Antibacterial effects of adipose mesenchymal stromal cell-derived exosomes *in vitro* and in an *Escherichia coli*-induced acute lung injury mouse model

by

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Abstract

Antibacterial effects and immunomodulatory properties of mesenchymal stromal cells (MSCs) and their secretome are an emerging area of research. The antibacterial effects of MSC-derived secretome are attributed to antimicrobial peptides, and later speculated that these antimicrobial peptides were packaged within exosomes. It is also shown that, stimulation of MSCs with polyinosinic:polycytidylic acid (poly I:C), a toll-like receptor 3 (TLR 3) agonist, enhanced the antimicrobial and immunomodulatory properties and therapeutic effects. Exosomes, MSC-derived bilipid layered and nanosized secretome components, are shown to have immunomodulatory properties similar to MSCs. We hypothesized that exosomes derived from MSC have similar antibacterial activities as MSCs alone. In this study, we evaluated antibacterial effects of equine adipose MSC (ASC)-derived exosomes against Gram negative (*Escherichia coli* and *Klebsiella pneumoniae*) and Gram positive (*Staphylococcus aureus* and *Rhodococcus equi*) bacteria and investigated whether TLR 3 agonist stimulated ASC-derived exosomes enhanced the antibacterial activity. The therapeutic efficacy of ASC-derived exosomes was investigated in an *Escherichia coli*-induced acute lung injury mouse model. Equine ASCs were isolated and cultured in low-glucose DMEM medium containing 10% fetal bovine serum. Adipose stromal cells were stimulated with TLR 3 agonist (poly I:C) at 10 µg/ml concentration for 24 hours and exosomes were collected from both TLR 3 agonist stimulated and non-stimulated ASCs by differential ultracentrifugation. Characterization and quantification of exosomes were done by transmission electron microscopy, nanoparticle tracking analysis and bicinchoninic acid (BCA) protein assay. The *in vitro* antibacterial effects were determined by culturing bacteria with and without ASCs and ASC-derived exosomes, stimulated or non-

stimulated by TLR 3 agonist and bacterial growth was assessed by measuring optical density at 0, 12, and 24-hour incubations and by determining bacterial cell concentration at 24 hours by spread plate method. An acute lung injury mouse model elicited by intratracheal injection of *E. coli* to evaluate the therapeutic efficacy of ASC-derived MSC exosomes. Bronchoalveolar lavage (BAL) fluid was collected for assessment of bacterial load and alveolar protein permeability. *In vitro*, ASCs and ASC-derived exosomes significantly inhibited growth of both Gram negative and Gram-positive bacterial species. Adipose MSC-derived exosomes showed a dose-dependent inhibition of bacterial growth. Toll-like receptor 3 stimulated ASCs enhanced the number of exosomes released by two-fold. In addition, TLR 3 stimulated ASCs and ASC-derived exosomes showed significant bacterial inhibition compared to the control groups. In the mouse model, ASCs and ASC-derived exosomes enhanced the bacterial clearance and alveolar permeability. In summary, ASC exosomes exhibited antibacterial activities similar to ASCs in both *in vitro* and *in vivo*. Both ASCs and ASC-derived exosomes exhibited antibacterial effects in a dose dependent manner. This is the first study to demonstrate the antibacterial activities of MSC-derived exosomes *in vitro* against Gram negative and Gram-positive bacteria, and *in vivo* in a mouse acute lung injury model.

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Chapter 1 - Review of Literature

Introduction

Acute lung Injury (ALI) is a major cause of acute respiratory failure with high mortality rate in critically ill patients. The most common causes of ALI are bacterial, viral and mechanical/stress causing factors (Loy et al., 2019; Lucas et al., 2009; Wu et al., 2014). The inflammatory response cause damage to the alveolar endothelium and epithelium leads to increased protein permeability and reduced alveolar fluid clearance result in pulmonary edema (Lee et al., 2019; Lucas et al., 2009). Although there are conventional antibiotics and supportive care available, long term usage causes various adverse effects. Antibiotic resistance is a challenge for treating several bacterial infections and new therapeutic approach is urgently needed for treatment (Gupta et al., 2012; Jackson et al., 2016; Krasnodembskaya et al., 2010; Russell et al., 2020). Recent studies are focusing on cells release compounds that can kill bacteria such as antimicrobial peptides (AMPs) (Harman et al., 2017; X. Zhao & Zhang, 2020).

Mesenchymal stromal cells (MSCs) evolved as a potential cell therapy for treating a wide range of inflammatory diseases through immunomodulatory and tissue regeneration properties (Aggarwal & Pittenger, 2005; Gao et al., 2016; Gimble et al., 2013; Petri et al., 2017; Yoo et al., 2009). The therapeutic properties of MSCs demonstrated in multiple clinical studies including graft Vs host disease in acute hepatitis (Fang et al., 2007), multiple sclerosis (Hosseini Shamili et al., 2019), acute renal failure (Morigi et al., 2016), systemic lupus (Sun et al., 2010), myocardial infarction (Bai et al., 2010), cystic fibrosis (Sutton et al., 2016), acute lung injury (Islam et al., 2012), traumatic brain injury (Zhou et al., 2019), amyotrophic lateral sclerosis (Bonafede & Mariotti, 2017) and hindlimb ischemia (Moon et al., 2006). Even though much is known about characteristics and properties of MSCs, their use in therapy is limited due to strict FDA

regulations attributed to adverse reactions from limited scalability, and lack of homogeneity between the treatment groups (Bongso & Fong, 2013; Musiał-Wysocka et al., 2019; Prockop, 2017; Tatsumi et al., 2013). Due to limitations in application of MSCs, the focus of regenerative medicine has been largely shifted towards use of cell free therapy using MSC derived secretome. Multiple studies have shown that the therapeutic effect of MSCs is due to soluble factors released from MSCs during culture called secretome, which include cytokines, growth factors and numerous extracellular vesicles which constitute exosomes, microvesicles, and apoptotic bodies (Cai et al., 2020; Gupta et al., 2012; Jackson et al., 2016; Khatri et al., 2018; Krasnodembskaya et al., 2010; Monsel et al., 2015). Amongst these, exosomes, which are nanosized (50-150 nm) lipid bilayer vesicles that carry significant amounts of microRNA, noncoding RNA, DNA, proteins, and lipids were shown to exert similar therapeutic properties as MSCs (Blazquez et al., 2014; Favaro et al., 2014; Martin-Rufino et al., 2019; Rosenberger et al., 2019; Villatoro et al., 2019; Zhang et al., 2016)

Mesenchymal stromal cells

Mesenchymal Stromal Cells (MSCs) constitute an important source of cells for regenerative approaches for the past 30 years and has shown therapeutic potential for treating a wide range of inflammatory diseases due to their unconventional therapeutic versatility and promising clinical outcomes. These cells were initially reported as colony forming fibroblasts (CFU-F) by Friedenstein et al. in 1960s which were hematopoietic cells in bone marrow (Friedenstein et al., 1966). Later the term mesenchymal stem cell was coined by Caplan in early 1990's which have the property of self-renewal and multilineage differentiation (Caplan, 1991). The sources of MSCs include bone marrow, adipose tissue, umbilical cord tissue, cord blood, placenta, menstrual blood, cornea, spleen, hair, skin, brain, dental pulp, intestines, liver, and

skeletal muscles (Crisan et al., 2008; Hass et al., 2011; in ’t Anker et al., 2004; Zuk et al., 2002). International Society of Cell Therapy (ISCT) characterized MSCs based on their capacity of plastic adherence, multilineage differentiation and surface marker expressions. In 2006, ISCT established a minimum criterion for identifying cells as MSCs, this criterion states that MSCs grow as tissue culture plastic adherent; can differentiate into multiple lineages such as adipose, cartilage, and bone tissues; and stain positively for surface markers CD105, CD73, and CD90, but do not stain for surface markers CD45, CD34, CD14, or CD11b, CD79a, or CD 19, and human leukocyte antigen-antigen D related (HLA-DR) (Dominici et al., 2006). This definition has been used to identify MSCs for past 14 years. These MSCs are widely used for cell therapy over embryonic stem cells due to their ease of harvest without ethical issues and were considered safe for use in cell therapy due to low tumorigenic potential (Luna et al., 2014). The therapeutic potential of MSCs is well documented in a wide range of inflammatory diseases including in preventing graft Vs host disease (Fang et al., 2007), multiple sclerosis (Shamili et al., 2019), acute renal failure (Morigi et al., 2016), systemic lupus (Sun et al., 2010), myocardial infarction (Bai et al., 2010), cystic fibrosis (Sutton et al., 2016), acute lung injury (Islam et al., 2012), traumatic brain injury (Zhou et al., 2019), amyotrophic lateral sclerosis (Bonafede & Mariotti, 2017) and hindlimb ischemia (Moon et al., 2006). In addition, recently MSCs gained significant importance due to their antimicrobial properties against both viruses and bacteria such as influenza A H5N1-associated acute lung injury (Chan et al., 2016; Khatri et al., 2018; Loy et al., 2019), *E. coli* induced pulmonary pneumonia (Alcayaga-Miranda et al., 2017; Krasnodembskaya et al., 2010; Monsel et al., 2015). These therapeutic properties were shown to be mediated through immunomodulation and direct cellular interactions (Johnson et al., 2017; S. Zhang et al., 2016).

Several studies demonstrated that adipose tissue represents an alternative source of MSCs and have shown promising results in regenerative medicine. Adipose mesenchymal stromal cells (ASCs) were first characterized in early 2000's and since widely used as a major source of MSCs (Bora & Majumdar, 2017). Studies showed that the immunomodulatory and therapeutic potential of ASCs were similar to MSCs derived from bone marrow and umbilical cord, in addition ASCs have added advantages over other sources of MSCs. The main advantages of ASCs are they can be easily harvested with minimal invasive procedures, abundant sources of availability, and they can be easily cultured with high proliferation capacity (Gimble et al., 2013; Luna et al., 2014). Studies showed that adipose tissue yields MSCs 5-10% of the nucleated cells which is 500 times more compared with bone marrow, whereas bone marrow aspirate yields MSCs 0.001% of the nucleated cell population (Bourin et al., 2013; Freitag et al., 2020). Previous studies characterized that ASCs show positive for stromal markers such as CD10, CD13, CD73, CD90, CD105, VEGFR-1 and Nanog and ASCs do not express for surface markers CD45, CD34 and CD11b (Bourin et al., 2013; Luna et al., 2014). Studies showed that ASCs have more immune evasive and immunomodulatory potential compared to bone marrow MSCs such as low expression levels of HLA class-1 molecule, suppress dendritic cell, NK cell differentiation, and has shown to promote the Treg generation compared with the bone marrow MSCs makes them better alternative for clinical trials (Aggarwal & Pittenger, 2005; Caplan & Dennis, 2006; Cortés-Araya et al., 2018; Jimenez-Puerta et al., 2020; Sangiorgi & Panepucci, 2016; Wang et al., 2014).

Mesenchymal stromal cell properties

It is well established that MSCs have therapeutic potential due to their self-renewable, multilineage differentiation, tissue regeneration, anti-inflammatory, and immunomodulatory properties. Mesenchymal stromal cells are capable of self-renewable and are shown to differentiate into multiple lineages, such as adipocytes, chondrocytes, osteocytes, endothelial cells, skin, muscle, and neurons (Crisan et al., 2008; Prockop, 2017; Romanov et al., 2005; Zuk et al., 2002). Studies showed that MSCs have the ability to migrate towards the affected tissue by chemotactic signals released due to tissue damage and promote tissue regeneration (Baraniak & McDevitt, 2010). The tissue repair properties of MSCs were exerted due to secretion of anti-apoptotic factors such as vascular endothelial growth factor (VEGF), insulin growth factor (IGF), hepatic growth factor (HGF), neurotrophin-3, and nerve growth factors as well as through paracrine signaling (Gao et al., 2016; Rogers et al., 2020; Villatoro et al., 2019; Yoo et al., 2009). Previous studies demonstrated that MSCs have immunomodulatory potential by altering both innate and adaptive responses. These immunomodulatory properties were mediated through shift in pro-inflammatory cytokines including IL-1β, IL-6, MIP-2, CXCL-1, CXCL-2, TNF-β, IL-17, and protease like MMP-9 and MMP-12 to anti-inflammatory status by releasing prostaglandin E2 (PGE2), IL-10, Human Leukocyte Antigen-G (HLA-G), indoleamine 2,3-dioxygenase (IDO), and chemokines, as CXCL9, CXCL10, and CXCL11 (Jimenez-Puerta et al., 2020). These cytokines, mainly IDO enable the macrophages to produce transforming growth factor-β (TGF-β) and promote regulatory T cells (Treg) production. Mesenchymal stromal cells are capable to inhibit both adaptive cells, such as T and B cell, and innate immune cells, such as dendritic cells, macrophages through release of anti-inflammatory cytokines (Chow et al., 2020). In addition, MSCs have the capability to change the phenotype of macrophages from M1 pro-inflammatory

to M2 anti-inflammatory phenotype with high phagocytic capacity. Several studies showed that therapeutic properties of MSCs were shown due to their paracrine activity by releasing growth factors, cytokines, chemokines, and extracellular vesicles (EVs) into their surroundings (Park et al., 2018).

Furthermore, antimicrobial properties of MSCs were demonstrated in both *in vitro* and *in vivo* clinical models. These antimicrobial activities exhibited through the secretion of antimicrobial peptides (AMPs), such as cathelicidin peptide LL-37, hepcidin, β-defensin 2, and lipocalin 2 (Chow et al., 2020; Harman et al., 2017). These AMPs are capable of destroying both bacterial and virus by altering their membrane integrity (Harman et al., 2017). Khatri et al., 2018 demonstrated that MSCs have anti-inflammatory and anti-viral properties through transfer of RNAs. Jackson et al., 2016 described that mitochondrial transfer from MSCs to innate immune cells via tunneling nanotubes leads to enhancement of phagocytic capacity of macrophages and bacterial clearance.

Mesenchymal stromal cell-derived exosomes

Recent studies were focusing on using MSC secretome as a cell free regenerative therapy. Mesenchymal stromal cells proved to mediate their therapeutic effects by releasing secretome components into their extracellular environments (Bonafede & Mariotti, 2017). Secretome contains growth factors like HGF, tumor necrosis factor-inducible gene 6 protein (TSG6), VEGF, fibroblast growth factor (FGF), insulin-like growth factor 1 (IGF-1), CCL-2, epidermal growth factor (EGF), platelet derived growth factor (PDGF), cytokines, such as IL-10, TGF-β, prostaglandin E2 (PGE2), IDO, and stromal cell-derived factor 1 (SDF-1) (Jimenez-Puerta et al., 2020). In addition to these, MSCs release extracellular vesicles includes, exosomes, microvesicles, and apoptotic bodies. These EVs carry the MSC cargo such as microRNA,

mRNA, proteins, and lipids (Varderidou-Minasian & Lorenowicz, 2020). Exosomes are small extracellular vesicles of size range from 50-200 nm and formed by inward budding of plasma membrane. Microvesicles (MVs) are heterogenous population with size ranges from 200-1000 nm and are released by outward budding of plasma membrane (Shen et al., 2018). These secretome components were shown to mediate their therapeutic effects *via* paracrine signaling.

Exosomes are small EVs of size ranging from 50 nm to 200 nm with a phospholipid bilayer membrane (Kao & Papoutsakis, 2019; Marote et al., 2016; Shen et al., 2018; Witwer & Théry, 2019). These exosomes are formed through endocytosis by in ward budding of plasma membrane as multiple vesicular bodies and are released into extracellular environment. They were shown to release from all types of cells and are identified in many biological fluids, such as cerebrospinal fluid (CSF), urine, saliva, blood, lymph, and amniotic fluid (Bonafede & Mariotti, 2017). Initially, exosomes were considered as discarding metabolic waste products of cells. But studies showed that, they are carrying the cell cargo which includes, mRNAs, microRNA, proteins, and lipids from which they originated (Kao & Papoutsakis, 2019; Marote et al., 2016; Shen et al., 2018; Witwer & Théry, 2019). After releasing into extracellular environment, these exosomes will reach the target cells by paracrine signaling and enters the cells by endocytosis or by adhesion with the help of transmembrane surface protein markers such as CD9, CD63, CD81and Alix (Blazquez et al., 2014; Martin-Rufino et al., 2019; Rosenberger et al., 2019). Later exosomes will release the cargo into the target cells and implicate the therapeutic role in cells. Exosomes released from the specific cells were thought to show therapeutic effects of the cells from which they originated due to their cell specific cargo. Similarly, exosomes released from MSCs were able to show similar therapeutic effects as MSCs. In the past decade, several

researchers were comparing the therapeutic effects of exosomes with MSCs for providing the cell free therapy (Bandeira et al., 2018; Cai et al., 2020).

Applications of exosomes

Exosomes were shown to have lower safety risk due to their nano size and non-self-replicative properties compared to MSCs (Shen et al., 2018). They can be scalable and easily stored at -80°C in phosphate buffered saline (PBS) without any specific cryopreservation media such as dimethyl sulfoxide (DMSO) which make them safe and off the shelf products (Baharlooi et al., 2020; Bonafede et al., 2016; Kao & Papoutsakis, 2019; Lai et al., 2015; Marote et al., 2016; Stahl & Raposo, 2019). Exosomes reach target tissues by paracrine signaling and are proved to cross the microvasculature and blood-brain barrier due to their nanosized diameter (Bonafede et al., 2016; Ying Zhou et al., 2013). Different groups of mRNAs, microRNAs carried by MSC-derived exosomes were shown to have therapeutic properties against acute hepatitis (Li et al., 2015), myocardial ischemia (Zhang et al., 2016), acute kidney injury (Zhou et al., 2013), bleomycin induced pulmonary fibrosis (Mansouri et al., 2019), wound healing (Nakamura et al., 2015), neurodegeneration (Xin et al., 2017), experimental silicosis (Bandeira et al., 2018), and multiple sclerosis (Shamili et al., 2019; Yin et al., 2019). The protein and lipid cargo present in exosomes were proved to have angiogenic, anti-apoptotic properties and helps in tissue regeneration and wound healing (Varderidou-Minasian & Lorenowicz, 2020). Exosomes were shown to have anti-inflammatory and immunomodulatory properties by altering the M1 phenotype to M2 phenotype macrophages and increase in Treg cells which increase the anti-inflammatory cytokine levels such as IL- 10, PGE2, and IDO (Martin-Rufino et al., 2019). Pachler et al., 2017 showed that the MSC-derived exosomes have immunomodulatory potential by inhibiting the T- cell proliferation when treated with mitogens. Recently, researchers were

showing that exosomes can be used to deliver or over express specific bio-active factors by engineering their cargo for therapeutic purposes. Studies also described that exosomes can be used as diagnostic markers for cancer prognosis and detection based on their cargo (Shen et al., 2018).

Isolation and characterization of exosomes

Several methods were in use to isolate and characterize the exosomes. Among them differential ultracentrifugation used as gold standard for several *in vivo* clinical trials (Klymiuk et al., 2019; Théry et al., 2006). The other methods include density gradient ultracentrifugation based on different floating densities, size exclusion chromatography (SEC), ultrafiltration separates based on particle size, polyethylene glycol method based on precipitation and immune affinity capture based method based on surface protein expression (Klymiuk et al., 2019; Patel et al., 2019). No single isolation procedure was an ideal for isolation of exosomes, if yield is high then the purity will be low and if purity is high yield is low. For ensuring better yield and purity, International Society for Extracellular Vesicles recommends combining different methods of isolation (Patel et al., 2019; Varderidou-Minasian & Lorenowicz, 2020). Several methods were used for characterization and quantification of exosomes, such as scanning or transmission electron microscopy (SEM or TEM), dynamic light scattering (DLS), nanoparticle tracking analysis (NTA), protein quantification methods using Bicinchoninic acid assay or Bradford assay and western blot analysis (Chopra et al., 2019; Gupta et al., 2018). Among these no single method will give accurate analysis of exosomes sample. Hence, different techniques were used to evaluate the exosomes. Specific challenges must be targeted for isolation and characterization of exosomes for better yield and clinical translation of exosomes for therapeutic properties.

Varderidou-Minasian et al., 2020, discussed about the advantages and disadvantages of various methods used for isolation and characterization of extracellular vesicles.

MSC-derived exosome clinical trials

Exosomes derived from various MSC sources such as bone marrow, umbilical cord, adipose tissue, and embryonic stem cells were used in wide range of clinical studies (Cai et al., 2020). However, most of the studies were done using human MSC-derived exosomes and very few studies were done on animal tissue MSC-derived exosomes. The clinical trials using MSC-derived exosomes were focused on variety of diseases including acute hepatitis (Li et al., 2015), rheumatoid arthritis (Zhang et al., 2016)), myocardial ischemia (Zhou et al., 2013), acute kidney injury (Zhou et al., 2013), bleomycin induced pulmonary fibrosis (Mansouri et al., 2019), wound healing (Nakamura et al., 2015), neurodegeneration (Xin et al., 2017) and amyotrophic lateral sclerosis (Bonafede & Mariotti, 2017). For faster clinical translation of exosomes, better isolation and characterization exosomes are needed to be addressed and also production of exosomes by altering the growth conditions or stimulating the cells using various methods and growing MSCs in bioreactors (Haraszti et al., 2018; Thippabhotla et al., 2019). In a renal ischemia study, exosomes from MSCs were showed protect through different pathways and improved the reperfusion injury (Zhou et al., 2013). Researchers found that MSC-derived exosomes showed to have wound healing properties by increasing gene expression levels of N-cadherin, cyclin-1, and collagen levels (Hu et al., 2016). Previous studies have showed that MSC derived exosomes were shown to suppress the liver fibrosis and improve the regeneration (Rong et al., 2019). MSC-derived exosomes with overexpression of miR-375 were shown to improve bone regeneration (Chen et al., 2019). In a recent study, researchers showed that combination of MSC-derived exosomes with a polylactic acid glycolic acid (PLGA) scaffold protects from skull

defects (Cai et al., 2020; Li et al., 2018). Mendt et al., 2018 described that engineering of exosomes derived from MSCs by injecting siRNA exogenously to target Kras oncogene helps in cancer reduction and improved the survivability of mice. However, antimicrobial properties of MSC-derived exosomes were still unclear and needed to be performed.

Stimulation/preconditioning of MSCs

Even though, MSCs were shown to have therapeutic properties against several diseases, clinical trials demonstrated that large number of MSCs are required for obtaining better therapeutic effects. Recent studies showed that MSCs have dose dependent clinical responses for variety of diseases and optimal number of MSCs will give faster recovery and better healing properties. To obtain large number of MSCs in millions, MSCs needed to be repeatedly passaged which leads to loss of multilineage and immunomodulatory properties of MSCs and cell senescence will be caused as number of passages increased (Domenis et al., 2018; Mastri et al., 2012). To address these issues, recent studies targeted towards improving the efficacy of MSCs by altering their microenvironment with stimuli to enhance the immunomodulatory properties of MSCs. The type of stimulation will determine the immunomodulatory effects of MSCs and increase the efficacy of MSCs in preclinical models by mimicking the inflammatory disease model compared to the non-stimulated MSCs. Based on this, recent studies have been proposed to stimulate the MSCs with inflammatory cytokines, hypoxia, chemical compounds, or toll-like receptors (TLR) to improve their therapeutic potential (Martin-Rufino et al., 2019). These stimulatory mechanisms are targeted based on the microenvironment to which MSCs are exposed upon infusion in clinical trials with systemic inflammation or altered immune responses. These altered immunomodulatory and therapeutic properties due to stimulation were also imparted to the MSC secretome and extracellular vesicles (Domenis et al., 2018). These

stimulatory factors were shown to enhance MSC properties by a) increase in the inflammatory cytokine secretion such as IL-10, TGF-β, and IDO which increase Treg cell population, b) carrying the specific microRNA which involved in polarization of macrophages to M2 anti-inflammatory phenotype with increased phagocytic capacity and c) increase growth factor secretion such as VEGF, HGF, IGF, KGF, and SDF1 through different signaling pathways (Martin-Rufino et al., 2019). Mastri M et al., 2012 showed that, stimulation of MSCs with TLR 3 agonist [poly(I:C)] effectively regenerated the hamster heart at 1 million cells/kg concentration, whereas, untreated MSCs were able to show these effects at 40 million cells/kg concentration. This cardiac improvement was associated with increase in angiogenesis, reduction in fibrosis and apoptosis through multiple mitogen-activated protein kinase pathways. Giuliani et al., 2014 demonstrated that TLR 3 stimulation protects the MSCs from lysis by NK cells. Ti et al., 2015 demonstrated that LPS preconditioned MSC derived extracellular vesicles will enhance the M2 polarization of macrophages by increase in let-7b miRNA expression through upregulation of signal transducer and activator of transcription 3 (STAT3) and AKT pathways. Varkouhi et al., 2019 showed that interferon-γ primed MSC extracellular vesicles were effectively treated the *E. coli* induced acute lung injury than non-treated MSC extracellular vesicles through change in phagocytic capacity of macrophages. Lombardo et al., 2009 showed that TLR 3 agonist stimulation, but not TLR 4 enhanced the anti-inflammatory factors such as IDO activity. Monsel et al., 2015 described that TLR 3 pre-stimulated MSCs further enhanced the therapeutic effects of MSC derived microvesicles against mice acute lung injury by increasing the expression levels of cyclooxygenase 2 (COX2) and IL-10.

Summary

As outlined, MSCs and MSC-derived exosomes have properties that make them a potential therapeutic option in regenerative medicine. With their added advantages, exosomes are a better choice to use as a cell free therapy. Developing exosome-based treatments relies on better understanding their secretion into the secretome, growth conditions for optimal number and standard isolation procedures. Addressing these drawbacks can dramatically help in using exosomes as better therapeutic option compared to MSCs. The homing and mechanism of action for exosomes is not completely understood, but it is known that they will reach target tissue by paracrine signaling and release their cargo into the cells leads to immunomodulatory and anti-inflammatory properties. Exosomes derived from MSCs are known to show their immunomodulatory properties by altering both innate and adaptive immune cells, changing phenotype of macrophages to M2 phenotype, increase in Treg cells, and other secreted growth factors. Stimulation of MSCs with TLR 3 agonist alters the secretion of pro-inflammatory cytokines to anti-inflammatory cytokines and enhance immunomodulatory and antimicrobial activities. But effect of stimulation on exosomes is still unclear. As a result, MSC-derived exosomes are considered as a therapeutic option for several disease models. Current study evidence that MSC-derived exosomes have antibacterial effects both *in vitro* and *in vivo* studies. This is the first study to demonstrate the antibacterial effects of exosomes against both Gram negative and Gram-positive bacteria *in vitro* and also in *E. coli* induced acute lung injury mouse model. Further research is needed to be conducted using in animal models to obtain a more comprehensive picture of using exosomes as safe cell free therapy and efficacy of exosomes in *in vivo* clinical trials and role of TLR 3 stimulation in altering the immune responses.

Chapter 2 - Antibacterial effects of mesenchymal stromal cell-derived exosomes *in vitro* and in an *Escherichia coli*-induced acute lung injury mouse model

Introduction

Acute lung Injury (ALI) is a major cause of acute respiratory failure with high mortality rate in critically ill patients. The most common causes of ALI are bacterial, viral and mechanical/stress causing factors (Loy et al., 2019; Lucas et al., 2009; Wu et al., 2014). The inflammatory response cause damage to the alveolar endothelium and epithelium leads to increased protein permeability and reduced alveolar fluid clearance result in pulmonary edema (Lee et al., 2019; Lucas et al., 2009). The standard of care that is available has limited therapeutic potential as evidenced by long term deleterious effects like antibacterial resistance, loss of lung function secondary to interstitial fibrosis (Gupta et al., 2012; Jackson et al., 2016; Krasnodembskaya et al., 2010; Russell et al., 2020). Recent studies are focusing mainly on compounds that can directly kill bacteria such as antimicrobial peptides (AMPs) rather than altering their metabolic pathways (Harman et al., 2017; Zhao et al., 2013).

Mesenchymal Stromal Cells (MSCs) constitute an important source of cells for regenerative approaches for the past 30 years. Mounting evidence in the form of numerous publications has shown therapeutic potential against multiple inflammatory diseases. Mesenchymal Stromal Cells can be isolated mainly from bone marrow, adipose tissue, umbilical cord tissue, cord blood, placenta, menstrual blood, cornea, spleen, hair, skin, brain, dental pulp, intestines, liver, and skeletal muscles (Crisan et al., 2008; Hass et al., 2011; in ’t Anker et al., 2004; Zuk et al., 2002). They are self-renewable, plastic adherent, fibroblast like cells with

multilineage differentiation potential (Caplan, 1991). They express surface markers positive for CD105, CD73, and CD90 and negative for surface markers CD45, CD34, CD14, CD11b, CD79a, and CD 19. Currently, more than 600 clinical trials are ongoing to evaluate the safety and efficacy of MSCs (www.clinicaltrials.gov). The therapeutic potential of MSCs is well documented in a wide range of inflammatory diseases including graft versus host disease (Fang et al., 2007), multiple sclerosis (Shamili et al., 2019), acute renal failure (Morigi et al., 2016), systemic lupus (Zhou et al., 2020), myocardial infarction (Bai et al., 2010), cystic fibrosis (Sutton et al., 2016), acute lung injury (Islam et al., 2012), traumatic brain injury (Zhou et al., 2019), amyotrophic lateral sclerosis (Bonafede et al., 2016) and hindlimb ischemia (Moon et al., 2006). In addition, recently MSCs gained significant importance due to their antimicrobial properties against influenza A H5N1-associated acute lung injury (Chan et al., 2016; Cortés-Araya et al., 2018; Khatri et al., 2018; Loy et al., 2019), *E. coli* induced pulmonary pneumonia (Alcayaga-Miranda et al., 2017; Chow et al., 2020; Gupta et al., 2012; Jeyaseelan et al., 2007; Krasnodembskaya et al., 2010; Monsel et al., 2015). These therapeutic properties were shown to be mediated through immunomodulation and direct cellular interactions (Johnson et al., 2017). They have the potential to suppress both T cell and B cell proliferation and increase regulatory T (Treg) cell population (Chow et al., 2020).

Adipose tissue derived mesenchymal stromal cells (ASCs) are represented as better alternative source for adult MSCs as they can be obtained by minimal invasive procedures (Bora & Majumdar, 2017). Studies have showed that the immunomodulatory and therapeutic potential of ASCs were similar to other sources of MSCs, in addition ASCs have added advantages over MSCs derived from bone marrow and umbilical cord. The main advantages of ASCs are they can be easily harvested with minimal invasive procedures, abundant sources of availability, and they

can be easily cultured with high proliferation capacity (Gimble et al., 2013; Luna et al., 2014). Studies showed that adipose tissue yields MSCs at 500- fold higher than from bone marrow (Bourin et al., 2013; Freitag et al., 2020). Previous studies characterized that ASCs show positive for stromal markers such as CD10, CD13, CD73, CD90, CD105, VEGFR-1 and Nanog and ASCs do not express for surface markers CD45, CD34 and CD11b (Bourin et al., 2013; Luna et al., 2014). Studies showed that ASCs have more immune evasive and immunomodulatory potential compared to bone marrow MSCs such as low expression levels of HLA class-1 molecule, suppress dendritic cell, NK cell differentiation, and has shown to promote the Treg generation compared with the bone marrow MSCs makes them better alternative for clinical trials (Aggarwal & Pittenger, 2005; Caplan & Dennis, 2006; Cortés-Araya et al., 2018; Jimenez-Puerta et al., 2020; Sangiorgi & Panepucci, 2016; Wang et al., 2014).

Recent studies are showing that these therapeutic properties of MSCs were mainly due to the secretome released into the extracellular environment rather than their tissue engraftment ability (Bonafede & Mariotti, 2017). After injecting MSCs in clinical trials, they were shown to release secretome components which helps in tissue regeneration. Secretome contains growth factors like hepatocyte growth factor (HGF) , tumor necrosis factor-inducible gene 6 protein (TSG6), vascular endothelial growth factor (VEGF), fibroblast growth factor (FGF), insulin-like growth factor 1 (IGF-1), C-C chemokine ligand – 2(CCL-2), epidermal growth factor (EGF), platelet derived growth factor (PDGF), cytokines, such as IL-10, TGF-β, prostaglandin E2 (PGE2), Indoleamine 2,3-dioxygenase (IDO), and stromal cell-derived factor 1 (SDF-1) (Jimenez-Puerta et al., 2020). In addition to these, MSCs release extracellular vesicles (EVs) which includes exosomes, microvesicles (MVs), and apoptotic bodies (Varderidou-Minasian & Lorenowicz, 2020). These secretome contents can reach the target tissue by paracrine signaling.

It has been widely accepted that MSC secretome has wide variety of bioactive factors that have similar apoptotic, angiogenic, tissue regenerative and differentiation properties (Martin-Rufino et al., 2019). Due to their paracrine mechanism *via* secretome Caplan, 2017 proposed to rename MSCs as “medicinal signaling cells”. Studies in the last few years had been mainly focusing on using EVs which includes exosomes and microvesicles.

Exosomes are nanosized phospholipid bilayered small EVs that carry the cargo of MSCs such as microRNAs, RNAs, proteins and lipids (Shen et al., 2018). They express surface proteins CD63, CD9, CD81 and Alix (Martin-Rufino et al., 2019). After releasing from the MSCs, exosomes reach target cells/injured tissue by paracrine mechanism and enter into the cells by endocytosis to release the cargo (Shen et al., 2018). Exosomes have several advantages over MSCs, due to nano size and they cannot self-replicate in the host tissues. In addition, they can be scalable to the required concentrations and easily stored at -80°C in PBS without any cryopreservation media which will make them off the shelf cell free therapeutics (Bonafede & Mariotti, 2017). Due to their smaller size compared to cells, exosomes were shown to cross the microvasculature and blood brain barrier (BBB) (Zhou et al., 2019). The reports showed that MSC-derived exosomes have therapeutic effects against acute hepatitis (Li et al., 2020), myocardial ischemia (Zhang et al., 2016), acute kidney injury (Zhou et al., 2019), bleomycin induced pulmonary fibrosis (Mansouri et al., 2019), wound healing (Nakamura et al., 2015), neurodegeneration (Xin et al., 2017), experimental silicosis (Bandeira et al., 2018), and autoimmune disorders (Baharlooi et al., 2020). Previous studies showed that these therapeutic effects mediated through the cell specific exosome cargo. The proteins and functional microRNAs present in exosomes proved to have tissue repair, regeneration, angiogenic, and immunomodulatiory properties similar to MSCs (Bandeira et al., 2018; Cai et al., 2020;

Varderidou-Minasian & Lorenowicz, 2020; Yin et al., 2019). They can also be genetically engineered to deliver desired cargo to the specific disease models (Baraniak & McDevitt, 2010; Cassatella et al., 2011; Mastri et al., 2012; Varderidou-Minasian & Lorenowicz, 2020). In this study, we proposed to evaluate the antibacterial effects of ASC-derived exosomes and demonstrate that the antimicrobial properties of MSCs are exosome derived.

Producing MSCs that can meet clinical applications is considered as a challenge in regenerative medicine. Recent studies showed that MSCs can be stimulated by altering their microenvironment with certain inflammatory cytokines and toll like receptors to enhance their therapeutic effects. These stimulatory factors will create the inflammatory microenvironment and enhance the immunomodulatory and therapeutic properties of MSCs to particular pathogen type (Varkouhi et al., 2019; Chen et al., 2014; Krasnodembskaya et al., 2010; Monsel et al., 2015). Studies showed that the therapeutic effects can be achieved with less number MSCs by stimulation, which make them safer to use in clinical trials (Mastri et al., 2012). Recently, MSCs are shown to express toll-like receptors which can recognize pathogen associated molecular patterns (Petri et al., 2017; Rashedi et al., 2017; Sangiorgi & Panepucci, 2016). Stimulation of MSCs with a TLR 3 agonist were shown to secrete anti-inflammatory cytokines IL-10, PGE2, and TGF- β. Toll-like receptor 3 agonist stimulation also enhances the Treg cells and increase the secretion of VEGF, IGF, HGF and IDO (Baraniak & McDevitt, 2010; Jimenez-Puerta et al., 2020; Martin-Rufino et al., 2019). This will also alter the phenotype of macrophages from pro-inflammatory M1 to anti-inflammatory M2 phenotype with high phagocytic capacity (Martin-Rufino et al., 2019). Studies showed that stimulation of MSCs will also enhance immunomodulatory and therapeutic properties of MSC-EVs (Domenis et al., 2018). Monsel et al. 2015 demonstrated that stimulation MSCs with TLR 3 agonist enhanced the bacterial clearance

in mice pneumonia study. Accordingly, we postulated that stimulation of ASCs with TLR 3 agonist might enhance the antibacterial activity of ASC-derived exosomes.

The irrelevant usage of antibiotics and gene mutations are increasing the incidence of antibiotic resistant bacterial infections (Chow et al., 2020). Recent studies are focusing on alternative therapies in place of conventional antibiotics. Due to their anti-inflammatory and immunomodulatory role MSCs are considered as better alternative therapy. Hence, it is important to evaluate the pathways underlying MSC antimicrobial activity. Mesenchymal stromal cells are able to enhance the phagocytic capacity of macrophages and escape the neutrophils from apoptosis and enhance their migration(Devaney et al., 2015). Studies showed that MSCs can produce antimicrobial peptides that kill the bacteria by disrupting the integrity of membrane or by altering the immune responses. Mesenchymal stromal cells release AMPs such as cathelicidin peptide LL-37, hepcidin, β-defensin and lipocalins that are able to control the chronic bacterial infections (Alcayaga-Miranda et al., 2017; Gupta et al., 2012; Harman et al., 2017; Krasnodembskaya et al., 2010). Previous studies demonstrated that antimicrobial effects of MSCs and MSC-EVs were also mediated through transfer of mitochondria to target cells and alter their cytokine gene expression and secretion and increase phagocytosis of macrophages (Islam et al., 2012; Jackson et al., 2016). This antibacterial effect of MSCs and MSC secretome were also shown to have therapeutic properties in *in vivo* clinical trials (Cortés-Araya et al., 2018; Harman et al., 2017; Krasnodembskaya et al., 2010; Monsel et al., 2015). However, the antimicrobial properties of MSC-derived exosomes still remain unknown.

In this study, for the first time we investigated the antibacterial effects of ASC-derived exosomes in both *in vitro* and *in vivo* models. We hypothesized that ASC-derived exosomes will show antibacterial activity similar to their parent ASCs against Gram negative (*Escherichia coli*

and *Klebsiella pneumoniae*) and Gram positive (*Staphylococcus aureus* and *Rhodococcus equi*) bacteria in an *in vitro* model. Monsel et al. 2015 showed that, TLR 3 agonist stimulation of human MSCs enhanced the antibacterial effects of human MSC-derived extracellular vesicles. Hence, we also hypothesized that TLR 3 agonist stimulation will enhance the antibacterial activity of ASC exosomes. We assessed the antibacterial activity by measuring bacterial growth using optical density readings and culture counts by spread plate method. An acute lung injury mouse model elicited by intratracheal injection of *E. coli* to evaluate the therapeutic efficacy of ASC-derived MSC exosomes. Bronchoalveolar lavage (BAL) fluids were collected to assess the bacterial clearance and alveolar protein permeability. This is the first study to demonstrate the antibacterial activities of MSC-derived exosomes in Gram negative and Gram-positive bacteria and in a mouse acute lung injury model.

Material and methods

Isolation and culture ASCs

Adipose mesenchymal stromal cells (ASCs) used in this study were isolated from three donor horses using aseptic technique at Kansas State University Manhattan, Kansas, USA. The fat tissue was collected in a sterile 1X Dulbecco's phosphate buffered saline (DPBS) with 1% antibiotic-antimycotic and processed within 24 hours after collection. The tissue was initially transferred to petri plates to mechanically separate muscle and fascia and washed with 1X DPBS to remove blood. The tissue was weighed and minced into manageable pieces using sterile scissors inside a biosafety cabinet. The tissue was digested using 0.1% collagenase solution for 30-40 minute at 37°C in a water bath with occasional shaking. After the fat tissue was digested, excess digestion was neutralized using 10 ml of 10% fetal bovine serum (FBS) culture media. The digested fat tissue was centrifuged at 720 g for 10 minute and the supernatant was discarded.

The cell suspension was washed twice with 1 X DPBS and strained through 100 µm cell strainers. Cell counts for the final stromal vascular fraction were obtained using the LUNA-STEM™ (Logos Biosystem, South Korea) with Acridine Orange/Propidium Iodide (AO/PI) staining. The cells were plated at 250,000/cm^2 density in 10% FBS culture medium composed of low glucose DMEM containing 1% antibiotic-antimycotic and 10% fetal bovine serum. The culture flasks were incubated at 37°C with 95% relative humidity and 5% carbon dioxide. The culture media was replaced every 48 hours. Cells were passaged by using 0.1 % trypsin EDTA when they reached 80%-85% confluence. Manual cell counts were obtained using a Hemocytometer with trypan blue staining and plated at 10,000 cells/cm^2. The antibiotic-antimycotic was removed from the culture media after passage 2. The cells used in this study were from passage 3-7. The culture media was collected and stored at -80°C for exosome isolation.

Cryopreservation and thawing of ASCs

At passage 2 to 4, cells were cryopreserved using a 1:1 dilution of 10% FBS culture media and 2X cryomedia composed of 40% FBS, 40% low glucose DMEM and 20% dimethyl sulfoxide (DMSO). Cryotubes were placed in a Mr. Frosty freezing device and transferred to -80°C freezer immediately after adding freeze medium. Within 24 hours, the cryotubes were taken out from Mr. Frosty and placed in -80°C freezer box and within one week transferred to liquid nitrogen tank for long term storage.

For thawing of the cells, cryotubes were placed up to the middle of the tube into a water bath at 37°C for approximately 1 minute. The cryotube was transferred to biosafety cabinet when small ice crystal was still noticed and immediately added 1 ml of 10% FBS culture media to the cryotubes. After 3 minutes, cells in the cryotube were added in a drop wise manner into 10 ml of

10% FBS culture medium prewarmed to 37°C. Cells were centrifuged for 5 minutes 200 x g at room temperature. After centrifugation, supernatant was discarded, and cells were resuspended in 1 mL of 10% FBS culture medium. The cells were counted using 0.4% trypan blue staining method. All cell lines were expanded post thaw with high recovery and viability rates.

Stimulation of ASCs

Previous studies described that MSCs express the TLR 3 mRNA and stimulation of MSCs with TLR 3 agonist enhance the anti-inflammatory and immunomodulatory role of MSCs (Sangiorgi & Panepucci, 2016). Monsel et al. 2015 showed that pre-stimulation of MSCs increased the expression of anti-inflammatory cytokines and enhanced the bacterial clearance. For TLR 3 stimulation, ASCs were plated at a density of 20,000 cells/cm^2 in T-175 plate in culture media for 24 hours. Cells were treated with polyinosininc-polycytidylic acid (poly I:C LMW), a TLR 3 agonist at 10 µg/ml concentration for 24 hours (InvivoGen, San Diego, CA). After 24 hours, the culture media was discarded and replaced with fresh culture media. The culture media was collected after 48 hours for collection for stimulated ASC exosomes.

Isolation of exosomes

Exosomes were isolated by differential ultracentrifugation method. Briefly, the collected conditioned media was thawed to 4°C and centrifuged at 2000 X g for 20 minutes at 4°C. The supernatant was collected, and dead cell pellet was discarded. The collected supernatant was centrifuged at 10,000 X g for 30 minutes at 4°C. The supernatant was collected, and cell debris pellet was discarded. The supernatant was filtered through 0.22 µm filter to eliminate larger particles and cellular debris. The filtered conditioned media was subjected to ultracentrifugation at 100,000 X g for 70 minutes at 4°C. The supernatant was discarded, and the invisible pellet was washed with large volume of PBS. The suspended pellet was again centrifuged at 100,000 X

g for 70 minutes at 4°C to remove contaminated proteins (Théry et al., 2006). The supernatant was discarded, and the pellet was resuspended in 1 X DPBS. The exosomes were aliquoted and stored at -80°C for further studies.

Characterization and quantification of exosomes

Exosomes were characterized by using transmission electron microscopy (TEM) which will give the shape and size distribution. Nanoparticle tracking analysis was used to measure the size distribution and number of particles per ml of PBS diluted exosome sample. Bicinchoninic acid assay was used to quantify the protein concentration of the exosome sample (Théry et al., 2006; Villatoro et al., 2019). In this study, quantity of exosomes used was determined based on the protein concentration (Liu et al., 2019; Monsel et al., 2015).

Bacterial cultures

In this study, two Gram positive and two Gram negative bacteria were used to determine the antibacterial activity of ASCs, and ASC-derived exosomes. Gram positive bacteria are *Staphylococcus aureus* American Type Culture Collection (ATCC) 25923 strain and *Rhodococcus equi* Clinical bacterial isolate obtained from Kansas State Veterinary Diagnostic Laboratory (KSVDL). Gram negative *Escherichia coli* ATCC 25922 strain and *Klebsiella pneumoniae* ATCC 13883 strains were used. The bacterial isolates were stored as frozen stocks at -80°C.

Antibacterial assays *in vitro*

Bacteria from stock cultures were streaked on blood agar plates and incubated at 37°C for overnight to obtain single colonies. 100 µl of each treatment group described below was added to each in a 96 well microtiter plate. The treatment groups include 1. 1X DPBS as saline positive control, 2. mouse embryonic fibroblasts (MEFs) as cell control, 3. MEF derived exosomes (20

µg) as exosome control, 4. ASCs (32,000 cells), 5. TLR 3 stimulated AMSCs (32,000 cells), 6&7. ASC-derived exosomes at 2 µg (low) dose and 20 µg (high) dose and 8&9. TLR 3 stimulated ASC-derived exosomes at 2 µg (low) dose and 20 µg (high) dose were used. The isolated single bacterial colonies were used to prepare the initial bacterial suspension in Mueller Hinton II (MH II) broth using 0.5 McFarland standard to obtain 1-2 X 10^8 colony forming units (CFU)/ ml. Bacterial suspension was diluted to obtain approximately 1 million CFU/ml. From this final bacterial suspension 100 µl (100,000CFU) was added to each well to make the final volume 200 µl. After inoculation of bacteria, the plates were incubated in a shaker incubator at 37°C for 24 hours with continuous shaking at 100 RPM speed. Pilot studies were completed to determine the dosage of exosomes using 0.5 µg, 1 µg, 2 µg, 4 µg, 10 µg, 20 µg and 40 µg concentrations and OD readings were recorded at 0, 6, 12- and 24-hour incubations to assess the bacterial inhibition. The 2 µg concentration of exosomes exhibited significant decrease in absorbance compared to other lower doses and was chosen to use in this assay as the low dose. Monsel et al. 2015 showed using MSC-MVs secreted from 10-fold number of MSCs had comparable antibacterial effects similar to MSCs. Hence, we also used 20µg concentration to compare results with previous studies and to evaluate dose dependency of antibacterial effects. The *in vitro* antibacterial assays were replicated three times for all four bacteria using ASCs obtained from three different horses. Each time all treatment groups were co-cultured with three different bacterial cultures.

OD readings and culture counts

Optical density (OD) readings at 600 nm wavelength were recorded at 0, 12- and 24-hour incubation periods to see the growth characteristics of the bacteria. Colony counts were measured at 24-hour incubation time points. Bacterial cultures were serially diluted in MH II

broth based on OD readings and plated on blood agar plates. The blood agar plates with 30 to 300 colony forming unit were counted and the final bacterial counts were obtained based on the dilution factor.

Antibacterial assays *in vivo*

Animals

Male C57BL/6J mice (8-10 weeks, 25-30 grams) were purchased from The Jackson Laboratories, USA. All animal study protocols were approved by Institutional Animal Care and Use Committee (IACUC) of Kansas State University (KSU). Mice were housed in rodent cages in ABSL-2 comparative medicine group facilities with 24-hour access to adequate food and water. The mice study was performed twice. In the first study we used 6 mice per treatment group. Due to lack of profound experience in BAL fluid collection, we were not able to collect from all the animals uniformly, and we lose samples of few animals in each treatment group. We repeated the entire mice study using 4 mice per treatment group to have valid number of samples from experimentally sound animals and also to strengthen our results in this study. The data was analyzed using only valid samples and combined data from both studies was used for results analysis.

Escherichia coli-induced acute lung injury mouse model

The mice were anesthetized using Ketamine (100 mg/kg) and Xylazine (10 mg/kg) given intraperitoneal route. The anesthetized mice were placed in inclined supine position ~60° on an mice intubation platform. Optical light source was used for direct visualization of trachea. Mice were inoculated with approximately 1 million CFU/20 µl of *E. coli* ATCC 25922 strain intratracheal route (IT) using polyethylene – 90 tubing (PE-90). After *E. coli* inoculation, mice were randomized among different cages. After 4 hours of infection, mice were re-anaesthetized

using Ketamine and Xylazine and treated IT with 60 µl of seven different treatment groups, includes 1) PBS- as positive control, 2) MEF as cell control (1 million cells), 3) MEF exosomes (20 µg) as exosome control, 4) ASCs (1million cells), 5) TLR 3 stimulated ASCs (St. ASCs) (1 million), 6) ASC exosomes (20 µg), and 7) TLR 3 stimulated ASC exosomes (20 µg). Mice were monitored every 12 hours for 48 hours. After 48 hours, mice were sacrificed using CO2 euthanasia. Bronchoalveolar lavage (BAL) fluids were collected using 25-gauge catheter for measuring bacterial counts and protein concentration (van Hoecke et al., 2017). Briefly, post-euthanasia an incision was made along the ventral neck area near the trachea. Then, sternohyoid muscles were incised to expose the trachea. Trachea was punctured carefully in the middle using 25 G needle. A small incision was made into the trachea and a catheter made of PE-50 tubing was inserted using a 25 G needle. The catheter was passed about 0.5 cm and secured using a nylon suture material around the trachea. One ml of ice-cold PBS was injected into the lungs each time and aspirated using 1 ml syringe and repeated same procedure for three times using total of 3 ml. Samples with more than 2 ml of PBS recovery were considered as valid sample. Aliquots of BAL fluid was serially diluted and cultured on blood agar plates at 37°C for 24 hours. Colony forming unit counts were recorded. Bronchoalveolar lavage fluid was centrifuged at 1000 X rpm for 10 minutes and the supernatant was used for measuring protein concentration using Bicinchoninic protein assay.

Statistical analysis

Numerical values were expressed as mean ± standard error. Results were tested for normality distribution and compared between the groups. Comparisons between the two groups were done by using unpaired t test. Comparisons between the multiple groups were done by using one-way analysis of variance (ANOVA) test using Bonferroni or Sidak correction for

multiple comparisons. All the statistical analysis was performed by using GraphPad Prism 8.4.3. software.

Results

Isolation and culture of ASCs

The adipose tissue samples were collected aseptically from three horses of ages between 5-14 years. Approximately 10 grams of adipose tissue were used to obtain from each horse for ASCs isolation. Adherence of the stromal cell population was started within the first 24 hours after plating stromal vascular fraction cells. The ASCs started proliferating rapidly and fibroblast-like colonies were observed within 2-3 days and reached 80% confluence with homogenous monolayer of MSCs within 6-7 days. The cell growth, viability and morphology of the cells remained the same from passage 3 to 7 and post cryopreservation (Figure 1A).

Exosomes isolation, characterization and quantification

Morphological analysis using TEM revealed that the isolated exosomes were spheroid in shape and within ideal size distribution. The quantity of number of particles per ml and quality of size distribution of isolated exosomes were analyzed by nanoparticle tracking analysis. The size distribution of exosomes with mean size at 145.35 ± 2.7 nm. The number of exosomes release per million ASCs were $28.4x10^9 \pm 2.06x10^7$. The protein concentration of exosomes was determined by using bicinchoninic acid assay method. The protein concentration of one million ASC-derived exosomes was 9.316 ± 0.28 µg (Figure 2).

Equine ASCs and ASC-derived exosomes inhibits bacteria growth *in vitro*

The antibacterial effect of ASCs and ASC-derived exosomes was investigated in an *in vitro* model by co-culturing bacteria with different treatment groups. Adipose mesenchymal stromal cells significantly inhibited bacterial growth compared with MEFs and positive control.

Adipose mesenchymal stromal cells inhibited the growth of *E. coli* by 80-fold. Whereas, for other bacteria *K. pneumoniae, S. aureus* and *R. equi,* ASCs greatly inhibited the growth of bacteria (Figure 1). Exosomes at 2 µg dose inhibited the growth of *E. coli, K. pneumonia, S. aureus* and *R. equi* by 2-,3-,18-, and 500-fold, respectively. Exosomes at 20 µg concentration inhibited growth of *E. coli* by 100- fold and greatly inhibited the growth of *K. pneumoniae, S. aureus* and *R. equi*. ASC exosomes at 20 µg high dose resulted in bacterial growth inhibition similar to the ASCs cell treatment.

Stimulation of ASCs with TLR 3 agonist *in vitro*

Stimulation of ASCs with TLR 3 agonist increased the number of exosomes released per million cells by 2-fold. Measurement of protein concentration also revealed that stimulation of ASCs enhanced exosomes secretion by 2-fold (Figure 2). Toll-like receptor 3 agonist stimulated ASCs and ASC exosomes significantly inhibited the growth of *E. coli, K. pneumonia, S. aureus* and *R. equi* compared to positive control. However, TLR 3 agonist stimulation did not show significant changes in the antibacterial effects compared to non-stimulated ASCs and ASC exosomes (Figure 4).

Both ASCs and ASC exosomes reduced bacterial load in acute lung injury mouse model

The bacterial counts in BAL fluid reduced significantly in ASCs and ASC exosome treated groups compared to positive control, MEFs cell control and MEF exosomes control groups (Figure 7). Adipose mesenchymal stromal cells and TLR 3 stimulated ASCs (1million cells) reduced BAL fluid *E. coli* bacterial counts significantly by 50-fold and 54-fold compared to positive control, respectively. Exosomes at 20 µg concentration inhibited the *E. coli* count by

17-fold. Toll-like receptor 3 stimulation did not show any significant bacterial inhibition compared to non-stimulated ASCs and ASC- exosomes (Figure 8).

Protein concentration in BAL fluid decreased in ASCs and ASC exosome treatment groups

Treatment with ASCs and ASC exosomes significantly lowered the protein concentration levels of BAL fluid (Figure 8). Both ASCs and ASC-exosomes showed significant decrease in protein concentration 0.71± 0.23 mg/ml and 0.79± 0.28 mg/mL, respectively compared to positive control 1.39± 0.17 mg/ml. Mouse embryonic fibroblast cell control and MEF-derived exosome control treatment groups did not show any significant difference from positive control group (Figure 9).

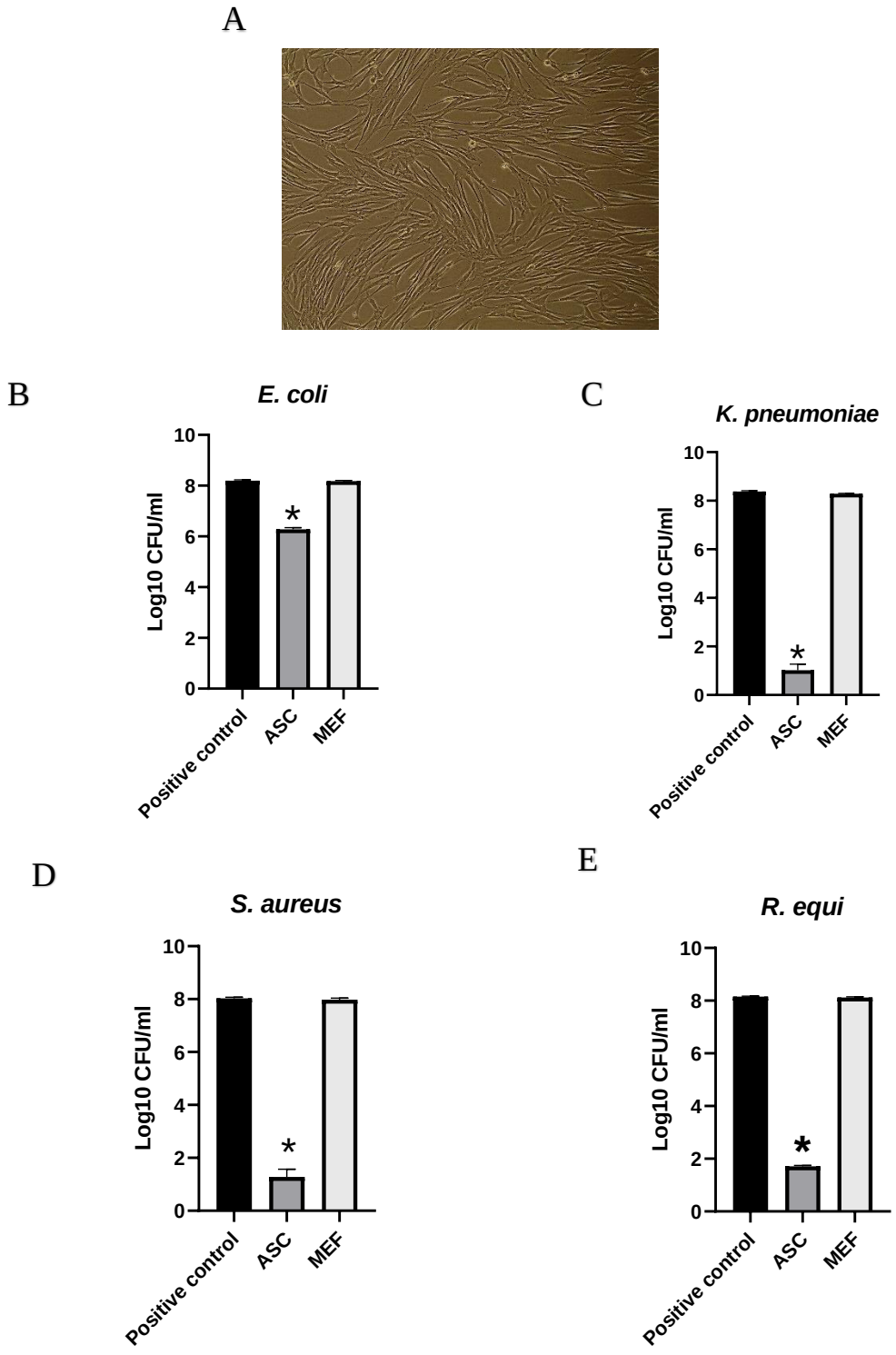

Figure 1. *In vitro* evaluation of antibacterial effects of ASCs.

(A) ASCs culture image at 10X. Antibacterial effects of ASCs against E. coli (B), K. pneumoniae (C), S. aureus (D), and R. equi (E) after 24-hour incubation. Data are presented as mean + SE and n=3. * means significantly difference with $p < 0.0001$ versus control groups by one-way ANOVA.

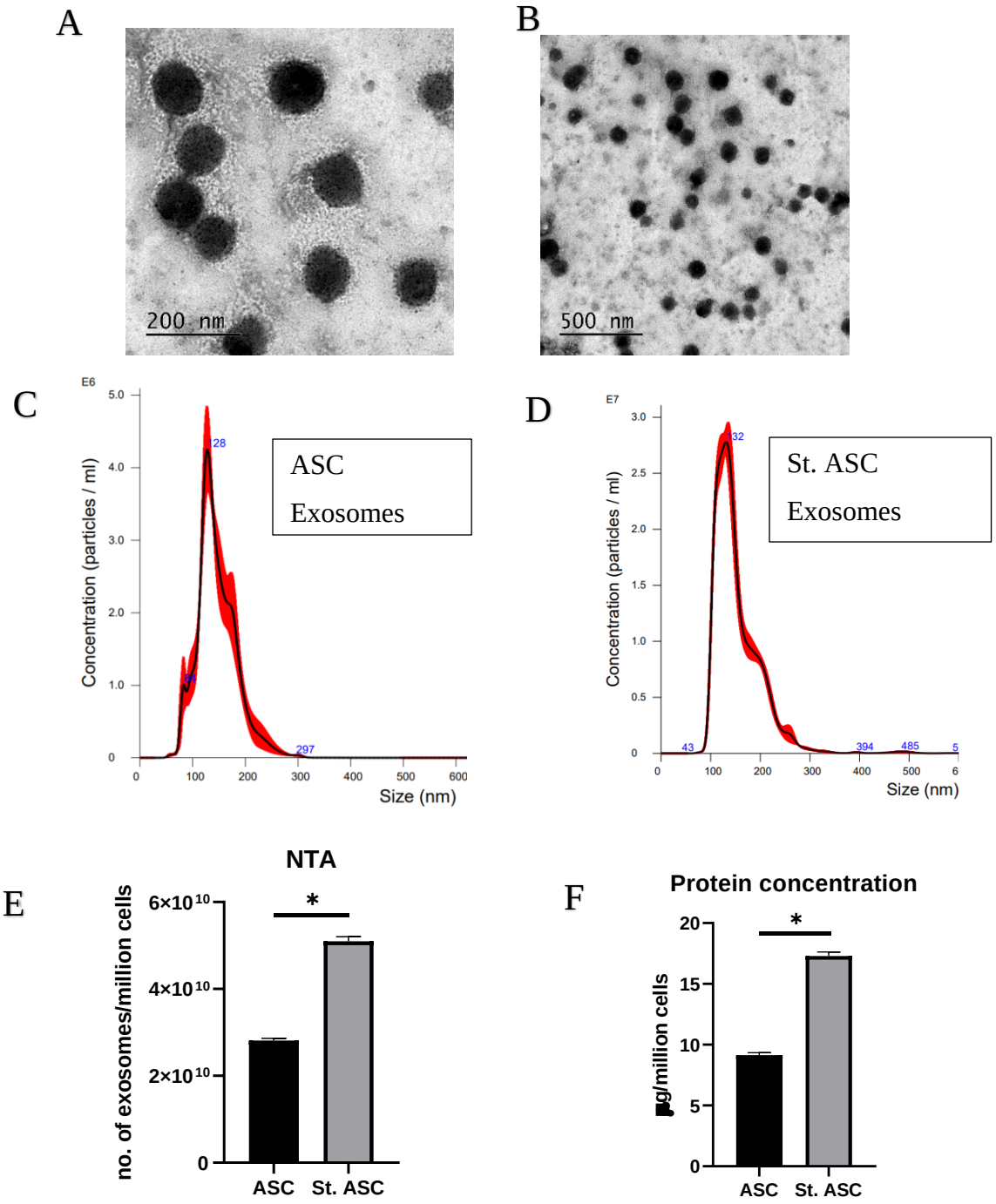

Figure 2. Characterization and quantification of ASC exosomes.

(A) TEM images of ASC exosomes with 200 nm bar and (B) 500 nm bar. (C) NTA analysis for size distribution of ASC exosomes and (D) TLR 3 stimulated ASC (St. ASC) exosomes. (E) Concentration of exosomes per million cells using NTA. (F) Protein concentration of exosomes per million cells using BCA assay. Data are presented as mean + SE and n=5. * means significantly difference with $p < 0.0001$ between groups by unpaired t test.

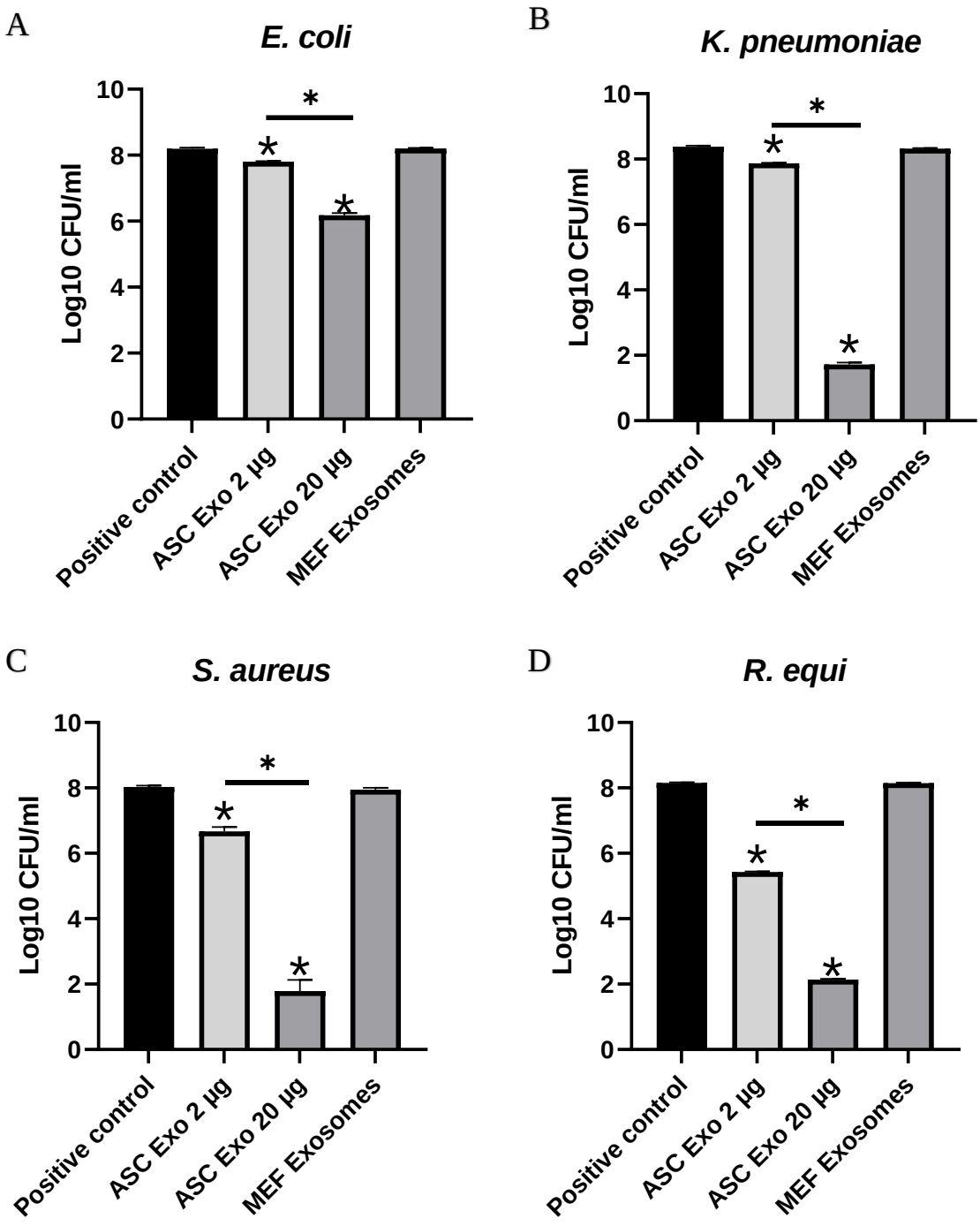

Figure 3. ASC-derived exosomes inhibited bacterial growth *in vitro*.

Co-culture of exosomes derived from ASCs inhibited growth of *E. coli* (A), *K. pneumoniae* (B), *S. aureus* (C), *and R. equi* (D) in a dose dependent manner. Data are presented as mean + SE and n=3. * means significantly difference with $p < 0.0001$ versus control groups by one-way ANOVA.

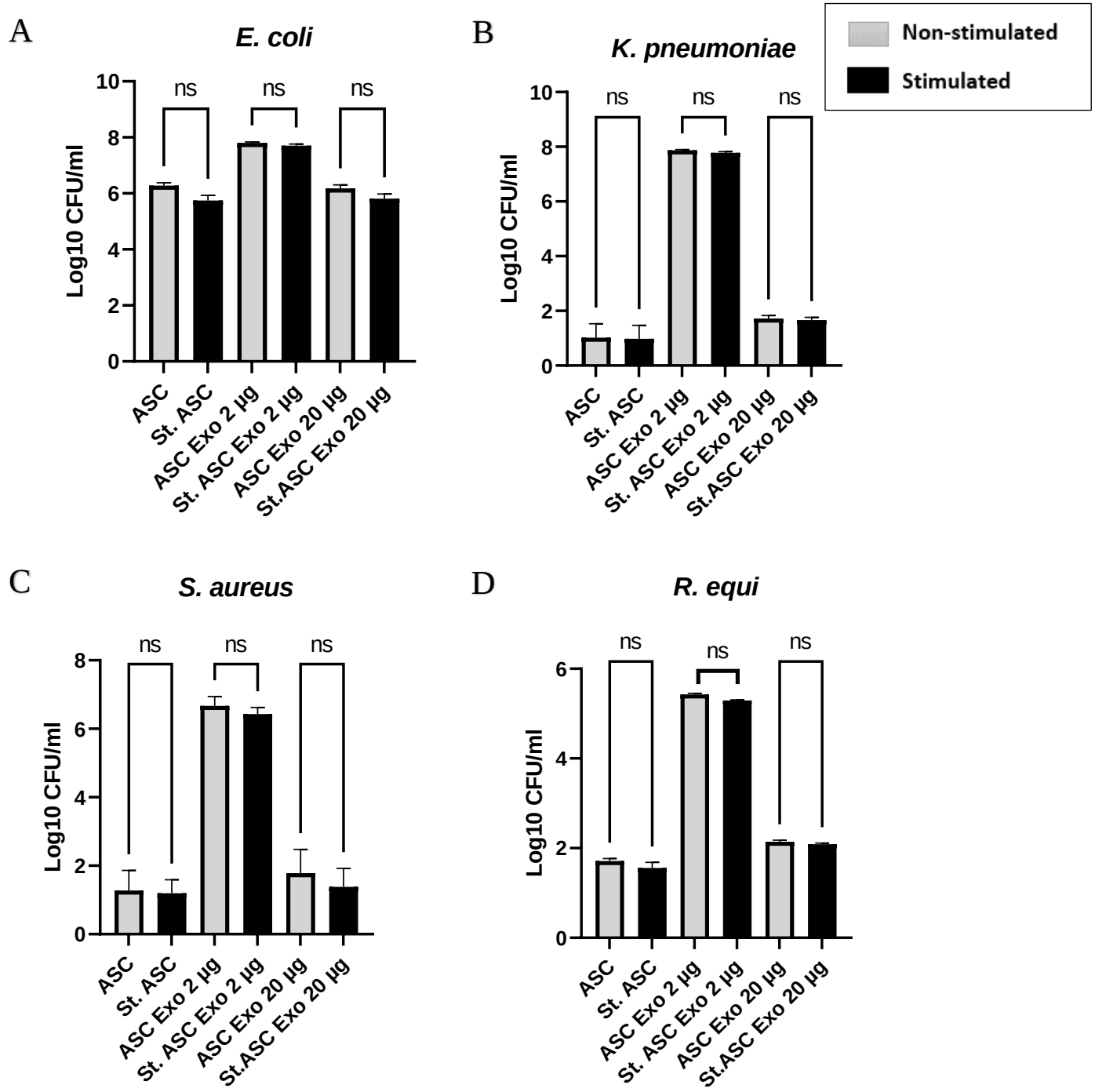

Figure 4. Role of TLR 3 agonist stimulation in *in vitro* bacterial inhibition of ASCs.

Comparison between TLR 3 agonist stimulated and non-stimulated ASCs and ASC exosomes bacterial inhibition against *E. coli* (B), *K. pneumoniae* (C), *S. aureus* (D), and *R. equi* (E) after 24-hour incubation. Data are presented as mean + SE and n=3. 'ns' means no significant difference between compared groups by one-way ANOVA.

Table 1. Bacterial CFU/ml counts with different treatment groups after 24 hours of incubation.

Treatment Group	***E. coli*** **CFU/ml**	***K. pneumoniae*** **CFU/ml**	***S. aureus*** **CFU/ml**	***R. equi*** **CFU/ml**
Positive control	1.61E+08	2.46E+08	1.11E+08	1.44E+08
MEFs (Cell control)	1.54E+08	1.96E+08	1.01E+08	1.35E+08
MEF Exosomes (Exosome control)	1.62E+08	2.02E+08	9.49E+07	1.41E+08
ASC	2.11E+06	2.31E+01	5.02E+01	5.27E+01
TLR 3 Stimulated ASC	7.11E+05	2.02E+01	2.84E+01	3.98E+01
ASC Exosomes Low (2 µg)	6.38E+07	7.62E+07	6.39E+06	2.70E+05
ASC Exosomes High (20 µg)	1.67E+06	5.70E+01	2.04E+02	1.40E+02
TLR 3 Stimulated ASC Exosomes Low (2 µg)	5.16E+07	6.31E+07	3.19E+06	1.97E+05
TLR 3 Stimulated ASC Exosomes High (20 µg)	7.78E+05	4.90E+01	5.71E+01	1.24E+02

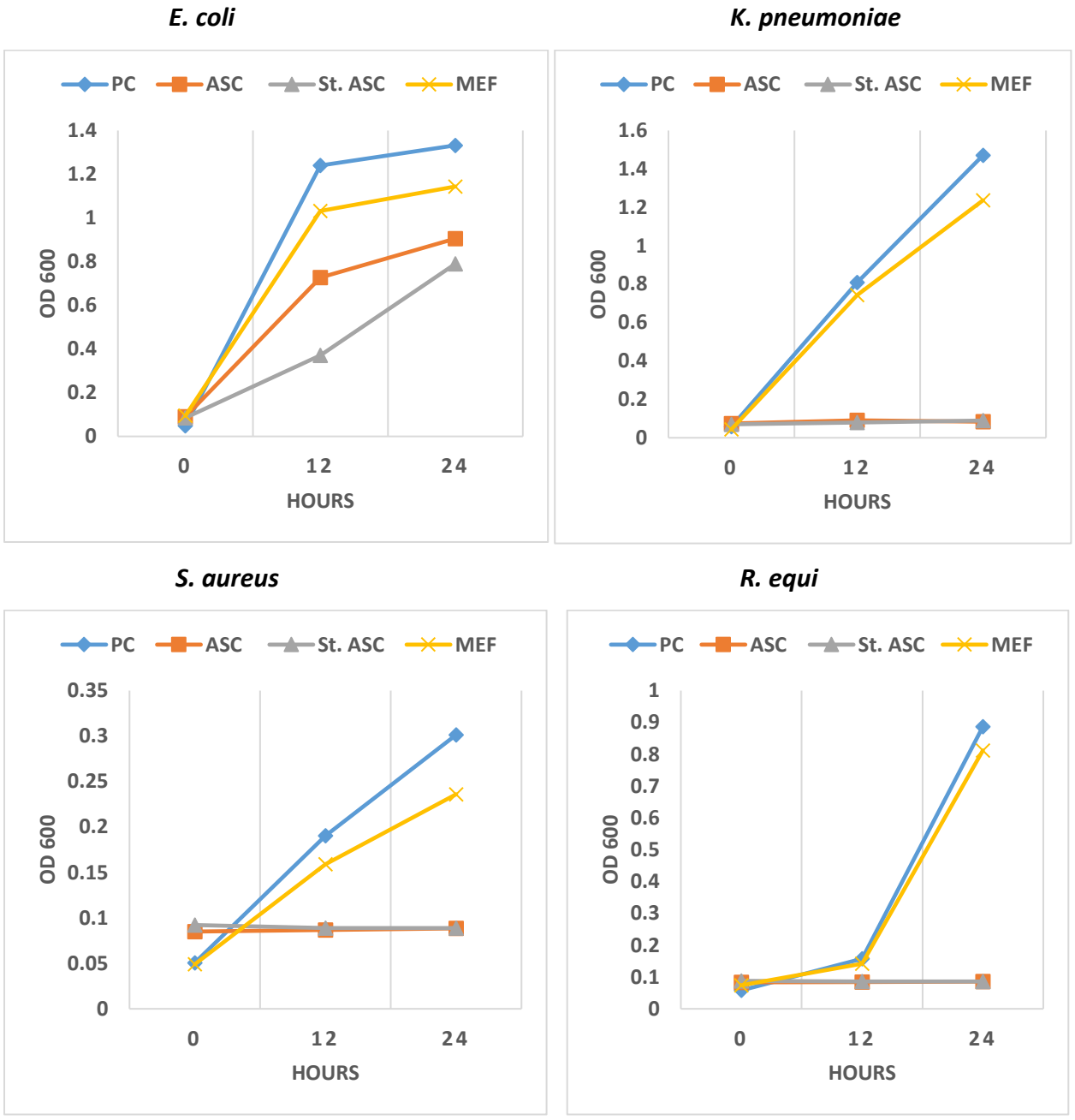

Figure 5. Optical density readings at 600 nm of bacterial with cell treatment groups.

The OD reading measurements at 600 nm for bacterial cultures treated with ASCs and TLR 3 agonist stimulated ASCs compared with positive control (PC) and mouse embryonic fibroblasts (MEF) at 0, 12, and 24-hour incubation periods. Data are presented as mean and n=3.

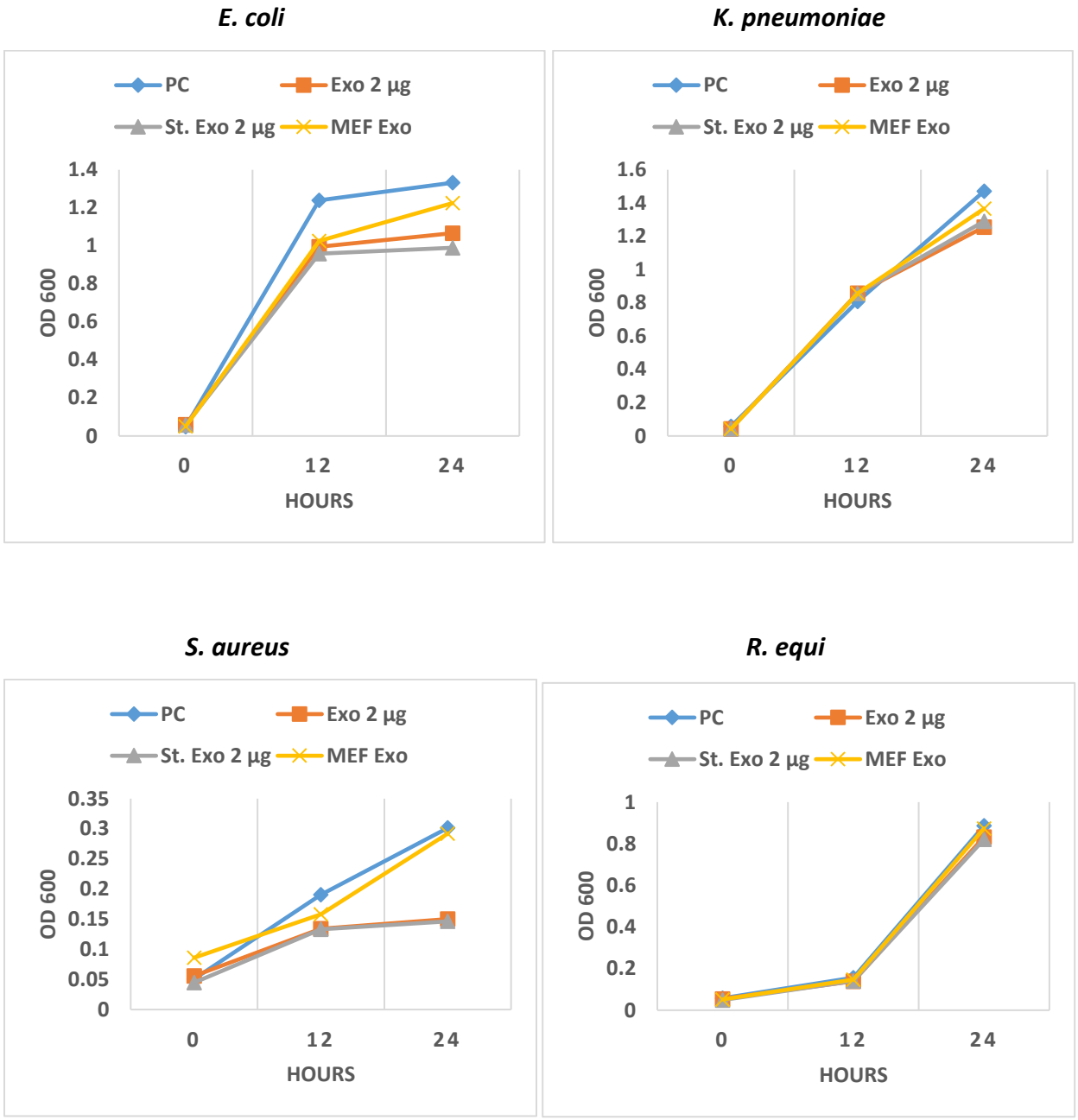

Figure 6. Optical density readings at 600 nm of bacterial with exosome treatment groups at 2 µg concentration.

The OD reading measurements at 600 nm for bacterial cultures treated with ASC-derived exosomes (Exo) and TLR 3 agonist stimulated ASC exosomes (St. Exo) at 2 µg concentration compared with positive control (PC) and mouse embryonic fibroblast exosomes (MEF Exo) at 0, 12, and 24-hour incubation periods. Data are presented as mean and n=3.

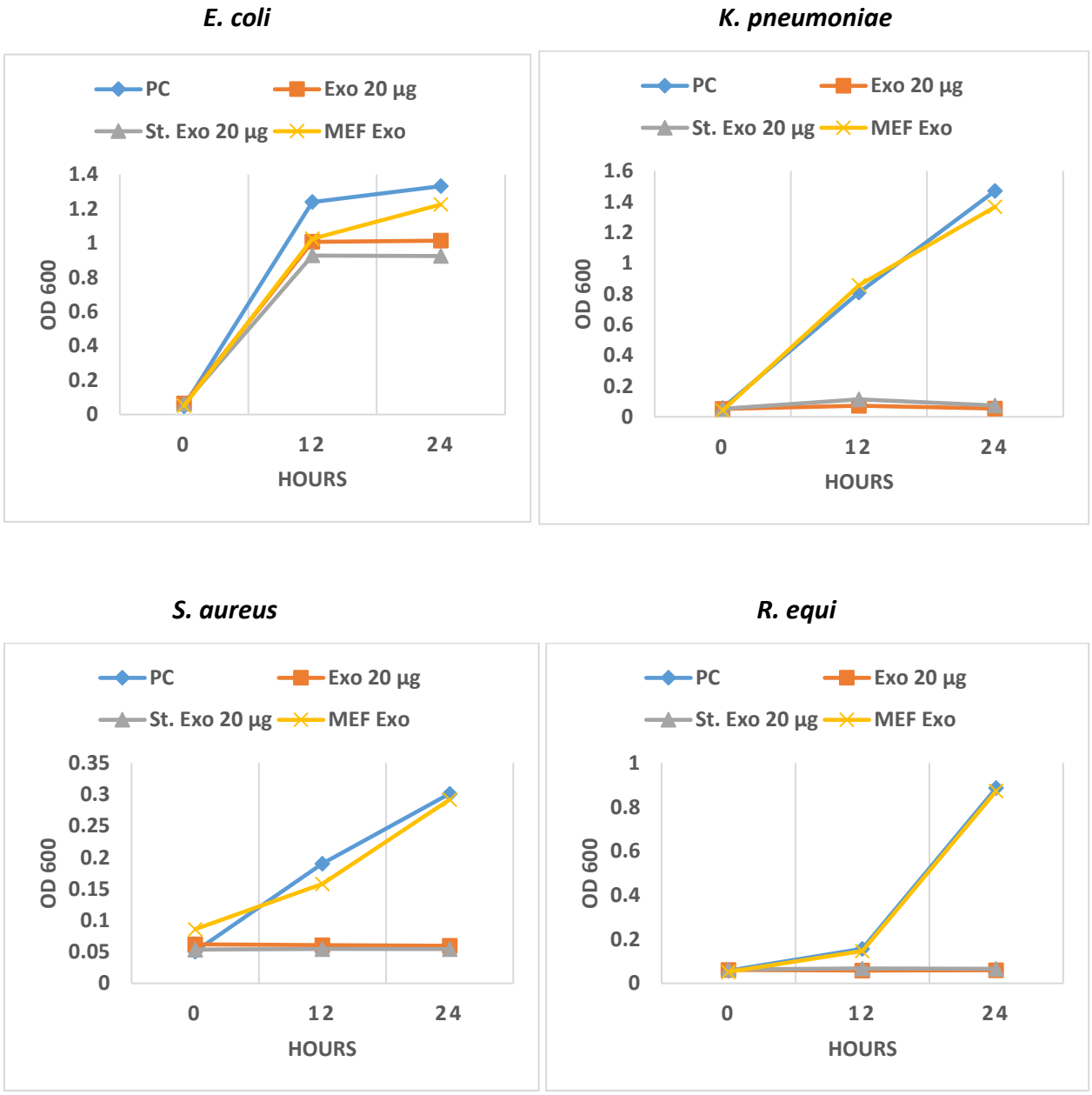

Figure 7. Optical density readings at 600 nm of bacterial with exosome treatment groups at 20 µg concentration.

The OD reading measurements at 600 nm for bacterial cultures treated with ASC-derived exosomes (Exo) and TLR 3 agonist stimulated ASC exosomes (St. Exo) at 20 µg concentration compared with positive control (PC) and mouse embryonic fibroblast exosomes (MEF Exo) at 0, 12, and 24-hour incubation periods. Data are presented as mean and n=3.

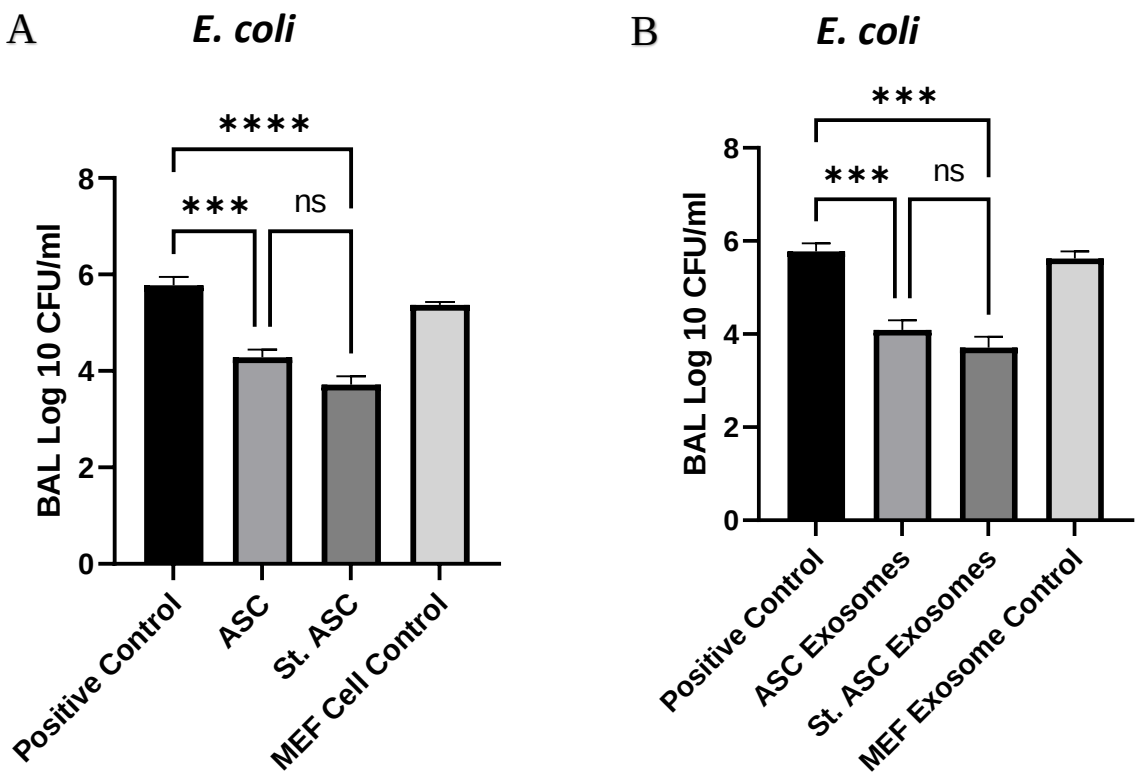

Figure 8. *In vivo* evaluation of antibacterial effects of ASCs and ASC-exosomes.

Bacterial counts of *E. coli* BAL fluid *E. coli* expressed in log10 CFU/mL for (A) cell treatment groups of Positive Control (n=7), ASCs (n=7), stimulated ASCs (St. ASC) (n=7), and MEF cell control (n=5). (B) Exosome treatment groups of ASC Exosomes (n=7), Stimulated ASC exosomes (St. Exo) (n=7), and MEF exosome control (n=7). Data are presented as mean + SE. ***, p<0.001 and ****, $p < 0.0001$ versus control groups by one-way ANOVA.

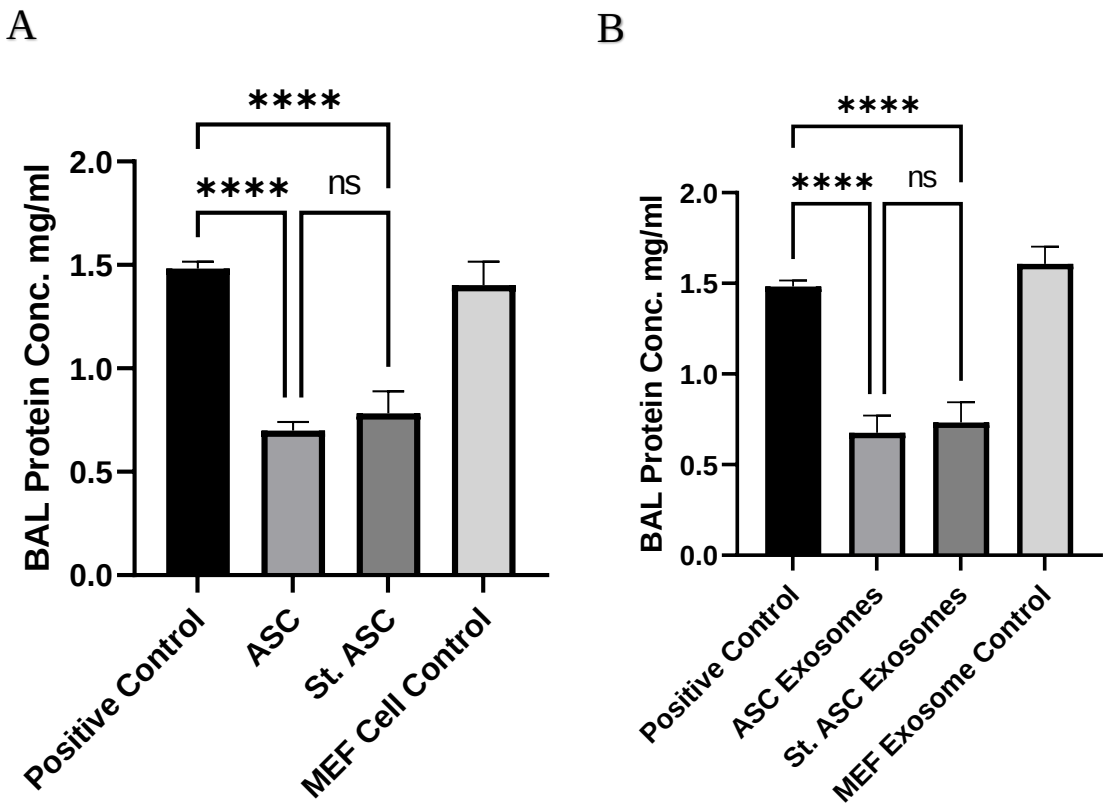

Figure 9. Protein concentration levels of BAL fluid in acute lung injury mouse model.

Protein levels expressed in mg/mL for BAL fluid collected from (A) cell treatment groups, Positive Control (n=7), ASCs (n=6), Stimulated ASCs (St. ASC) (n=6), and MEF cell control (n=5), and (B) Exosome treatment groups of ASC Exosomes (n=6), Stimulated ASC (St. ASC) exosomes (n=6), and MEF exosome control (n=6). Data are presented as mean + SE. ****, $p <$ 0.0001 versus control groups by one-way ANOVA.

Table 2. *Escherichia coli* colony forming unit counts of BAL fluid with different treatment groups in acute lung injury mouse model.

Treatment Group	***E. coli* CFU/ml Mean ±SE**
Positive control	758000±456,190
MEFs (Cell control)	287000±24500
MEF Exosomes (Exosome control)	337000±34510
ASCs	15857±2159
TLR 3 Stimulated ASCs	14214±3838
ASC Exosomes (20 µg)	22917±2105
TLR 3 Stimulated ASC Exosomes (20 µg)	19093±2450

Discussion

The major aim of this study is to evaluate the antibacterial properties of ASC exosomes in both *in vitro* and *in vivo* models. For the first time we demonstrated that ASC exosomes have antibacterial activity against both Gram negative and Gram-positive bacteria *in vitro* and in *E. coli* induced acute lung injury mouse model. The major findings of the *in vitro* study include, 1) ASCs showed significant inhibition of both Gram negative and Gram-positive bacteria compared to positive control fibroblast cell control. 2) ASC exosomes inhibited bacterial growth similar to ASCs alone. 3) ASC exosomes exhibited antibacterial effects in a dose dependent manner. 4) TLR 3 agonist stimulated ASCs enhanced the release of exosomes by 2-fold. However, stimulation did not show significant changes antibacterial of effects of ASCs and ASC exosomes. In the *in vivo* study we demonstrated that, 1) ASCs and ASC exosomes showed significant decrease in bacterial counts compared to control groups. 2) Protein concentrations of BAL fluids was significantly decreased in ASCs and ASC exosomes treated groups compared to control groups. In summary, we demonstrated that ASC-derived exosomes have antibacterial effects against both Gram negative and Gram-positive bacteria *in vitro* and, have significant antibacterial effects in *E. coli* induced acute lung injury mice model.

Mesenchymal stromal cells have been widely used in treating wide range of chronic and acute inflammatory diseases, and it is believed to be that the therapeutic effect is due to their immunomodulatory and tissue regenerative properties. In addition, it was recently shown that MSCs derived from bone marrow, umbilical cord, endometrium, and adipose tissue has antimicrobial properties against various bacteria and viruses in *in vivo* and *in vitro* studies of human, equine and canine studies (Alcayaga-Miranda et al., 2017; Chan et al., 2016; Cortés-Araya et al., 2018; Jackson et al., 2016b; Johnson et al., 2017; Loy et al., 2019; Sutton et al.,

2016). In this study, ASCs were used to evaluate these *in vitro* antibacterial effects due to their easy accessibility, higher yield, and higher proliferation rate compared to MSCs derived from bone marrow and umbilical cord (Gimble et al., 2013; Luna et al., 2014). This study also showed that ASCs exhibited antibacterial effects against both Gram negative and Gram-positive bacteria *in vitro* similar to previous studies (Figure 1). *In vitro* antibacterial effects of ASCs were determined by measuring culture counts and optical density readings of bacterial cultures. The main advantage of measuring bacterial culture counts over OD measurements is, it will enable us to know live bacteria present in culture, whereas OD measurements are based on the culture density which includes both live and dead bacteria. These antibacterial effects of MSCs was shown to be mediated by release of AMPs (Harman et al., 2017). These AMPs released were shown to act on bacteria by membrane disruption and bacterial lysis. Recent studies demonstrated that these antibacterial effects were mediated through MSC secretome and majorly through extracellular vesicles that carry MSC cargo includes nucleic acids, lipids and AMPs (Bonafede et al., 2016; Chow et al., 2020; J. Park et al., 2019). Hence, we hypothesized that ASC- exosomes have antibacterial effects comparable to ASCs alone.

The use of MSC cell therapy has gained significance in treating multiple inflammatory diseases, however there are inherent challenges associated with cell therapy which include lack of homogeneity between the passages and among different sources of MSCs, decreased viability due to cryopreservation, lack of consistency between cell treatments (Bongso & Fong, 2013; Musiał-Wysocka et al., 2019; Tatsumi et al., 2013). Previous studies demonstrated that anti-inflammatory, anti-apoptotic, antimicrobial and immunomodulatory properties of MSCs were exerted through the secretome which contains AMPs, cytokines and extracellular vesicles secreted by MSCs (Bonafede & Mariotti, 2017; Chen et al., 2014; Martin-Rufino et al., 2019;

Zhang et al., 2016; Zhou et al., 2019). Previous studies demonstrated that these antimicrobial properties were mainly due to the presence extracellular vesicles secreted by MSCs (Khatri et al., 2018; Monsel et al., 2015; Zhu et al., 2014). Extracellular vesicles include exosomes, microvesicles and apoptotic bodies which contain cargo of MSCs, such as AMPs, proteins, microRNAs, and lipids, which were shown to mediate immunomodulatory and therapeutic properties of MSCs through paracrine signaling (Bonafede et al., 2016; Chen et al., 2018; Hikita et al., 2018; Martin-Rufino et al., 2019; Shen et al., 2018). Use of exosomes have several advantages compared to the MSC treatment, they can be scalable, do not need any specific cryopreservation, can be directly stored at -80°C in saline, and also, they can cross the microvasculature and biological barrier such BBB which makes them safe and off the shelf products (Bonafede et al., 2016; Shen et al., 2018). The role of exosomes in antimicrobial activity remains unknown. Here for the first time we investigated the antibacterial effects of ASC exosomes against both Gram negative and Gram-positive bacteria *in vitro.*

In this study, we isolated exosomes by differential ultracentrifugation which considered as a standard isolation protocol for therapeutic application of exosomes (Théry et al., 2006). Here we used two different doses to verify the efficacy of exosomes against bacteria *in vitro*, i.e., 2 µg (low dose) and 20 µg (high dose). Pilot studies revealed that 2 µg concentration had significant bacterial inhibition compared to other low doses of exosomes. Hence, we used exosomes at 2 µg concentration as initial low dose of exosomes. Monsel et al. 2015 demonstrated that using microvesicles derived from 10-fold number cells had comparable antimicrobial effects. Therefore, we used 20 µg concentration of ASC exosomes as high dose to compare results with previous studies and evaluate the dose dependent antibacterial activity of exosomes. In this study, for the first time we demonstrated *in vitro* that, ASC-derived exosomes at 2 µg dose

significantly inhibited the bacterial growth compared to the positive control and MEF-derived exosome control. Whereas increase in the dose of exosomes by 10-fold i.e., 20 µg dose, significantly enhanced the bacterial inhibition compared to 2 µg dose. Exosomes at 20 µg dose significantly inhibited the growth of *E. coli* by 100- fold and greatly inhibited the growth of *K. pneumonia*, *S. aureus* and *R. equi*. These results demonstrated that ASC exosomes at 20 µg i.e., 10-fold increase have showed antibacterial effects comparable to ASCs (Fig. 3). The underlying mechanisms for antibacterial effects of ASC-derived exosomes are further needed to be demonstrated.

Although several studies showed that MSCs and MSC-derived extracellular vesicles have therapeutic potential against various inflammatory diseases, they however require large-scale production of MSCs and repeated passaging which leads to cell senescence and decreased therapeutic potential. Recent studies demonstrated that pretreating of MSCs with inflammatory cytokines, stress factors and toll-like receptors will enable them to achieve maximum therapeutic effect with a fewer number of cells compared to untreated cells (Alcayaga-Miranda et al., 2017). Waterman et al. 2010 demonstrated that TLR 3 agonist treated MSCs increased immunosuppressive activity by producing anti-inflammatory cytokines such as IL-10, PGE2 and indolamine 2,3-dioxygense (IDO) and also by changing the phenotype of MSCs into anti-inflammatory phenotype. Recent studies also described that TLR 3 activated MSCs will enhance the survival of neutrophils and macrophages and increase their phagocytic capacity (Cassatella et al., 2011). Mastri et al., 2012 demonstrated that therapeutic effects of MSCs were obtained by suboptimal dose of MSCs stimulated with TLR 3 agonist than untreated cells. They reported that TLR 3 agonist stimulation enhanced the release of relevant trophic factors such as stromal-derived factor, hepatocyte growth factor and vascular endothelia growth factors. Recent studies

demonstrated that preconditioning of MSCs with inflammatory cytokines such as INF-γ and TLRs such TLR 3 and TLR 4 increased the production of extracellular vesicles and also enhanced the therapeutic potential of EVs by carrying specific microRNAs such as KGF and Let-7b (Varkouhi et al., 2019; Monsel et al., 2015; Ti et al., 2015). Monsel et al. 2015 demonstrated that pre-stimulation of MSCs with TLR 3 agonist further enhanced the therapeutic effects of MSC-derived microvesicles compared to untreated MSC-MVs. In this study, we proposed to stimulate the ASCs with poly I:C @10 µg/ml (a TLR 3 agonist) to evaluate the antibacterial effects of stimulation.

We demonstrate that stimulation of ASCs with TLR 3 agonist significantly enhanced the release of exosomes from ASCs by 2-fold. Both NTA and BCA protein assay revealed that increase in number of exosomes released per million ASCs (Figure 2). But, TLR 3 agonist stimulation did not show any significant changes in antibacterial effects of both ASCs and ASC-derived exosomes compared to non-stimulated ASCs and ASC-derived exosomes against *E. coli*, *K. pneumonia, S. aureus* and *R. equi* (Figure 4). The mechanisms underlying the antibacterial effects by stimulation needed to be addressed in further studies.

Since we demonstrated that ASCs and ASC exosomes have antibacterial effects *in vitro* against both Gram negative and Gram-positive bacterial species, we hypothesized to determine their effects in *in vivo* by using *E. coli* induced acute lung injury mouse model. Although, it was thought that MSCs were primarily immunosuppressive and it might deleteriously affect the host immune system and bacterial clearance in clinical therapy, but several studies showed that MSCs treatment enhanced the bacterial clearance and increased the survival rate in animal studies (Gupta et al., 2012; Krasnodembskaya et al., 2010; Monsel et al., 2015). The potential antibacterial effects of MSCs were mainly due to the secretion of AMPs such as cathelicidin,

lipocalin 2, cystatin C and β-defensin and also by enhancing the enhancing host phagocytic capacity by altering from pro inflammatory to anti-inflammatory cytokine responses (Cortés-Araya et al., 2018; Harman et al., 2017; Jackson et al., 2016). In agreement with these studies, current study also demonstrates that ASCs enhanced the bacterial clearance and maintained alveolar protein permeability in *E. coli* induced acute lung injury mouse model. We infected C57BL/6J male mice with *E. coli* (ATCC 25922 strain 1-2 X 10^6 CFU/mice) *via* intratracheal route. Administration of ASCs and TLR 3 agonist stimulated ASCs (1 million cells/mice) *via* intratracheal route after *E. coli* infection was associated with a reduction of bacterial counts in the BAL fluid by 50-fold and 54-fold compared to positive control group, respectively (Figure 8A). The protein concentration of BAL fluid represents the capillary endothelial hyperpermeability associated with a reduced alveolar fluid clearance caused by *E. coli* triggered infection. Protein concentration was low in ASCs and TLR 3 agonist stimulated ASCs compared to positive control which represents ASCs mitigates the damage caused due to *E. coli* infection and maintained protein concentration in lungs by maintaining alveolar endothelial protein permeability (Figure 9A).

Several studies showed that MSC-derived secretome had similar antimicrobial effects as MSCs (Chen et al., 2014; Gupta et al., 2012; Jackson et al., 2016; Krasnodembskaya et al., 2010). Monsel et al. 2015 demonstrated that human MSC-derived microvesicles (MVs) also enhanced the bacterial clearance and improved the survival of mice by increasing the phagocytic capacity of alveolar macrophages. In this study, similar to ASCs, administration of exosomes enhanced the bacterial clearance and reduced endothelial protein permeability. Both ASC- and stimulated ASC-derived exosomes showed 33- and 40-fold bacterial inhibition compared to positive control treatment groups, respectively (Figure 8B). Protein concentration of BAL fluid

was also significantly low in ASC-exosomes and TLR 3 agonist stimulated ASC-exosomes treated groups compared to control groups (Figure 9B). However, TLR 3 agonist stimulation did not show any significant changes in bacterial clearance and protein concentrations in BAL fluid compared to non-stimulated ASCs and ASC-derived exosomes. Further studies needed to be done to determine the underlying mechanisms of exosomes in bacterial clearance and to evaluate the role TLR 3 agonist stimulation.

Limitations

This study provides the antibacterial effects of exosomes derived from both ASCs and TLR 3 agonist stimulated ASCs and gave insights for future clinical use of MSC-derived exosomes. However, this study has certain limitations that should be considered. First, the number of exosomes released from the 2D culture methods were low and better cell culture methods are needed for collection of exosomes such as tangential flow filtration, 3D culture methods and bioreactors (Haraszti et al., 2018; Thippabhotla et al., 2019). Second, the differential ultracentrifugation isolation yield is low by this method compared to other methods such as size exclusion chromatography, ultrafiltration, sucrose cushion ultracentrifugation and density gradient isolation which were showed to give high yield of exosomes. However, exosomes derived from differential ultracentrifugation are considered as gold standard for clinical trials due to their high purity and cost effective compared to other methods (Klymiuk et al., 2019; Patel et al., 2019). To address this limitation, we used high dose of exosomes released from 10-fold number of cells compared to normal dose. Third, dose of exosomes was determined based on protein concentration of exosome samples, which might also have some extracellular proteins. Additional dose calculation methods such as number of particles by NTA and studies

using multiple concentrations would provide further insights regarding MSC-derived exosomes. Fourth, characterization of exosomes cargo would enable the insights of antibacterial activities.

Summary

We demonstrated that ASC-derived exosomes exhibited antibacterial activity against both Gram negative and Gram-positive bacterial species in *in vitro* study. These antibacterial effects of exosomes were showed in a dose-dependent inhibition of bacterial growth. Toll-like receptor 3 agonist stimulation enhanced the exosomes released by 2-fold. In addition, TLR 3 stimulated ASCs and ASC-derived exosomes showed significant bacterial inhibition compared to the control groups. In the mouse model, ASCs and ASC-derived exosomes enhanced the bacterial clearance and alveolar permeability. More importantly, ASC-derived exosomes at 10-fold dose were as effective as ASCs. Overall, this study showed that increase in the dose of ASC-derived exosomes were as effective as ASCs in bacterial inhibition suggesting a potential cell free therapeutic alternative for bacterial diseases both *in vitro* and *in vivo*.

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