs

We next asked whether TBBE cells released extracellular vesicles that can attach to or be ingested by bovine PMNs. TBBE cells were labelled with a red fluorescent cell linker (PKH26), incubated with *H. somni* or its LOS for 6 h and the CM collected. Extracellular vesicles were isolated from the CM by centrifugation, labelled with anti-von Willebrand Factor antibody and Annexin V, and then incubated with bovine PMNs. The latter were then evaluated by confocal microscopy and flow cytometry. As shown in Fig. 5, bovine PMNs exposed to extracellular vesicles from *H. somni*-stimulated TBBE cells bovine PMNs became positive for the endothelial cell marker von Willebrand Factor and Annexin V staining, and displayed the red fluorescent dye PKH26 (Fig. 5A). Approximately 20% of bovine PMNs were positive for PKH26 after incubation with extracellular vesicles from *H. somni* stimulated TBBE cells *versus* 5% of PMNs incubated with extracellular vesicles from control TBBE cells (Fig. 5B). The above findings suggest PMN acquisition of TBBE cell extracellular vesicles.

3.5. CM from H. somni-stimulated TBBE cells increases PMN tissue factor activity

We then incubated bovine PMNs with serial dilutions of CM from *H. somni* stimulated TBBE cells. Tissue factor activity was determined by measuring activation of factor X into factor Xa, which is the product that marks convergence of the intrinsic and extrinsic coagulation pathways. As shown in Fig. 6, bovine PMNs exhibited dose-dependent tissue factor activity following incubation with CM from *H. somni* exposed TBBE cells. Interestingly, exposure to *H. somni* LOS, *Salmonella* LPS or phorbol myristate acetate (PMA) did not induce pro-coagulant activity on bovine PMNs.

3.6. Extracellular vesicles from H. somni-stimulated TBBE cells increase bovine PMN pro-coagulant activity

We next sought to determine whether *H. somni*-stimulated TBBE

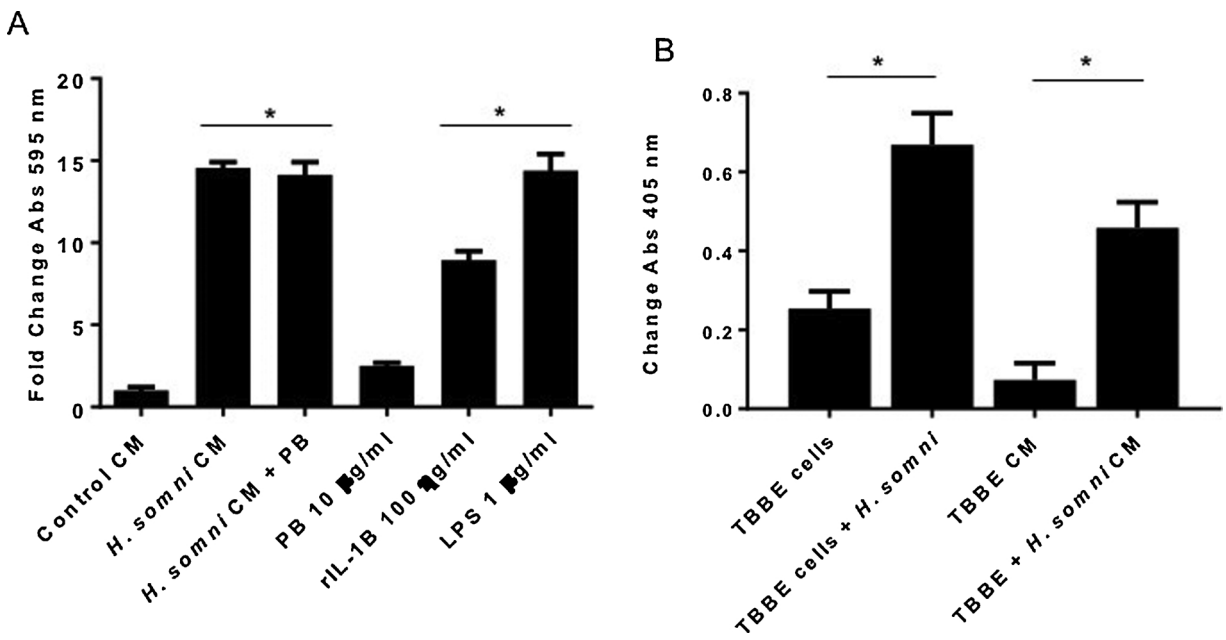

Fig. 4. Exposure to *H. somni* increases tissue factor activity in TBBE cell CM and lysates. A) TBBE cells were grown to confluence in triplicate wells in a 96 well plate. CM from *H. somni*-stimulated or control TBBE cells were added at a 50% final concentration (v\v). The plate was incubated for 24 h at 37 °C with 5% CO_2. Cells were washed 3x with Ca^{2+} and Mg^{2+} free PBS, and suspended in 50 µl phenol red-free Ca^{2+} and Mg^{2+} free HBSS. Cells were lysed with 3x rapid freeze-thaw cycles, and 50 µl of fresh citrated bovine plasma was added per well. The plasma was re-calcified with warm $CaCl_2$ (final concentration of 8.3 mM), and fibrin clot formation at 37 °C determined by measuring absorbance at 595 nm. Controls included CM with polymixin B (10 µg/ml), recombinant IL-1β, or *S typhosa* LPS. Data represent the mean ± SEM fold change in absorbance at 595 nm for three independent experiments. * $p < 0.05$, compared to control CM. B) TBBE cells were incubated for 6 h with *H. somni* (25:1 MOI) at 37 °C with 5% CO_2. CM were collected, the cells washed 2x with PBS, and then detached by gentle scraping. Cells were suspended in 50 µl of phenol red Mg^{2+} and Ca^{2+} free HBSS and lysed by 3x freeze thaw cycles. Tissue factor activity was determined for cell lysates and CM by measuring factor Xa substrate cleavage. Data represent the mean ± SEM change absorbance at 405 nm for three independent experiments. * $p < 0.05$.

cells released extracellular vesicles that were responsible for the increased pro-coagulant activity of bovine PMNs. Extracellular vesicles were isolated from the CM of *H. somni*-stimulated and control TBBE cells and then incubated with bovine PMNs. Fibrin clot formation by PMNs then was assessed by a one-step fibrin deposition assay as described previously. As shown in Fig. 7, removing extracellular vesicles from TBBE cell CM decreased bovine PMN pro-coagulant activity. Conversely, adding TBBE cell extracellular vesicles directly to bovine PMNs increased their pro-coagulant activity comparable to unfractionated CM.

4. Discussion

PMNs generally are the first cells to arrive at a site of inflammation. Here we demonstrate that bovine PMNs exhibited greater adherence to *H. somni*-stimulated TBBE cells than unstimulated control TBBE cells. PMN adhesion was proportional to the number of *H. somni* cells added to the TBBE cell monolayers. However, H. *somni* cells were not absolutely required for PMN adherence to endothelial cell monolayers, because exposure to CM from *H. somni*-stimulated TBBE cells resulted in comparable PMN adhesion. Adding polymixin B to neutralize endotoxin in the CM did not decrease PMN adhesion to TBBE cell monolayers. This observation suggests that LOS carryover has a minimal effect, although we cannot completely exclude a small contribution to the observed response. It was previously reported that TBBE cells release pro-inflammatory cytokines in response to *H. somni* (Behling-Kelly et al., 2007). We speculate that TBBE cells incubated with *H. somni* release pro-inflammatory cytokines into the CM that drives PMN adherence to TBBE cells. However, the molecules responsible for PMN adherence to TBBE cells were not evaluated in the present study. We estimated the number of adherent PMNs by quantifying MPO in cell lysates. Although a well-established method, it is possible that some PMNs were dislodged. If this were the case our values would under-estimate to some extent the number of adherent PMNs.

In addition, we observed increased fibrin deposition when PMNs were incubated with CM from *H. somni*-stimulated TBBE cells. PMNs alone incubated with CM had comparable fibrin deposition to PMNs co-incubated with CM and TBBE cells (Fig. 3A). This suggests that PMNs were responsible for most of the fibrin deposition observed in TBBE cell-PMN co-cultures. Furthermore, CM alone had no direct effect on TBBE cell pro-coagulant activity at 6 h.

One mechanism by which neutrophils can actively participate in blood clotting is through the effects of PMN derived serine proteases. For example, neutrophil elastase and cathepsin G can degrade Tissue Factor Pathway Inhibitor, and activate coagulation factors (Massberg et al., 2010). In addition, the nucleic acids and histones present in neutrophil extracellular traps (NETs) bind to and activate Factor XII leading to fibrin formation (Fuchs et al., 2012). Therefore, neutrophils could trigger fibrin deposition through both the extrinsic and intrinsic coagulation pathways (Fuchs et al., 2012). However, in our experiments stimulating bovine PMNs to form NETs, by adding PMA or *H. somni*, did not increase fibrin deposition by PMNs (data not shown). Nor did adding *S. typhosa* LPS or TNF-α, induce bovine PMN pro-coagulant activity as evaluated by one step fibrin deposition assay. These findings are consistent with a previous report in which bovine peripheral blood PMNs stimulated with PMA or LPS failed to activate plasma fibrin clot formation *in vitro* (Car et al., 1991).

Previous work from our laboratory reported increased tissue factor expression and activity in endothelial cells exposed to *H. somni* cells *in vitro* (Behling-Kelly et al., 2007; Kuckleburg et al., 2008). In the present study, we observed increased tissue factor activity in *H. somni* stimulated TBBE cell lysates and CM, as evaluated by factor Xa activity. These findings suggest bovine brain endothelial cells express and release active tissue factor following stimulation by *H. somni*. These findings are in accordance with other reports of tissue factor release in extracellular vesicles from human endothelial cells stimulated with endotoxin or

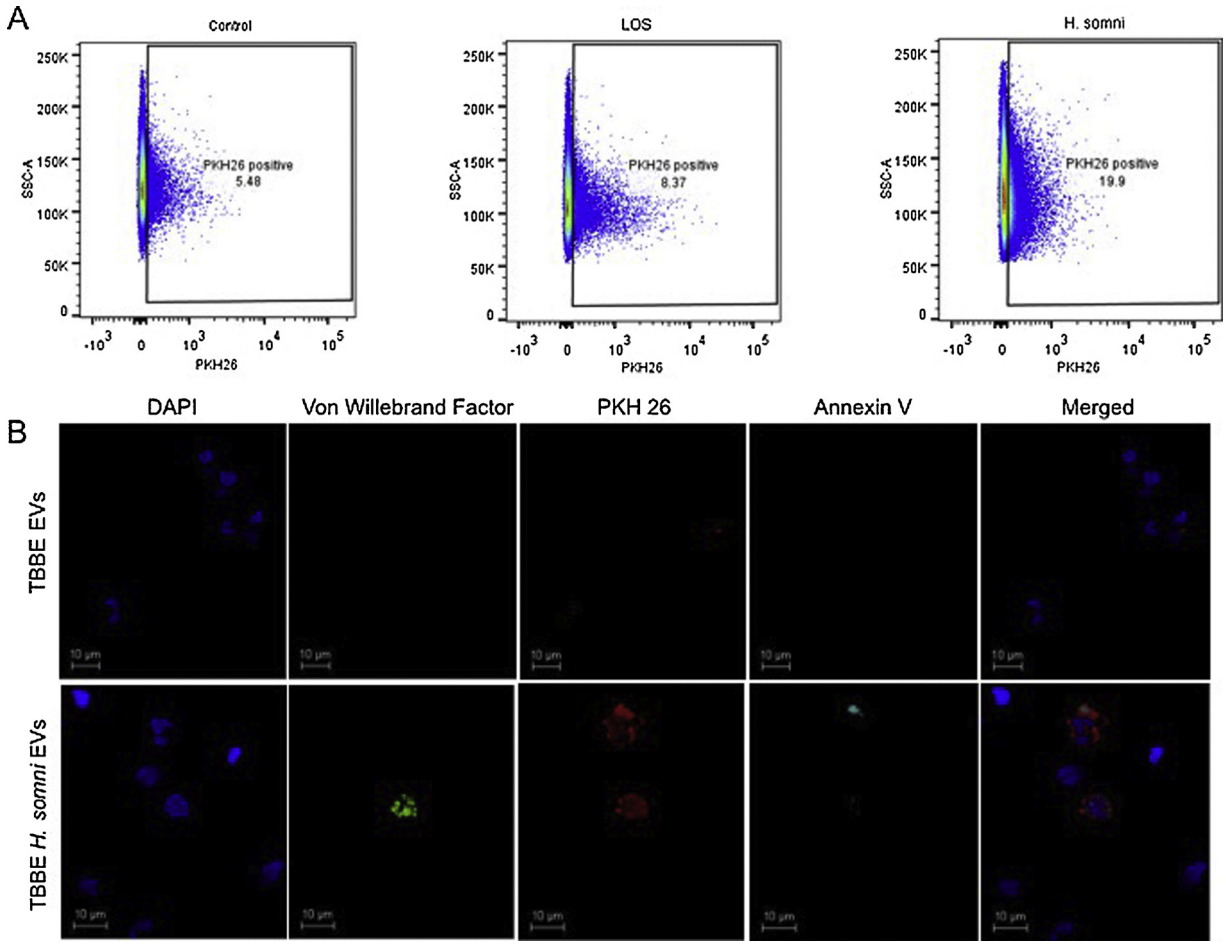

Fig. 5. Bovine PMNs bind extracellular vesicles from *H. somni* stimulated TBBE cells. A) TBBE cells were labeled with PKH26 Red Fluorescent Cell Linker and incubated with *H. somni* for 6 h at 37 °C 5% with CO_2. Extracellular vesicles (EVs) were collected, labeled with FITC-anti-von Willenbrand factor antibody and APC-Annexin V. Vesicles were washed 2x, added to bovine PMNs and incubated for 3 h at 37 °C with 5% CO_2. PMNs were washed, fixed and mount on glass coverslips with antifade mounting medium ProLong Gold with DAPI. PMNs were examined by confocal microscopy. PMNs incubated with EVs from *H. somni* stimulated TBBE cells were positive for PKH26, von Willebrand Factor and Annexin V. Confocal micrographs representative of three independent experiments, 63x magnification. B) TBBE cells were labeled with PKH26 red fluorescent cell linker and stimulated with *H. somni* or its LOS for 6 h at 37 °C with 5% CO_2. Extracellular vesicles from unstimulated and stimulated TBBE cells were isolated as described on the method section. Freshly isolated bovine PMNs were exposed to extracellular vesicles from unstimulated (Control), *H. somni* lipooligosaccharide (LOS) or *H. somni* stimulated (*H. somni*) TBBE cells for 3 h at 37 °C with 5% CO_2. As negative and positive control, bovine PMNs were incubated with media, or directly labeled with PKH26 red fluorescent cell linker, respectively. Cells were washed and analyzed by flow cytometry. Representative density dot plots show percent of PKH26 positive bovine PMNs from one representative experiment (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

TNF-α (Combes et al., 1999; Kagawa et al., 1998; Kushak et al., 2005).

Interestingly, Hellum et al. (2014) observed increased tissue factor activity in extracellular vesicles isolated from plasma of human meningococcal sepsis patients. Extracellular vesicle concentration in peripheral blood has been used as a prognostic indicator in patients with severe meningococcal septic shock; elevated concentrations reflect a poor to grave prognosis (Hellum et al., 2014). The clinical presentation of CNS infection with *H. somni* in cattle is similar to meningococcal meningitis in humans, consisting of marked inflammatory cell activation, disseminated intravascular coagulation, and vascular compromise (Little, 1986; MacDonald et al., 1973; Zeerleder et al., 2003). However, the contributions of extracellular vesicles to the coagulopathy observed in cattle with Histophilosis have not been evaluated.

Previous studies have shown that extracellular vesicles can mediate intercellular transfer of bioactive molecules including cell surface receptors, messenger molecules, RNA and DNA (Gross, 2005; Horstman et al., 2009; Kushak et al., 2005; Nieuwland et al., 2000; Thaler et al., 2014). These bioactive molecules can be integrated into recipient cells, rendering them responsive to new stimuli (Gross, 2005). For example, extracellular vesicles from activated endothelial cells express tissue factor, E-selectin, PECAM-1and ICAM-1 (Hugel, 2005; Jimenez et al., 2003). Acquisition of these vesicles by neutrophils could potentiate adhesion to neutrophil counter receptors (integrins and selectins) on endothelial cells. In the present study, bovine PMNs became positive for von Willenbrand Factor, Annexin V and PKH26 after exposure to extracellular vesicles from *H. somni* stimulated TBBE cells (Fig. 5). PMN acquisition of extracellular vesicles, released from activated endothelial cells also increased PMN fibrin deposition (Fig. 7). Because preparation of the microvesicles (conditioned medium filtered through 0.22 μm filter and supernatant centrifuged at 10,000 x *g*) removes *H. somni* cells, we believe it excludes the possibility the procoagulant effect of microvesicles is due to direct stimulation of PMNs by intact *H. somni* cells. In further support of this inference we found that the direct interaction of PMNs with *H. somni* cells does not stimulate procoagulant activity (Supplemental Fig. 1).

Previous reports indicated that human neutrophils activated by certain inflammatory stimuli expressed tissue factor (Maugeri et al., 2006). Some investigators suggested human neutrophils acquire tissue factor from monocytes (Hellum et al., 2014; Kushak et al., 2009). However, that seems unlikely in our study as the number of

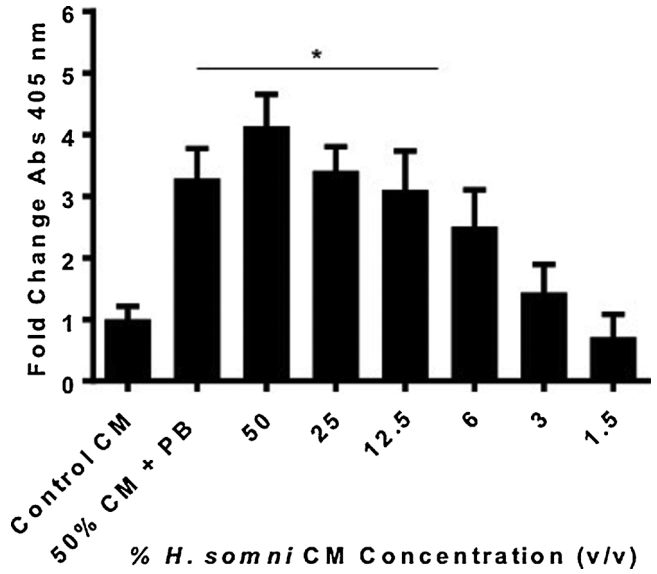

Fig. 6. Tissue factor activity by bovine PMNs after incubation with various concentrations of *H. somni* stimulated TBBE cell CM. Bovine PMNs were incubated with various concentration of CM from *H. somni*-stimulated or unstimulated control TBBE cells. PMNs were washed 2x with PBS, and tissue factor activity assessed by cleavage of para-nitroaniline substrate. Polymixin B (10 μg/ml) was added to some wells to neutralize LOS. Data represent the mean ± SEM fold change in Abs at 405 nm for three independent experiments. * $p < 0.05$, compared to control CM.

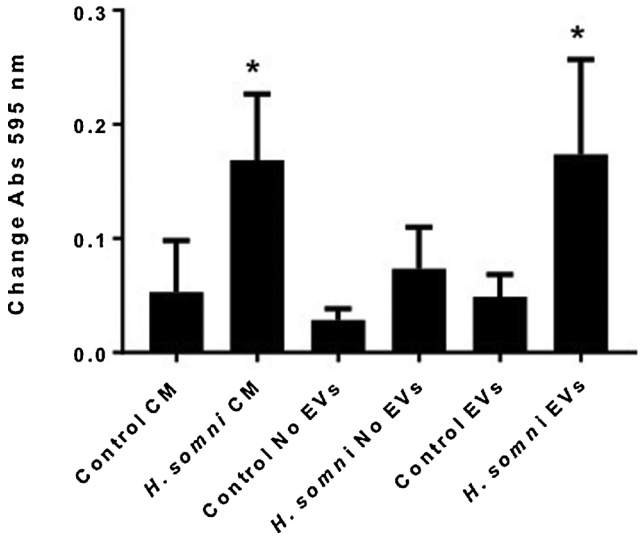

Fig. 7. Extracellular vesicles (EVs) from *H. somni*-stimulated TBBE cells increase bovine PMN pro-coagulant activity. Bovine PMNs were seeded in round bottom 96 well plates at 5×10^5 cells/well. The following was added to bovine PMNs and incubated for 6 h at 37 °C 5% CO_2: conditioned media from unstimulated and *H. somni*-stimulated TBBE cells (CM), conditioned medium that was centrifuged at high speed to remove extracellular vesicles (no EVs), and extracellular vesicles isolated from conditioned medium (EVs). Bovine PMNs were centrifuged at 250 x *g* 5 min and washed 2x with HBSS without $Ca^{2+}Mg^{2+}$. Platelet poor plasma and 50 μL of 25 mM $CaCl_2$ were warmed at 37 °C and added to the wells in rapid succession. The reaction was incubated at 37 °C and the change in absorbance (595 nm) of one step fibrin clot formation was determined using a DTX880 plate reader. Data are representative of three independent experiments, mean ± SEM change absorbance 595 nm. *$p < 0.05$, compared to control CM or control MPs.

mononuclear cells in our neutrophil preparations was very low. Nor did we detect tissue factor activity on bovine neutrophils when they were activated by *H. somni*, endotoxin or PMA.

In summary, this study shows that bovine PMNs exhibit properties that could make them active players in the thrombus formation that characterizes TME. A previous report showed that *H. somni* induces bovine PMN adhesion and transmigration across TBBE cells *in vitro* (Tiwari et al., 2009). In the present study, bovine PMNs amplified the pro-coagulant activity of activated bovine endothelial cells through acquisition extracellular vesicles containing tissue factor. Delineating the sequence of events and the mediators involved in the dysfunction of vascular homeostasis could help identify new potential therapeutic targets aimed at inhibiting intravascular thrombosis during H. somni infection.

Acknowledgements

We would like to thank Dr. Thomas J. Inzana from Virginia-Maryland College of Veterinary Medicine for generously providing the *H. somni* LOS. We would also like to acknowledge Dr. Erica Behling-Kelly from Cornell University College of Veterinary Medicine for her technical support. This work was supported by Pfizer Animal Health, the USDAAFRI 2010-2010-03509, US Public Health Service and Institutional Training GrantT32 RR023916-06, NIH S10 Shared Instrumentation Grant1S100OD018202-01 Special BD LSR Fortessa, and the Walter and Martha Renk Endowed Laboratory in Food Safety.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.vetimm.2019.03.009.

References

Arfors, K.-E., Lundberg, C., Lindbom, L., Lundberg, K., Beatty, P.G., Harlan, J.M., 1987. A monoclonal antibody to the membrane glycoprotein complex CD18 inhibits polymorphonuclear leukocyte accumulation and plasma leakage in vivo. Blood 69, 338–340.

Behling-Kelly, E., Kim, K.S., Czuprynski, C.J., 2007. *Haemophilus somnus* activation of brain endothelial cells: potential role for local cytokine production and thrombosis in CNS infection. Thromb. Haemost. 98, 823–830. https://doi.org/10.1160/TH06-11-0665.

Bertina, R.M., 2009. The role of procoagulants and anticoagulants in the development of venous thromboembolism. Thromb. Res. 123, S41–S45.

Car, B.D., Suyemoto, M.M., Neilsen, N.R., Slauson, D.O., 1991. The role of leukocytes in the pathogenesis of fibrin deposition in bovine acute lung injury. Am. J. Pathol. 138, 1191.

Combes, V., Simon, A.-C., Grau, G.-E., Arnoux, D., Camoin, L., Sabatier, F., Mutin, M., Sanmarco, M., Sampol, J., Dignat-George, F., 1999. *In vitro* generation of endothelial microparticles and possible prothrombotic activity in patients with lupus anticoagulant. J. Clin. Invest. 104, 93–102.

Cooray, R., 1994. Use of bovine myeloperoxidase as an indicator of mastitis in dairy cattle. Vet. Microbiol. 42, 317–326.

Corbeil, L.B., 2007. *Histophilus somni* host–parasite relationships. Anim. Health Res. Rev. 8, 151–160. https://doi.org/10.1017/S1466252307001417.

Doring, Y., Weber, C., Soehnlein, O., 2013. Footprints of neutrophil extracellular traps as predictors of cardiovascular risk. Arterioscler. Thromb. Vasc. Biol. 33, 1735–1736. https://doi.org/10.1161/ATVBAHA.113.301889.

Fuchs, T.A., Brill, A., Wagner, D.D., 2012. Neutrophil extracellular trap (NET) impact on deep vein thrombosis. Arterioscler. Thromb. Vasc. Biol. 32, 1777–1783. https://doi.org/10.1161/ATVBAHA.111.242859.

Goel, M.S., 2003. Neutrophil cathepsin G promotes prothrombinase and fibrin formation under flow conditions by activating fibrinogen-adherent platelets. J. Biol. Chem. 278, 9458–9463. https://doi.org/10.1074/jbc.M211956200.

Gross, P.L., 2005. Leukocyte-versus microparticle-mediated tissue factor transfer during arteriolar thrombus development. J. Leukoc. Biol. 78, 1318–1326. https://doi.org/10.1189/jlb.0405193.

Harding, M., Kubes, P., 2012. Innate immunity in the vasculature: interactions with pathogenic bacteria. Curr. Opin. Microbiol. 15, 85–91. https://doi.org/10.1016/j.mib.2011.11.010.

Hellum, M., Øvstebø, R., Brusletto, B.S., Berg, J.P., Brandtzaeg, P., Henriksson, C.E., 2014. Microparticle-associated tissue factor activity correlates with plasma levels of bacterial lipopolysaccharides in meningococcal septic shock. Thromb. Res. 133, 507–514. https://doi.org/10.1016/j.thromres.2013.12.031.

Higuchi, D.A., Wun, T.-C., Likert, K.M., Broze, G.J., 1992. The effect of leukocyte elastase on tissue factor pathway inhibitor. Blood 79, 1712–1719.

Horstman, L.L., Jy, W., Bidot, C.J., Nordberg, M.L., Minagar, A., Alexander, J.S., Kelley, R.E., Ahn, Y.S., 2009. Potential roles of cell-derived microparticles in ischemic brain disease. Neurol. Res. 31, 799–806. https://doi.org/10.1179/

016164109X12445505689526.
Hugel, B., 2005. Membrane microparticles: two sides of the coin. Physiology 20, 22–27. https://doi.org/10.1152/physiol.00029.2004.
Jimenez, J.J., Jy, W., Mauro, L.M., Soderland, C., Horstman, L.L., Ahn, Y.S., 2003. Endothelial cells release phenotypically and quantitatively distinct microparticles in activation and apoptosis. Thromb. Res. 109, 175–180. https://doi.org/10.1016/S0049-3848(03)00064-1.
Kagawa, H., Komiyama, Y., Nakamura, S., Miyake, T., Miyazaki, Y., Hamamoto, K., Masuda, M., Takahashi, H., Nomura, S., Fukuhara, S., 1998. Expression of functional tissue factor on small vesicles of lipopolysaccharide-stimulated human vascular endothelial cells. Thromb. Res. 91, 297–304.
Kaplan, M.J., Radic, M., 2012. Neutrophil extracellular traps: double-edged swords of innate immunity. J. Immunol. 189, 2689–2695. https://doi.org/10.4049/jimmunol.1201719.
Kuckleburg, C.J., McClenahan, D.J., Czuprynski, C.J., 2008. Platelet activation by *Histophilus somni* and its LOS induces endothelial cell pro-inflammatory responses and platelet internalization. Shock 29, 189.
Kushak, R.I., Nestoridi, E., Lambert, J., Selig, M.K., Ingelfinger, J.R., Grabowski, E.F., 2005. Detached endothelial cells and microparticles as sources of tissue factor activity. Thromb. Res. 116, 409–419. https://doi.org/10.1016/j.thromres.2005.01.013.
Levi, M., 2010. The coagulant response in sepsis and inflammation. Hamostaseol. 30, 10.
Little, P.B., 1986. *Haemophilus somnus* complex: pathogenesis of the septicemic thrombotic meningoencephalitis. Can. Vet. J. 27, 94.
MacDonald, D.W., Christian, R.G., Chalmers, G.A., 1973. Infectious thromboembolic meningoencephalitis: literature review and occurrence in Alberta, 1969-71. Can. Vet. J. 14, 57.
Massberg, S., Grahl, L., von Bruehl, M.-L., Manukyan, D., Pfeiler, S., Goosmann, C., Brinkmann, V., Lorenz, M., Bidzhekov, K., Khandagale, A.B., Konrad, I., Kennerknecht, E., Reges, K., Holdenrieder, S., Braun, S., Reinhardt, C., Spannagl, M., Preissner, K.T., Engelmann, B., 2010. Reciprocal coupling of coagulation and innate immunity via neutrophil serine proteases. Nat. Med. 16, 887–896. https://doi.org/10.1038/nm.2184.
Maugeri, N., Brambilla, M., Camera, M., Carbone, A., Tremoli, E., Donati, M.B., De Gaetano, G., Cerletti, C., 2006. Human polymorphonuclear leukocytes produce and express functional tissue factor upon stimulation1. J. Thromb. Haemost. 4, 1323–1330.
Nogami, K., Ogiwara, K., Matsumoto, T., Nishiya, K., Takeyama, M., Shima, M., 2011. Mechanisms of human neutrophil elastase-catalysed inactivation of factor VIII(a). Thromb. Haemost. 105, 968–980. https://doi.org/10.1160/TH10-12-0777.
Pérez, D.S., Bretschneider, G., Pérez, F.A., 2010. *Histophilus somni*: pathogenicity in cattle. An update. An. Vet. Murcia 26, 5–21.
Rivera-Rivas, J.J., Kisiela, D., Czuprynski, C.J., 2009. Bovine herpesvirus type 1 infection of bovine bronchial epithelial cells increases neutrophil adhesion and activation. Vet. Immunol. Immunopathol. 131, 167–176. https://doi.org/10.1016/j.vetimm.2009.04.002.
Tiwari, R., Sullivan, J., Czuprynski, C.J., 2009. PECAM-1 is involved in neutrophil transmigration across *Histophilus somni* treated bovine brain endothelial cells. Microb. Pathog. 47, 164–170. https://doi.org/10.1016/j.micpath.2009.06.001.
Zeerleder, S., Zenklusen, R.Z., Hack, C.E., Wuillemin, W.A., et al., 2003. Disseminated intravascular coagulation in meningococcal sepsis. Hämostaseology 23, 125–130.
Zekarias, B., O'Toole, D., Lehmann, J., Corbeil, L.B., 2011. Histophilus somni IbpA Fic cytotoxin is conserved in disease strains and most carrier strains from cattle, sheep and bison. Vet. Microbiol. 149, 177–185. https://doi.org/10.1016/j.vetmic.2010.10.012.