

EXTRACELLULAR VESICLES HAVING ANTI-BACTERIAL AND WOUND HEALING FUNCTIONS

TECHNICAL FIELD

5 The present disclosure relates to extracellular vesicles (EVs) exhibiting anti-bacterial and wound healing functions. Also provided are a method of manufacturing the EVs, compositions thereof, and uses of the EVs and compositions in the treatment of infections, wounds and aesthetic procedures.

10 BACKGROUND

 Infectious diseases, like chronic wounds, possess a significant threat to the public health systems due to increase in antimicrobial resistance (AMR), inefficacy of antibiotics, dearth of novel biologics and off- target effects leading to the normal flora damage. Chronic wound is a large and growing problem predominantly due to the emergence of multidrug resistant (MDR) bacterial pathogens with a reported annual
15 spending of US\$25 billion in the United States ^{1,2}. In addition, chronic wound presents a substantial economic burden to Singapore challenged with increasing occurrence of aging population and diabetes mellitus. Chronic wound causing bacteria are predominantly multidrug resistant (MDR) pathogens, which can easily survive standard
20 antibiotics treatments by gaining antimicrobial resistance (AMR) ³. As of today, antibiotics stand as the first line of treatment against wound infections. However, emergence of antibiotic resistant bacterial infections accompanied with the dearth of new antibiotics possess the biggest challenge ⁴.

 Several combination therapies of either AMPs or polymers with antibiotics have
25 garnered much attention to treat infections caused by MDR bacterial strains. Combination therapies have shown favorable effects in in vitro and animal studies ⁵⁻⁹.

 As an alternate approach, in the context of wound healing, exosomes derived from mesenchymal stem cells (MSCs) are reported as cell-free therapies for accelerated skin wound closure. However, exosomes are not reported to fundamentally exhibit an
30 antibacterial function essential for chronic wound management.

 Thus, there is a need for an alternative extracellular vesicle (EV) having antibacterial activity.

SUMMARY

In one aspect, there is provided an extracellular vesicle (EV) having anti-bacterial and wound healing properties, wherein the EV is derived/obtained/isolated from a mesenchymal stem cell (MSC) which has been primed with one or more species of live bacteria.

In one aspect, there is provided a method of manufacturing an EV as defined herein, comprising the steps of priming a mesenchymal stem cell (MSC) with one or more species of live bacteria; and isolating the EV.

In one embodiment, the priming of the MSC is performed in the absence of serum.

In one embodiment, the priming of the MSC is performed in the presence of a minimal medium comprising or consisting of a media, for example DMEM, such as KnockOut™ DMEM (Gibco™); and a buffer, such as HEPES.

In one embodiment, priming of the MSC is performed in the presence of one or more of the following:

- D-glucose, such as high (e.g. 4.5 g/l) or low (e.g. 1g/l) D-glucose;
- a serum, such as FBS, for example; 5 to 15% FBS, such as 10% FBS; and
- a buffer, such as HEPES, for example 10 to 30 mM HEPES, such as 20 mM HEPES.

In one embodiment, the MSC is cultured, for example grown for 2 days, prior to the priming step.

In one embodiment, the EV is isolated from MSC conditioned media (CM), *i.e. the media that the MSC has been primed in.*

In one embodiment, the CM is filtered prior to isolation of the EV, for example using a 0.2 µM syringe filter.

In one embodiment, the EV is isolated from the CM by centrifuging the CM, obtaining a pellet containing the EV, and reconstituting the EV in a suitable buffer, such as PBS.

In one embodiment, the MSC originates from an organism selected from the group comprising: a human, and mouse, in particular a human MSC.

In one embodiment, the MSC is obtained from one of more of the following: bone marrow, cord cells, adipose tissue, molar cells, amniotic fluid, muscle, and liver.

In one embodiment, the MSC is selected from the group comprising an adipose-derived stem cell (ASC), an epidermal stem cell (EPSC), a hair follicle stem cell (HFSC), and a hematopoietic stem cell (HSC).

In one embodiment, the MSC is an adipose-derived stem cell (ASC).

5 In one embodiment, differentiation of the ASC is induced with an adipogenic cocktail.

In one embodiment, the adipogenic cocktail comprises one of more of the following:

- 10 • dexamethasone, for example 0.5 to 1.5 mM dexamethasone, such as 1 mM dexamethasone;
- IBMX, for example 0.25 to 0.75 mM IBMX, such as 0.5 mM IBMX;
- Insulin, for example 125 to 200 nM insulin, such as 167 nM insulin; and
- Indomethacin, for example 50 to 150 mM indomethacin, such as 100 mM indomethacin.

15 In one embodiment, the MSC/ASC is an intermediate differentiating MSC/ASC, for example a Day 4 to 7, such as Day 4, 5, 6, or 7 intermediate differentiating MSC/ASC.

In one embodiment, the MSC/ASC is a Day 6 intermediate differentiating MSC/ASC.

In one embodiment, the EV is an exosome.

20 In one embodiment, the EV has a size in the range 80 to 140 nm, such as 80, 85, 90, 95, 100, 105, 110, 115, 120, 125, 130, 135 or 140 nm; or a size in the range of 95 to 105 nm, such as 95, 96, 97, 98, 99, 100, 101, 102, 103, 104 or 105 nm.

In one aspect, there is provided a composition, such as a pharmaceutical composition, comprising an EV as defined herein.

25 In one embodiment, the composition further comprises one of more of the following: a pharmaceutically acceptable excipient, a buffer or an additive.

In one aspect, there is provided a method of treatment, comprising administering a therapeutically effective amount of an EV or a pharmaceutical composition as defined herein to a patient in need thereof.

30 In one aspect, there is provided an EV or a pharmaceutical composition as defined herein, for use in the treatment of a patient.

In one aspect, there is provided a use of an EV or a pharmaceutical composition as defined herein, in the manufacture of a medicament for the treatment of a patient.

In one embodiment, the treatment is for one or more of the following: a wound, such as a chronic wound, a bacterial infection or a disease/condition caused by a bacterial infection caused by one or more species of bacteria.

In one embodiment, the treatment is for non-infective wounds or damaged skin.

5 In one embodiment, the treatment is for chronic wound healing.

In one embodiment, the chronic wound is selected from the group comprising a vascular ulcer, such as a venous ulcer or an arterial ulcer, a diabetic ulcer and a pressure ulcer, a surgical wound, and a traumatic wound, in particular a diabetic foot ulcer.

In one embodiment, the treatment is for a bacterial infection caused by one or
10 more species of bacteria, for example selected from the group comprising: *Acinetobacter baumannii*, *Bacillus anthracis*, *Bacillus cereus*, *Bartonella henselae*, *Bartonella quintana*, *Bordetella pertussis*, *Borrelia burgdorferi*, *Borrelia garinii*, *Borrelia afzelii*, *Borrelia recurrentis*, *Brucella abortus*, *Brucella canis*, *Brucella melitensis*, *Brucella suis*, *Campylobacter jejuni*, *Chlamydia pneumoniae*, *Chlamydia trachomatis*, *Chlamyophila psittaci*, *Clostridium botulinum*, *Clostridium difficile*, *Clostridium perfringens*, *Clostridium tetani*, *Corynebacterium diphtheriae*, *Enterobacter spp*, *Enterococcus faecalis*,
15 *Enterococcus faecium*, *Escherichia coli*, *Francisella tularensis*, *Hemophilus influenzae*, *Helicobacter pylori*, *Klebsiella pneumoniae*, *Legionella pneumophila*, *Leptospira interrogans*, *Leptospira santarosai*, *Leptospira weilii*, *Leptospira noguchii*, *Listeria monocytogenes*, *Mycobacterium leprae*, *Mycobacterium tuberculosis*, *Mycobacterium ulcerans*, *Mycoplasma pneumoniae*, *Neisseria gonorrhoeae*, *Neisseria meningitidis*,
20 *Pseudomonas aeruginosa*, *Rickettsia rickettsia*, *Salmonella typhi*, *Salmonella typhimurium*, *Shigella sonnei*, *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Staphylococcus saprophyticus*, *Streptococcus agalactiae*, *Streptococcus pneumoniae*,
25 *Streptococcus pyogenes*, *Treponema pallidum*, *Ureaplasma urealyticum*, *Vibrio cholerae*, *Yersinia pestis*, *Yersinia enterocolitica*, and *Yersinia pseudotuberculosis*.

In one embodiment, the one or more species of bacteria are selected from the group comprising *Salmonella Typhimurium*, *Staphylococcus aureus* and *Pseudomonas aeruginosa*.

30 In one embodiment, the treatment is for biofilm disruption and/or planktonic bacteria killing.

In one embodiment, the patient is a human, such as a diabetic patient.

In one aspect, there is provided an EV or pharmaceutical composition as defined herein, for use in an aesthetic procedure, such as a skin aesthetic procedure, for

example selected from the group comprising: the treatment of scars, skin laxity, wrinkles, liver spots, cellulite and skin discoloration.

DEFINITIONS

5 The term “extracellular vesicle” or “EV” as used herein to membrane-surrounded vesicles that are secreted by cells into the extracellular space. Their main function is to carry bioactive molecules for delivery to recipient cells. Extracellular vesicles generally have a diameter in the range 10nm to 5000nm.

10 As employed herein, the term “extracellular vesicle” includes exosomes, microvesicles (also referred to microparticles), ectosomes, matrix vesicles, calcifying vesicles, prostasomes, oncosomes, retrovirus-like particles, bacterial extracellular vesicles, intraluminal vesicles and apoptotic bodies. Of these, the three main types of extracellular vesicles are microvesicles, exosomes and apoptotic bodies, which are differentiated based on their biogenesis, release pathways, size, content and function.

15 Extracellular vesicles (EVs) are important mediators of long distance intercellular communication and are involved in a diverse range of biological processes across both prokaryotic and eukaryotic organisms, including immune responses, tissue repair and cancer progression.

20 EVs shed from infected cells (viral, bacterial or parasitic) contain material from these infectious agents. This material can vary from toxins to virulence factors to infectious virus (both enveloped and non-enveloped and may be a novel means to more effectively deliver viral populations rather than single viral particles. Consequently, their isolation could also provide rapid diagnostic insight into disease.

25 EVs can be derived/obtained/isolated from a wide variety of different cell types. These include immune cells, such as T cells, B cells, dendritic cells, macrophages and neutrophils; blood cells, such as platelets and red blood cells; neural cells, such as neurons, oligodendrocytes, schwann cells; epithelial cells, for example intestinal epithelial cells; cardiovascular cells, for example endothelial cells and cardiomyocytes; fibroblasts; adipocytes; and mesenchymal stem cells.

30 The term “exosome” as used herein refers to nanovesicles typically having a diameter in the range 30 to 150nm. Exosomes are generally produced inside multivesicular bodies and are released after fusion of the multivesicular body with the cytomembrane. Exosomes were originally thought to be a way for cells to dispose of unwanted material. However, exosomes have since been found to participate in cell-cell
35 communication, cell maintenance and tumour progression.

The term "mesenchymal stem cell" or "MSC" as used herein refers to a type of multipotent stromal cell which can differentiate into a variety of cells of the mesodermal lineage, including osteocytes, adipocytes and chondrocytes. Mesenchymal stem cells are predominantly isolated from the bone marrow, although they can also be derived
5 from other tissues such as fat, umbilical cord and dental pulp.

MSCs express a specific set of surface markers, including CD73, CD90 and CD105, while lacking the expression of hematopoietic markers such as CD34 and CD45. This unique marker profile allows MSCs to be isolated from other cell types.

In terms of function, MSCs have a high capacity for self-renewal while
10 maintaining their multipotency. They possess significant immunomodulatory properties, allowing them to play an important role in modulating immune responses and promoting tissue repair. As such, MSCs employed for the treatment of diseases are in clinical development around the world, in particular for treating autoimmune diseases, graft vs
host disease, Crohn's disease, multiple sclerosis, and systemic lupus erythematosus.

15 The term "adipose-derived stem cell" or "ASC" as used herein refers to a subset of mesenchymal stem cell which is derived from adipose tissue, for example white adipose tissue (WAT) or brown adipose tissue (BAT). Methods of obtaining adipose - derived mesenchymal stem cells (ASCs) are known in the art. Adipose tissues are minced and then undergo enzymatic digestion with collagenase type II. After
20 centrifugation, the resulting pellet is called the stroma vascular fraction (SVF). Approximately 2 to 6 million cells in stroma vascular fraction (SVF) can be obtained from one millilitre of lipoaspirate. Stroma vascular fraction (SVF) contains adipose -derived mesenchymal stem cells (ASCs), endothelial cells, endothelial progenitor cells, pericytes, smooth muscle cells, leukocytes, and erythrocytes. Adipose-derived
25 mesenchymal stem cells (ASCs) are obtained as the plastic -adherent population after overnight culturing.

The term "priming" as used herein refers to the process of preparing a cell for use. "Priming" therefore entails imbuing a cell with properties desirable for its intended use. In this respect, a cell that is ready for use is said to have been "primed". Thus, in
30 the context of the present disclosure, a mesenchymal stem cell that has been "primed" is an MSC which has been imbued with antibacterial/wound healing properties. Therefore, as used herein, A MSC which has been "primed with" one or more species of live bacteria is an MSC that has been imbued with anti-bacterial and/or wound healing properties by employing one or more live bacteria.

The term "differentiation" as used herein refers to a biological process that causes a stem cell to become mature into a more specialised cell types having a more distinct form and function. Differentiation of stem cells, such as mesenchymal stem cells can be induced in various ways, such as by exposing the stem cells to relevant growth factors, by specific gene transfer, and by treatment with chemical agents.

The term "intermediate differentiating" as used herein refers to a stem cell which is midway through the process of differentiation and has not yet become fully differentiated. Thus, an "intermediate differentiating" ASC is midway through the process of adipogenic differentiation and has not become a fully differentiated adipocyte.

Adipogenic differentiation timeline varies based on the species. For example, human ASC differentiation requires about 12-14 days; mice ASCs about 8-10 days. Thus, to generate intermediate stem cells undergoing mid- adipogenesis, the timeline would depend on the species. Accordingly, an intermediate differentiating human ASC refers to a human ASC which has been subjected to adipogenic differentiation for about 4 days to 7 days, such as 4, 5, 6, or 7 days.

The term "adipogenic cocktail" as used herein refers to a mixture which can be used to induce adipogenic differentiation in MSCs/ASCs. Typically, a 3-component cocktail is used, containing varying amounts of insulin, dexamethasone and IBMX.

The term "conditioned media" or "CM" as used herein refers to media containing components secreted by cells that were previously cultured in the media. Thus, in the context of the present disclosure, the CM is the media in which the MSCs/ASCs have been primed with the one or more species of live bacteria.

The term "aesthetic procedure" as used herein refers to any cosmetic procedure that is aimed at improving a person's physical appearance. Such procedures can be surgical or non-surgical and are intended to enhance, alter or reshape specific parts of the body for aesthetic reasons.

The term "chronic wound" as used herein refers to wound that does not proceed through an orderly and timely reparative process. A wound that does not heal within 3 months is typically considered chronic. Chronic wounds generally have one or more of the following characteristics:

- Failure to Progress: They do not proceed through an orderly or timely reparative process.
- Prolonged Inflammation: The majority of chronic wounds exhibit a prolonged or excessive inflammatory phase.

- Cell Responsiveness Impairment: Dermal or epidermal cells struggle to respond to reparative stimuli.
- Persistent Infections: Microbial squatters further complicate matters.

The majority of chronic wounds can be classified under three categories: venous, diabetic and pressure ulcers. Other less common categories include wounds due to radiation poisoning and ischemia.

Treatment strategies for chronic wounds are varied and include:

- Local care: For example, debridement to aggressively remove necrotic debris or non-viable tissue, and proper wound dressing.
- Addressing the underlying causes: For example, treating comorbidities such as diabetes; weight management since excess weight can impair wound healing; and reviewing the patient's existing treatments, for example long term use of immunosuppressants may hinder wound healing
- Pain management: chronic wounds are often associated with chronic pain
- Infection control: For example, by using anti-microbial agents.
- Wound closure: For example, employing skin grafts to help cover the wound

The term "biofilm" as used herein refers to a syntrophic assembly of microorganisms, such as bacteria, fungi and/or algae, that stick to each other and often adhere to surfaces. The adherent cells create a 3D structure, embedded within a slimy extracellular matrix composed of extracellular polymeric substances (EPSs).

Biofilms have been found to be involved in a wide range of microbial infections in the body and it has been suggested that around two-thirds of all bacterial infections in humans involve the formation of biofilms. Infectious processes in which biofilms have been implicated include common minor infections, such as urinary tract infections, middle-ear infections, the formation of dental plaque; as well as more serious and rarer infections. Such as endocarditis, infections in cystic fibrosis and infections of medical implants such as heart valves and joint prostheses.

Infections involving biofilms are particularly challenging to eradicate, due to the fact that mature biofilms display various traits such as antimicrobial tolerance and immune response evasions. In the context of chronic wounds, the presence of biofilms hinders wound healing, delay tissue repair and stimulate chronic inflammation at the

The terms "treating", "treat" and "therapy", and synonyms thereof refer to both therapeutic treatment and prophylactic or preventative measures, wherein the object is to prevent or slow down (lessen) a medical condition, which includes but is not limited to diseases, symptoms and disorders. A medical condition also includes a body's response

to a disease or disorder, e.g. inflammation. Those in need of such treatment include those already with a medical condition as well as those prone to getting the medical condition or those in whom a medical condition is to be prevented.

The term "subject" as used herein includes patients and non-patients. The term
5 "patient" refers to individuals suffering or are likely to suffer from a medical condition, while "non-patients" refer to individuals not suffering and are likely to not suffer from the medical condition. "Non-patients" include healthy individuals, non-diseased individuals and/or an individual free from the medical condition. The term "subject" includes humans and animals. Animals may include, but is not limited to, mammals (for example non-
10 human primates, canine, murine and the like), and the like. "Murine" refers to any mammal from the family Muridae and / or Leporidae, such as mouse, rat, rabbit, and the like.

The term "preventing" and/or "reducing the severity of symptoms" as used herein refers to process of delaying the onset, reducing the severity of symptoms, reducing
15 and/or preventing weight loss, preventing death, inhibiting deterioration, inhibiting further deterioration, and/or ameliorating at least one sign or symptom of a disease.

The term "and/or", e.g., "X and/or Y" is understood to mean either "X and Y" or "X or Y" and should be taken to provide explicit support for both meanings or for either meaning.

20 Further, in the description herein, the word "substantially" whenever used is understood to include, but not restricted to, "entirely" or "completely" and the like. In addition, terms such as "comprising", "comprise", and the like whenever used, are intended to be non-restricting descriptive language in that they broadly include elements/components recited after such terms, in addition to other components not
25 explicitly recited. For example, when "comprising" is used, reference to a "one" feature is also intended to be a reference to "at least one" of that feature. Terms such as "consisting", "consist", and the like, may in the appropriate context, be considered as a subset of terms such as "comprising", "comprise", and the like. Therefore, in embodiments disclosed herein using the terms such as "comprising", "comprise", and
30 the like, it will be appreciated that these embodiments provide teaching for corresponding embodiments using terms such as "consisting", "consist", and the like. Further, terms such as "about", "approximately" and the like whenever used, typically means a reasonable variation, for example a variation of +/- 5% of the disclosed value, or a variance of 4% of the disclosed value, or a variance of 3% of the disclosed value, a
35 variance of 2% of the disclosed value or a variance of 1% of the disclosed value.

Furthermore, in the description herein, certain values may be disclosed in a range. The values showing the end points of a range are intended to illustrate a preferred range. Whenever a range has been described, it is intended that the range covers and teaches all possible sub-ranges as well as individual numerical values within that range.

5 That is, the end points of a range should not be interpreted as inflexible limitations. For example, a description of a range of 1% to 5% is intended to have specifically disclosed sub-ranges 1% to 2%, 1% to 3%, 1% to 4%, 2% to 3% etc., as well as individually, values within that range such as 1%, 2%, 3%, 4% and 5%. It is to be appreciated that the individual numerical values within the range also include integers, fractions and

10 decimals. Furthermore, whenever a range has been described, it is also intended that the range covers and teaches values of up to 2 additional decimal places or significant figures (where appropriate) from the shown numerical end points. For example, a description of a range of 1% to 5% is intended to have specifically disclosed the ranges 1.00% to 5.00% and also 1.0% to 5.0% and all their intermediate values (such as 1.01%,

15 1.02% ... 4.98%, 4.99%, 5.00% and 1.1%, 1.2% ... 4.8%, 4.9%, 5.0% etc.) spanning the ranges. The intention of the above specific disclosure is applicable to any depth/breadth of a range.

Additionally, when describing some embodiments, the disclosure may have disclosed a method and/or process as a particular sequence of steps. However, unless

20 otherwise required, it will be appreciated that the method or process should not be limited to the particular sequence of steps disclosed. Other sequences of steps may be possible. The particular order of the steps disclosed herein should not be construed as undue limitations. Unless otherwise required, a method and/or process disclosed herein should not be limited to the steps being carried out in the order written. The sequence of steps

25 may be varied and still remain within the scope of the disclosure.

Furthermore, it will be appreciated that while the present disclosure provides embodiments having one or more of the features/characteristics discussed herein, one or more of these features/characteristics may also be disclaimed in other alternative embodiments and the present disclosure provides support for such disclaimers and

30 these associated alternative embodiments.

DESCRIPTION OF EMBODIMENTS

It will be appreciated by a person skilled in the art that other variations and/or modifications may be made to the embodiments disclosed herein without departing

35 from the spirit or scope of the disclosure as broadly described. For example, in the

description herein, features of different exemplary embodiments may be mixed, combined, interchanged, incorporated, adopted, modified, included etc. or the like across different exemplary embodiments. The present embodiments are, therefore, to be considered in all respects to be illustrative and not restrictive.

5 The present inventors previously established that conditioned media (CM) from bacteria-primed, intermediate differentiating adipose-derived stem cells exhibit robust planktonic bacterial killing, significant antibiofilm effects and wound healing properties (WO2023/219566). By performing HPLC fractionation and mass spectrometric analysis, they discovered ENO3 and SPARCL1 as novel antibacterial proteins secreted from these
10 cells. Subsequently, they identified active short sequences (<22 amino acid residues) of ENO3 (E1A) and SPARCL1 (S1A and S1B) exerting significant bactericidal and biofilm eradication potentials.

 As ASC-CM represents a crude mixture of proteins and its use as a direct intervention against chronic wound infections presents challenges, the present inventors
15 explored the possibility of purifying extracellular vehicles (EVs) from ASC-CM due to their stability, storage compatibility, small size, minimal immunogenicity and tumorigenicity.

 Thus, in one aspect, there is provided an extracellular vesicle (EV) having anti-bacterial and wound healing properties, wherein the EV is derived/obtained/isolated from a mesenchymal stem cell (MSC) which has been primed with one or more species of live
20 bacteria. Unexpectedly, the presently disclosed EVs exhibit dual antibacterial and wound healing properties. In contrast, other commercially available EVs have been shown to only have wound healing properties. In particular, the present inventors discovered that priming the MSCs with live bacteria is crucial given that EVs isolated from MSCs primed with heat-inactivated bacteria failed to elicit an antibacterial response, despite their
25 surface antigens being intact.

 The presently disclosed EVs have at least the following technical benefits over prior art EVs:

 First, because of the EVs' dual intrinsic antibacterial and wound healing properties, they can be employed as a monotherapy.

30 Second, the presently disclosed EVs can prevent acquisition of antimicrobial resistance (AMR) in contrast to combinatorial treatment of EVs with antibiotics.

 Third, the presently disclosed EVs are devoid of antimicrobial peptides (AMPs), and thus other novel components appear to contribute to the EVs' antibacterial properties. Hence, without being bound by theory, the EVs may be exhibiting their
35 antibacterial effects via novel mechanisms.

In one embodiment, the EV is secreted from a MSC which has been primed with one or more species of live bacteria.

In one aspect, there is provided a method of manufacturing an EV as defined above, comprising the steps of priming a mesenchymal stem cell (MSC) with one or more species of live bacteria; and isolating the EV. Advantageously, the presently disclosed
5 method provides an efficient way to generate the EVs of the present disclosure.

In one embodiment, the MSC has been primed with one or more species of live bacteria by prior exposure to the one or more species of live bacteria, for example wherein the MSC has been incubated/co-cultured with one or more species of live
10 bacteria. Thus, in one embodiment, the MSC has been incubated/co-cultured with one or more species of live bacteria.

In one embodiment, the step of priming a MSC with one or more species of live bacteria comprises exposing the MSC to one or more species of live bacteria, for example by incubating/co-culturing the MSC with one or more species of live bacteria.
15 Accordingly, in one embodiment, the priming step comprises incubating/co-culturing the MSC with one or more species of live bacteria.

In one embodiment, the ASC is derived from white adipose tissue (WAT). Advantageously, ASCs are significantly more abundant in WAT compared to brown adipose tissue (BAT). Thus, isolating ASC from WAT rather than BAT is considerably
20 easier and more efficient.

In one embodiment, the priming of the MSC is performed in the absence of serum. EVs can potentially originate from serum sources such as FBS. Hence, by priming in serum-free media, this helps to ensure that the EVs isolated originate from the bacteria primed MSCs rather than another unintended source.

25 The choice of media components and buffers differs depends on the cell line and the species the MSCs are derived from. Advantageously, the present inventors have determined the optimal media components and buffers for the isolation of EVs from human ASCs.

Thus, in one embodiment, the priming of the MSC is performed in the presence
30 of a minimal medium comprising or consisting of a media and a buffer. Advantageously, the buffer helps to prevent acidification.

In one embodiment, the media is DMEM, such as KnockOut™ DMEM (Gibco™).

In one embodiment, priming of the MSC is performed in the presence of one or more of the following: D-glucose, a serum, such as FBS and a buffer.

In one embodiment, the D-glucose is high (e.g. 4.5 g/l) or low (e.g. 1g/l) D-glucose.

In one embodiment, the FBS is 5 to 15% FBS, such as 10% FBS.

In one embodiment, the buffer is HEPES.

5 In one embodiment, the HEPES is 10 to 30 mM HEPES, such as 20mM HEPES.

In one embodiment, the MSC is cultured, for example grown for 2 days, prior to the priming step.

In one embodiment, the EV is isolated from MSC conditioned media (CM), i.e. the media that the MSC has been primed in. Advantageously, the EVs are secreted from
10 the MSCs into the conditioned media. Hence, the conditioned media provides a rich source of EVs ready for isolation.

In one embodiment, the CM is filtered prior to isolation of the EV, for example using a 0.2 μ M syringe filter. Advantageously, filtering the CM helps to eliminate any bacterial contamination.

15 In one embodiment, the EV is isolated from the CM by centrifuging the CM, obtaining a pellet containing the EV, and reconstituting the EV in a suitable buffer, such as PBS.

In one embodiment, the MSC originates from an organism selected from the group comprising: a human, and mouse.

20 In one embodiment, the MSC is a human MSC.

In one embodiment, the MSC is obtained from one of more of the following: bone marrow, cord cells, adipose tissue, molar cells, amniotic fluid, muscle, and liver.

In one embodiment, the MSC is selected from the group comprising an adipose-derived stem cell (ASC), an epidermal stem cell (EPSC), a hair follicle stem cell (HFSC),
25 and a hematopoietic stem cell (HSC).

In one embodiment, the MSC is an adipose-derived stem cell (ASC). Thus, in one embodiment, the EV is an ASC-EV. In comparison with bone marrow derived MSCs, ASCs have several advantages, including a greater ease of isolation from subcutaneous regions through a minimally invasive and painless procedure, the ability to maintain their
30 phenotype for longer periods in culture, and a greater proliferative capacity.

In one embodiment, the differentiation of the ASC is not induced with an adipogenic cocktail.

In one embodiment, differentiation of the ASC is induced with an adipogenic cocktail. This adipogenic cocktail provides a convenient and efficient means of inducing
35 differentiation of the ASCs.

In one embodiment, the adipogenic cocktail comprises one of more of the following: dexamethasone, IBMX, insulin and indomethacin.

The EV or method according to any preceding AS, wherein the dexamethasone is 0.5 to 1.5 mM dexamethasone, such as 1 mM dexamethasone.

5 In one embodiment, the IBMX is 0.25 to 0.75 nM IBMX, such as 0.5 mM IBMX.

In one embodiment, the insulin is 125 to 200 nM insulin, such as 167 nM insulin.

In one embodiment, the indomethacin is 50 to 150 mM indomethacin, such as 100 mM indomethacin.

In one embodiment, the MSC/ASC is an intermediate differentiating MSC/ASC.

10 Advantageously, EVs isolated from intermediate differentiating MSCs/ASCs exhibit greater antibacterial and wound healing function compared to EVs isolated from pre-differentiated or fully differentiated MSCs/ASCs/

In one embodiment, the MSC/ASC is a Day 4 to 7, such as Day 4, 5, 6, or 7 intermediate differentiating MSC/ASC.

15 In one embodiment, the MSC/ASC is a Day 6 intermediate differentiating MSC/ASC.

In one embodiment, the EV is an exosome. Characterization of the presently disclosed EVs by nanoparticle tracking analysis (NTA) performed by the present inventors showed that the EVs had size of <130nm, suggesting that the EV population
20 secreted by the ASCs are enriched for exosomes.

In one embodiment, the EV has a size in the range 80 to 140 nm, such as 80, 85, 90, 95, 100, 105, 110, 115, 120, 125, 130, 135 or 140 nm.

In one embodiment, the EV has a size in the range of 95 to 105 nm, such as 95, 96, 97, 98, 99, 100, 101, 102, 103, 104 or 105 nm.

25 In one aspect, there is provided an EV obtained by the method as defined above.

In one aspect, there is provided a composition comprising an EV as defined above.

In one aspect, there is provided a pharmaceutical composition comprising an EV as defined above.

30 In one embodiment, the composition further comprises one of more of the following: a pharmaceutically acceptable excipient, a buffer or an additive.

Pharmaceutically acceptable agents for use in the present pharmaceutical compositions include carriers, excipients, diluents, antioxidants, preservatives, colouring, flavouring and diluting agents, emulsifying agents, suspending agents,

solvents, fillers, bulking agents, buffers, delivery vehicles, tonicity agents, cosolvents, wetting agents, complexing agents, buffering agents, antimicrobials, and surfactants.

Pharmaceutically acceptable salts can be used, for example mineral acid salts, such as hydrochlorides, hydrobromides, phosphates and sulphates, or salts of organic acids, such as acetates, propionates, malonates and benzoates.

Pharmaceutically acceptable carriers in therapeutic compositions may additionally contain liquids such as water, saline, glycerol and ethanol. Additionally, auxiliary substances, such as wetting or emulsifying agents or pH buffering substances, may be present in such compositions. Such carriers enable the pharmaceutical compositions to be formulated as tablets, pills, dragees, capsules, liquids, gels, syrups, slurries and suspensions, for ingestion by the patient. A thorough discussion of pharmaceutically acceptable carriers is available in Remington's Pharmaceutical Sciences (Mack Publishing Company, N.J. 1991).

In one aspect, there is provided a method of treatment, comprising administering a therapeutically effective amount of an EV or a pharmaceutical composition according as defined above to a patient in need thereof.

In one aspect, there is provided an EV or a pharmaceutical composition according as defined above, for use in the treatment of a patient.

In one aspect, there is provided a use of an EV or a pharmaceutical composition as defined above, in the manufacture of a medicament for the treatment of a patient.

In one aspect, there is provided an EV or pharmaceutical composition according as defined above, for use in an aesthetic procedure. Advantageously, in view of their wound healing abilities, the presently disclosed EVs may be particularly suitable for damaged skin care and skin related cosmetic therapies.

In one embodiment, the aesthetic procedure is a skin aesthetic procedure.

In one embodiment, the skin aesthetic procedure is selected from the group comprising: the treatment of scars, skin laxity, wrinkles, liver spots, cellulite and skin discoloration.

In one embodiment, the treatment is for one or more of the following: a wound, such as a chronic wound, a bacterial infection or a disease/condition caused by a bacterial infection caused by one or more species of bacteria.

In one embodiment, the treatment is for wound healing, for example chronic wound healing.

In one embodiment, the treatment is for non-infective wounds or damaged skin.

In one embodiment, the treatment is for chronic wound healing.

In one embodiment, the chronic wound is selected from the group comprising a vascular ulcer, such as a venous ulcer or an arterial ulcer, a diabetic ulcer and a pressure ulcer, a surgical wound, and a traumatic wound.

In one embodiment, the chronic wound is a diabetic foot ulcer.

- 5 In one embodiment, the treatment is for a bacterial infection caused by one or more species of bacteria.

In one embodiment, the one or more species of bacteria are from a bacterial genus selected from the group comprising: *Bacillus*, *Bartonella*, *Bordetella*, *Borrelia*, *Brucella*, *Campylobacter*, *Chlamydia*, *Chlamydomphila*, *Clostridium*, *Corynebacterium*,
10 *Enterococcus*, *Escherichia*, *Francisella*, *Haemophilus*, *Helicobacter*, *Legionella*, *Leptospira*, *Listeria*, *Mycobacterium*, *Mycoplasma*, *Neisseria*, *Pseudomonas*, *Rickettsia*, *Salmonella*, *Shigella*, *Staphylococcus*, *Streptococcus*, *Treponema*, *Ureaplasma*, *Vibrio* and *Yersinia*.

In one embodiment, the one or more species of bacteria are selected from the
15 group comprising: *Acinetobacter baumannii*, *Bacillus anthracis*, *Bacillus cereus*, *Bartonella henselae*, *Bartonella quintana*, *Bordetella pertussis*, *Borrelia burgdorferi*, *Borrelia garinii*, *Borrelia afzelii*, *Borrelia recurrentis*, *Brucella abortus*, *Brucella canis*, *Brucella melitensis*, *Brucella suis*, *Campylobacter jejuni*, *Chlamydia pneumoniae*, *Chlamydia trachomatis*, *Chlamydomphila psittaci*, *Clostridium botulinum*, *Clostridium difficile*, *Clostridium perfringens*, *Clostridium tetani*, *Corynebacterium diphtheriae*,
20 *Enterobacter spp* *Enterococcus faecalis*, *Enterococcus faecium*, *Escherichia coli*, *Francisella tularensis*, *Hemophilus influenzae*, *Helicobacter pylori*, *Klebsiella pneumoniae*, *Legionella pneumophila*, *Leptospira interrogans*, *Leptospira santarosai*, *Leptospira weillii*, *Leptospira noguchii*, *listeria monocytogenes*, *Mycobacterium leprae*,
25 *Mycobacterium tuberculosis*, *Mycobacterium ulcerans*, *Mycoplasma pneumoniae*, *Neisseria gonorrhoeae*, *Neisseria meningitidis*, *Pseudomonas aeruginosa*, *Rickettsia rickettsia*, *Salmonella typhi*, *Salmonella typhimurium*, *Shigella sonnei*, *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Staphylococcus saprophyticus*, *Streptococcus agalactiae*, *Streptococcus pneumoniae*, *Streptococcus pyogenes*, *Treponema pallidum*,
30 *Ureaplasma urealyticum*, *Vibrio cholerae*, *Yersinia pestis*, *Yersinia enterocolitica*, and *Yersinia pseudotuberculosis*.

In one embodiment, the one or more species of bacteria are selected from the group comprising *Salmonella Typhimurium*, *Staphylococcus aureus* and *Pseudomonas aeruginosa*. Advantageously, the present inventors have demonstrated that EVs derived

from MSCs primed in these bacterial species have antibacterial and wound healing function.

In one embodiment, the one or more species of bacteria are selected from the group comprising *Staphylococcus aureus*, *Pseudomonas aeruginosa* and *Enterococcus faecalis*. Advantageously, these species of bacteria are amongst the most commonly
5 found ones in chronic wounds. Thus, by priming the MSCs with 1, 2 or all 3 of these species, the EV has the potential to be suitable for the treatment of a large proportion of chronic wounds typically found in patients.

In one embodiment, the treatment is for biofilm disruption. Surprisingly, the
10 present inventors have demonstrated that the presently disclosed EVs are able to effectively disrupt biofilms.

In one embodiment, the treatment is for planktonic bacteria killing.

In one embodiment, the EV or composition thereof is administered to the subject by mode of administrations known in the art, including but not limited to intramuscularly,
15 subcutaneously, intravenously, intraarterially, intraarticularly, intraperitoneally, intranasally, parenterally, and the like.

In one embodiment, the subject is a mammal, such as a monkey, rabbit, mouse, rat, pig or dog. In one embodiment, the subject is a human.

The EV or pharmaceutical composition for use, method, or use according to any
20 preceding AS, wherein the patient is diabetic. The advantage of treating diabetic patients is that such patients may develop diabetic foot ulcers (DFUs) which are challenging to treat. Thus, the presently disclosed EVs may provide a useful alternative treatment for this vulnerable patient group.

In one aspect, there is provided an EV, composition thereof, method or use
25 thereof substantially as described herein, for example with reference to the drawings.

BRIEF DESCRIPTION OF FIGURES

Figure 1 shows a schematic summarising the presently disclosed method of manufacturing the presently disclosed extracellular vesicle.

30 **Figure 2A** shows a schematic of the framework of adipogenic differentiation for obtaining ASC-EVs.

Figure 2B shows a graph demonstrating that EVs from intermediate differentiating ASCs depict significant killing of *P. aeruginosa* (PA) bacterial strain grown as per CLSI guidelines. Conditions showing >50% bacterial killing compared to media
35 control are outlined. Each value is the mean $\pm$ SEM (n=3).). All statistical significance

was assessed by using one-way ANOVA (Dunnett's multiple comparisons test), compared to the media control normalized to 100%, *** $p < 0.001$, **** $p < 0.0001$.

Figure 2C shows a photo of a representative spot-plating showing colonies of surviving PA upon treatments with ATCC, S23 and S29-derived EVs.

5 Figure 3 shows a graph showing the results of Crystal violet (CV) staining of 3-day old *P. aeruginosa* (PA) biofilm in response to different ASC-EVs as indicated. Each value is the mean $\pm$ SEM (n=3, biological replicates). All statistical significance was assessed by using one-way ANOVA (Dunnett's multiple comparisons test), compared to the media control normalized to 100%, **** $p < 0.001$.

10 Figure 4 shows a graph indicating the migration potential of human keratinocytes (HaCat cells) assessed in presence of bacteria- primed intermediate differentiating ASC-EVs by 2D scratch assay after 48 hours of treatment analyzed by ImageJ. The rate of change of wound area was calculated as indicated in the Methods section. Each value is the mean $\pm$ SD (n=2). All statistical significance was assessed by using one-way
15 ANOVA (Dunnett's multiple comparisons test), compared to the media control normalized to 100%, * $p < 0.05$, ** $p < 0.01$.

Figure 5 shows graphs of (A) *P. aeruginosa* (PA) and (B) *Staphylococcus aureus* (SA) biofilms in response to different ASC-EVs as indicated. Each value is the mean $\pm$ SEM (n=3, biological replicates). All statistical significance was assessed by using
20 one-way ANOVA (Dunnett's multiple comparisons test), compared to the media control normalized to 100%, **** $p < 0.001$.

Figure 6A shows a graph of viable counts (CFU%) of PA formed within the biofilms in presence of various treatments obtained by sonication and standard plate counts.

25 Figure 6B shows representative images of wound regions between non-infected and PA-infected pellicle as indicated. Wound margins are indicated in dashed line and arrows indicate the direction of migration.

Figure 6C shows as graph of the computation of the speed of keratinocyte indicated as % wound area decrease per hour. All statistical significance was assessed
30 by using Student's paired t-test, * $p < 0.05$.

Figure 7 shows representative images of wound regions between (A) non-infected and PA-infected pellicle, in presence of gentamicin, ATCC-EVs and (B) S23 and S29-derived EVs as indicated. Wound margins are indicated in dashed line and arrows indicate the direction of migration.

Figure 7C shows a graph of the computation of the speed of keratinocyte is indicated as % wound area decrease per hour. Negative migration speed indicates wound retraction resulting in widening of the wound area. All statistical significance was assessed by using Student's paired t-test, *p < 0.05; **p < 0.005.

5

EXAMPLES

Example embodiments of the disclosure will be better understood and readily apparent to one of ordinary skill in the art from the following discussions and if applicable, in conjunction with the figures. It should be appreciated that other modifications may be made without deviating from the scope of the invention. Example embodiments are not necessarily mutually exclusive as some may be combined with one or more embodiments to form new exemplary embodiments. The example embodiments should not be construed as limiting the scope of the disclosure.

10

Example 1 – Bacteria-priming triggers antibacterial and wound healing properties in ASC-EVs

Materials and Methods

ASC lines

White adipose tissue (WAT) was isolated from the subcutaneous (abdominal region) depot from all the human volunteers undergoing bariatric surgery with approval from the Domain Specific Review Board at National Healthcare Group, Singapore. ASCs were isolated and enriched by serial passage culture of stromal vascular fractions (SVF), as described previously^{10,11}. ASCs were obtained from either health donor (ATCC SCRC 4000) or obese patients (S29, 37M; S23, 34F).

25

Bacterial strains

Pseudomonas aeruginosa (ATCC 14213, PA) was obtained from The American Type Culture Collection (ATCC).

30

Isolation of EVs from ASC-CM

100k ASCs were seeded in 6-well plates and grown for 2 days until D0. ASCs were then induced with adipogenic cocktail for 12 days as described¹². Live PA was used to prime the ASCs at different multiplicity of infection (MOI) from 3-24 hours and the respective CM were obtained at D0 (undifferentiated stage), D6 (intermediate

35

differentiating) and D12 (fully differentiated). Such priming was performed in presence of either high (4.5g/l) or low (1g/l) D-glucose and $\pm 10\%$ FBS. To prevent acidification, 20mM of HEPES was added during bacteria priming of ASCs. ASC-CM was filtered using 0.2 μ M syringe filter to eliminate any bacterial contamination. EVs were isolated utilizing two distinct methodologies.

The first method involves centrifugation of the ASC-CM at 10,000 RCF, 30 min, 4 °C to remove cell debris followed by supernatant centrifugation at 400,000 RCF, 3 hours, 4 °C with a Sorvall™ MTX150 Micro-Ultracentrifuge (ThermoFisher, catalogue #46960) and a S110-AT fixed angle rotor (ThermoFisher, catalogue #45539). Then, the supernatant was discarded and the final pellets containing exosomes were reconstituted in PBS, pooled into a single mixture per ASC group and analyzed.

Characterization of EVs

Physical analysis of EVs was performed using Zetasizer Nano ZSP and NanoSight NS300 (Malvern Panalytical). Dilutions of each sample were prepared with 1x PBS (v/v), then 500 μ L of each was syringe-injected into the O-ring top plate-secured laser module of the NanoSight device. Measurements were acquired in triplicates using the NTA 3.1 software, using a 405 nm (violet) embedded laser, with capture settings of 60 sec, at 25 °C, under static flow, with manual focus, and maximized camera level with minimal background and saturation. Frames were processed after capture, with detection thresholds adjusted to match capture focus. Dilutions were prepared in water (1:10, v/v) from previous dilutions and loaded into disposable polystyrene cuvettes (catalogue #ZEN0040) for size measurement and later into folded capillary cells (catalogue #DTS107) for zeta potential. Both measures were acquired in triplicates using automated duration settings, with dispersant set for water, temperature at 25 °C, and data processing by general purpose analysis model. Protein concentration of each sample was quantified using a Pierce™ BCA Protein Assay Kit (ThermoFisher, catalogue #23225) by the microplate method as instructed by the supplier, with a 96-well plate (ThermoFisher, catalogue #167008).

Antibacterial assay

Examination of the potency of ASC-EVs against PA strain was performed by using the broth microdilution protocol from the Clinical and Laboratory Standards Institute (CLSI) guidelines (CLSI 2012) as also described in ¹³. Briefly, five freshly plated bacteria colonies were grown in cation adjusted MH2 broth (BD Biosciences catalogue #212322)

in a shaker incubator at 37 °C, 220 RPM. Cells were grown to an optical density (OD₆₀₀) of 0.20 using a Microplate Spectrophotometer (Molecular Devices Spectra Max Plus). 100 µL of microbial culture containing ~ 5×10⁵ CFU/mL of bacteria in the respective broths were introduced into each well containing 100 µL of ASC-CM or exosomes and
5 incubated overnight at 37 °C. Next, to check the bactericidal effects, 100 µL from each well was transferred and spread on sterile MH2 agar plates. The plates were incubated overnight at 37 °C and the number of colonies forming units (CFU) was observed the next day. Percentage of bacteria killing was calculated as (100 - % of bacterial survival), media normalized to 100%. Bacterial killing percentage was normalized to the particle
10 counts as indicated.

Estimation of biofilm disruption

Biofilms were grown in cation adjusted MH2 broth for 72 hours in 96-well clear bottom polystyrene plates (ThermoFisher Nunc™ MicroWell™, catalogue# 167008). The
15 biofilm inoculum was prepared by subculturing overnight bacterial culture for 90 minutes until OD₆₀₀ reaches ~ 0.3-0.4. 200 µL of biofilm inoculum was aliquoted into each well of the 96-well plate. After 72 hours of static incubation at 37 °C, the spent media was expelled, and the biofilms were treated with ASC-CM/exosomes for 24 hours. Post-treatment, the minimum biofilm inhibition concentration (MBIC) showing >50% reduction
20 was determined by quantifying the levels of remaining attached biofilm, by crystal violet staining method.

Briefly, all the contents in the 96-well plates were removed and the wells were washed thrice with 1x PBS (Phosphate Buffer Solution) to remove the non-adherent planktonic bacteria. The attached biofilms were fixed with 95% methanol for 5 minutes.
25 The wells were air-dried and stained with 0.1% crystal violet solution for 15 minutes at room temperature. The crystal violet solution was removed, and the wells were washed with water three times. The plates were again air-dried, and the stained biofilm was solubilized using 30% acetic acid. The absorbance of the biofilms were recorded at OD₅₉₅ using Micro xMark™ Microplate Absorbance Spectrophotometer (Bio-Rad laboratories,
30 CA, USA). Percentage of biofilm disruption was calculated as (100 - % of biofilm mass), media normalized to 100%. Biofilm disruption percentage was normalized to the particle counts present as indicated.

2D scratch assay

The Migration potential of human keratinocytes were assessed in presence of mASC-derived EVs in a 2D scratch assay at each timepoint by applying an ImageJ plugin ‘Wound Healing Size tool’ on phase contrast time lapse images. The rate of change of wound area directed by the lateral keratinocytes migration was calculated by obtaining a best fit line vs time plot as described in ¹³. The rate of change of wound area was used as a surrogate measure for speed of migrating keratinocytes.

Statistical analysis

Results were represented as means ± SEM. Student’s t test was used to determine differences in means between two groups. The p value was calculated using ANOVA for multiple comparisons with corrections and p < 0.05 being considered as significant.

Results

Bacteria-primed, intermediate adipogenic differentiating ASC-EVs exhibit significant antibacterial effects

The present inventors isolated EVs from ASC-CM as indicated in the methods section. Properties of EVs are summarized in Table 1. The EVs were purified from the CM obtained from the ASCs as shown in (Figure 2A). The present inventors then investigated the antibacterial properties of ASC-EVs from bacteria-primed intermediate differentiating ASC-CM derived from both healthy donor (ATCC SCRC 4000) and obese patients (S29 and S23) against PA compared to ASCs growth media without antibiotics. Indeed, EVs from ASCs at the intermediate stage of adipogenic differentiation (D6) exhibited >50% bacterial killing (Figure 2B). Bacteria spot assay (Figure 2C) showed visible colony changes in agreement to the killing counts (Figure 2B).

Table 1. Characterization of intermediate differentiating ASC-EVs

EVs (D6)	Size	Particle count
ATCC Un-primed	125.2	8.66E+10
ATCC PA-primed	98.1	1.356E+11
S29 Un-primed	103.6	7.84E+10
S29 PA-primed	98.9	6.78E+10

S23 Un-primed	92.3	5.64E+10
S23 PA-primed	98.3	7.96E+10

Intermediate adipogenic differentiating ASC-EVs exert significant biofilm disruption

EVs obtained from bacteria-primed, intermediate adipogenic differentiating ASC-
CM significantly disrupted the biofilm forming abilities of PA strain. Such activated EVs
exhibited >75% disruption of 3-day old PA biofilms grown in MHB (Figure 3).

EVs from bacteria-primed, intermediate adipogenic differentiating ASC-CM exhibit positive wound healing effects

Intermediate differentiating ASC-EVs upon bacteria-priming displayed a
significant increase in lateral migration of human keratinocytes highlighting accelerated
wound closure compared to media control (Figure 4). Thus, the data demonstrates that
besides antibacterial properties, bacteria primed intermediate EVs also contribute to
accelerated wound healing.

Cost-Effective derivation of EVs through CM minimization

To derive EVs in a cost-effective manner, the present inventors refined the
composition of the CM during the bacteria-priming stage. Investigations revealed that
serum, commonly included in the CM, was not essential for maintaining the medium's
efficacy. Further cost reductions were achieved by omitting the adipogenic cocktail, with
the resulting CM still demonstrating antibacterial properties. Ultimately, the optimized
bacteria-priming medium comprised solely of KnockOut™ DMEM (Gibco™)
supplemented with HEPES. This streamlined formulation significantly reduces the
expenses associated with EV production.

In conclusion, the present inventors have discovered robust antibacterial and
wound healing properties of intermediate differentiating ASC-EVs triggered by bacteria
priming, which has never been reported before to their knowledge. These findings
demonstrate the potential for bacterial primed ASC-EVs as promising cell-free
therapeutics against chronic wound infections.

Example 2 – ASC-EVs exhibit antibiofilm effects in a chronic *in vitro* wound model
Materials and Methods

Bacterial strains

Enterococcus faecalis (ATCC 49474, EF), *Pseudomonas aeruginosa* (ATCC 14213, PA), and *Komagataeibacter hansenii* 53582 were obtained from The American Type Culture Collection (ATCC).

5

Visualization of biofilms

To estimate the biofilm mass formed on the pellicles, calcofluor white staining and Syto 9 were used to detect extracellular polysaccharides (EPS) and bacteria respectively. Briefly 0.3 µl of Syto 9 (3.34 mM) (Invitrogen, L7012) was used in 100 µl of reaction mixture for 15 min under dark. Next, Syto 9 stained pellicles were placed on
10 clean glass slides and 1 drop of calcofluor white stain (Sigma-Aldrich 18909) was added followed by one drop of potassium hydroxide. Each stain was put for 1 min, and the excess dyes were removed by paper towels and immediately proceeded for imaging.

15 **Microscopy**

Time lapse microscopy was carried out to track the extent of wound regression due to bacterial pellicle loaded with bacterial strains. Image interval of 2 hours and 6 hours was used on the Olympus IX-83 and Nikon TiE microscopes, respectively. The images were auto-stitched to derive an image of an entire well of a 24-well plate. A
20 humidity chamber set to 37°C was used to contain the plates during imaging. To estimate migration potential of keratinocytes, the leading edge of the wound is monitored over time and scored for wound area changes by using 'wound healing size tool plugin' analysed by ImageJ¹⁴.

25 **Statistical analysis.**

Results were represented as means ± SEM. Student's t test was used to determine differences in means between two groups. The p value was calculated using ANOVA for multiple comparisons with corrections and p < 0.05 being considered as significant.

30

Development of bacteria-infected *in-vitro* chronic wound model**(i) Inoculation of bacteria into BC pellicles**

The BC pellicles were prepared based on¹⁵ and lyophilization using freeze dryer. BC pellicles were first cut into small pieces (<5mm diameter) and sterilized by
35 autoclaving. Overnight grown PA (5% inoculum) was inoculated into BC pellicles in MHB

for 48h to allow bacteria to form biofilms. The pellicles were then washed thrice in PBS to eliminate the non-adhered bacteria. To estimate the amount of PA immobilized, the pellicles were sonicated in a water bath sonicator for 30 min at 15mA, serially diluted and plated to obtain the viable counts. Post-treatment with ASC-EVs, the pellicles were
5 sonicated and plated to estimate the reduction of the viable counts as an effect of treatments.

(ii) Placing of 'fence-collar-well' device for migration assay.

Fence-barrier method of leading edge was used to quantify migration as shown
10 in ¹⁶ with modifications. (A) Fence-collar units were inserted into 24-well tissue culture plates, which formed inner-wells. 40,000 human keratinocytes (HaCat) cells were seeded into each outer-wells and grown for 3 days to have an overlay of cells surrounding the inner wells. (C) To obtain a cell-free area in the inner wells, TrypLE™ Express was added to the inner chambers and the cells were lysed by repeatedly
15 pipetting and washing with PBS.

(iii) Introduction of the BC pellicle containing pre-formed biofilms into the inner well wound site.

BC pellicles containing pre-formed PA biofilms were placed through the inner well
20 into the centred wound region. 100 µl of ASC-EVs were seeded, the fence-collar unit was removed, and the plate was immediately put inside an *Incucyte*® SX5 Live-Cell Analysis Instrument placed inside a 5% CO₂ chamber for 48 hours.

Treatment assessment.

25 (A) Biofilm disruption potential of EVs were measured by sonicating the BC pellicles to obtain viable counts post-treatment. (B) To estimate migration potential of keratinocytes, the leading edge of the wound is monitored over time and scored for wound area changes by using 'wound healing size tool plugin ¹⁴ analysed by ImageJ.

30 **Results**

A BC pellicle containing preformed PA biofilms was placed at the wound centre surrounded by human keratinocytes as indicated in the *Methods* section. First, viable count of PA in presence of bacteria primed EVs obtained from adipogenic differentiating ASCs (D6) showed significant decrease compared to un-primed or media control (**Figure
35 6A**), suggesting biofilm disruption abilities of ASC-EVs in a chronic wound setting.

Next, migration speed of keratinocytes was compared between non-infected and PA-infected pellicles to examine any possible migration delay in presence of bacteria at the wound site. Indeed, the present inventors observed negligible migration in PA-infected pellicle compared to non-infected one (**Figures 6B, C**). Thus, the present
5 inventors' model could detect evidence of migration delay due to presence of bacterial infection at the wound site. Thus, the present inventors' model shows co-existence and interdependency between bacterial infection and wound closure, which is not reported by others in an *in-vitro* setting.

Finally, EF-primed EVs from both ATCC and S23 enabled maximum migration of
10 keratinocytes (**Figure 7**). In contrast, the un-primed EVs showed wound retraction resulting in wound area increase (**Figure 7**). Thus, EF-primed ATCC and S23 EVs stand as ideal treatments against chronic wound infections. On the contrary in S29, EF primed-EVs showed migration delay compared to the un-primed EVs.

Hence, this example demonstrates the antibiofilm and wound healing properties
15 of EVs in the context an *in vitro* chronic wound model, thereby further substantiating the antibacterial and wound healing functions of the presently disclosed EVs.

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APPLICATIONS

The presently disclosed EVs have various applications such as the following:

- 30 Firstly, EVs derived from bacteria-primed ASCs retaining antibacterial and wound healing properties stand as direct intervention against chronic wound infections.

Secondly, such EVs can be purified from all type of stem cells undergoing differentiation commonly recruited at the wound site such as epidermal stem cells (EPSCs), hair follicle stem cells (HFSCs), hematopoietic stem cells (HSCs) and other

multipotent MSCs present in stromal cells. Such potent EVs could lead to an alternate treatment strategy with biologics against wound infections.

Thirdly, EVs could translate to a clinical trial involving subjects with advanced diabetic foot ulcer (DFU).

- 5 Finally, ASC-EVs owing to their wound healing abilities can be investigated for treating non-infective wounds, damaged skin care and as skin related cosmetics.

CLAIMS

1. An extracellular vesicle (EV) having anti-bacterial and wound healing properties, wherein the EV is derived/obtained/isolated from a mesenchymal stem cell (MSC) which has been primed with one or more species of live bacteria.
2. A method of manufacturing an EV according to any preceding claim, comprising the steps of priming a mesenchymal stem cell (MSC) with one or more species of live bacteria; and isolating the EV.
3. The EV or method according to any preceding claim, wherein the MSC has been primed by incubating/co-culturing the MSC with the one or more species of live bacteria.
4. The EV or method according to any preceding claim, wherein the priming of the MSC is performed in the absence of serum.
5. The EV or method according to any preceding claim, wherein the priming of the MSC is performed in the presence of a minimal medium comprising or consisting of a media, for example DMEM, such as KnockOut™ DMEM (Gibco™); and a buffer, such as HEPES.
6. The EV or method according to any preceding claim, wherein priming of the MSC is performed in the presence of one or more of the following:
 - D-glucose, such as high (e.g. 4.5 g/l) or low (e.g. 1g/l) D-glucose;
 - a serum, such as FBS, for example; 5 to 15% FBS, such as 10% FBS; and
 - a buffer, such as HEPES, for example 10 to 30 mM HEPES, such as 20 mM HEPES.
7. The EV or method according to any preceding claim, wherein the MSC is cultured, for example grown for 2 days, prior to the priming step.
8. The EV or method according to any preceding claim, wherein the EV is isolated from MSC conditioned media (CM), *i.e. the media that the MSC has been primed in.*
9. The EV or method according to any preceding claim, wherein the CM is filtered prior to isolation of the EV, for example using a 0.2 µm syringe filter.
10. The EV or method according to any preceding claim, wherein the EV is isolated from the CM by centrifuging the CM, obtaining a pellet containing the EV, and reconstituting the EV in a suitable buffer, such as PBS.

11. The EV or method according to any preceding claim, wherein the MSC originates from an organism selected from the group comprising: a human, and mouse, in particular a human MSC.
- 5 12. The EV or method according to any preceding claim, wherein the MSC is obtained from one of more of the following: bone marrow, cord cells, adipose tissue, molar cells, amniotic fluid, muscle, and liver.
- 10 13. The EV or method according to any preceding claim, wherein the MSC is selected from the group comprising an adipose-derived stem cell (ASC), an epidermal stem cell (EPSC), a hair follicle stem cell (HFSC), and a hematopoietic stem cell (HSC).
- 15 14. The EV or method according to any preceding claim, wherein the MSC is an adipose-derived stem cell (ASC), for example an ASC derived from white adipose tissue (WAT).
- 20 15. The EV or method according to any preceding claim, wherein differentiation of the ASC is induced with an adipogenic cocktail.
- 25 16. The EV or method according to any preceding claim, wherein the adipogenic cocktail comprises one of more of the following:
- dexamethasone, for example 0.5 to 1.5 mM dexamethasone, such as 1 mM dexamethasone;
 - IBMX, for example 0.25 to 0.75 mM IBMX, such as 0.5 mM IBMX;
 - Insulin, for example 125 to 200 nM insulin, such as 167 nM insulin; and
 - Indomethacin, for example 50 to 150 mM indomethacin, such as 100 mM indomethacin.
- 30 17. The EV or method according to any preceding claim, wherein the MSC/ASC is an intermediate differentiating MSC/ASC, for example a Day 4 to 7, such as Day 4, 5, 6, or 7 intermediate differentiating MSC/ASC.
- 35 18. The EV or method according to any preceding claim, wherein the MSC/ASC is a Day 6 intermediate differentiating MSC/ASC.
- 40 19. The EV or method according to any preceding claim, wherein the EV is an exosome.
- 45 20. The EV or method according to any preceding claim, wherein the EV has a size in the range 80 to 140 nm, such as 80, 85, 90, 95, 100, 105, 110, 115, 120, 125, 130, 135 or 140 nm; or a size in the range of 95 to 105 nm, such as 95, 96, 97, 98, 99, 100, 101, 102, 103, 104 or 105 nm.
21. A composition, such as a pharmaceutical composition, comprising an EV according to any preceding claim.

22. The composition according to claim 21, further comprising one of more of the following: a pharmaceutically acceptable excipient, a buffer or an additive.
- 5 23. A method of treatment, comprising administering a therapeutically effective amount of an EV or a pharmaceutical composition according to any preceding claim to a patient in need thereof.
- 10 24. An EV or a pharmaceutical composition according to any one of claims 1 to 23, for use in the treatment of a patient.
25. Use of an EV or a pharmaceutical composition according to any one of claims 1 to 22, in the manufacture of a medicament for the treatment of a patient.
- 15 26. The EV or pharmaceutical composition for use, method, or use according to any one of claims 23 to 25, wherein the treatment is for one or more of the following: a wound, such as a chronic wound, a bacterial infection or a disease/condition caused by a bacterial infection caused by one or more species of bacteria.
- 20 27. The EV or pharmaceutical composition for use, method, or use according to any one of claims 23 to 26, wherein the treatment is for non-infective wounds or damaged skin.
- 25 28. The EV or pharmaceutical composition for use, method, or use according to any one of claims 23 to 26, wherein the treatment is for chronic wound healing.
- 30 29. The EV or pharmaceutical composition for use, method, or use according to any one of claims 26 to 28, wherein the chronic wound is selected from the group comprising a vascular ulcer, such as a venous ulcer or an arterial ulcer, a diabetic ulcer and a pressure ulcer, a surgical wound, and a traumatic wound, in particular a diabetic foot ulcer.
- 35 30. The EV or pharmaceutical composition for use, method, or use according to any one of claims 23 to 29, wherein the treatment is for a bacterial infection caused by one or more species of bacteria, for example selected from the group comprising: *Acinetobacter baumannii*, *Bacillus anthracis*, *Bacillus cereus*, *Bartonella henselae*, *Bartonella quintana*, *Bordetella pertussis*, *Borrelia burgdorferi*, *Borrelia garinii*, *Borrelia afzelii*, *Borrelia recurrentis*, *Brucella abortus*, *Brucella canis*, *Brucella melitensis*, *Brucella suis*, *Campylobacter jejuni*, *Chlamydia pneumoniae*, *Chlamydia trachomatis*, *Chlamyophila psittaci*, *Clostridium botulinum*, *Clostridium difficile*, *Clostridium perfringens*, *Clostridium tetani*, *Corynebacterium diphtheriae*, *Enterobacter spp*, *Enterococcus faecalis*, *Enterococcus faecium*, *Escherichia coli*, *Francisella tularensis*, *Hemophilus influenzae*, *Helicobacter pylori*, *Klebsiella pneumoniae*, *Legionella pneumophila*, *Leptospira interrogans*, *Leptospira santarosai*, *Leptospira weilli*, *Leptospira*
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- 5 *noguchii*, *listeria monocytogenes*, *Mycobacterium leprae*, *Mycobacterium tuberculosis*, *Mycobacterium ulcerans*, *Mycoplasma pneumoniae*, *Neisseria gonorrhoeae*, *Neisseria meningitidis*, *Pseudomonas aeruginosa*, *Rickettsia rickettsia*, *Salmonella typhi*, *Salmonella typhimurium*, *Shigella sonnei*,
10 *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Staphylococcus saprophyticus*, *Streptococcus agalactiae*, *Streptococcus pneumoniae*, *Streptococcus pyogenes*, *Treponema pallidum*, *Ureaplasma urealyticum*, *Vibrio cholerae*, *Yersinia pestis*, *Yersinia enterocolitica*, and *Yersinia pseudotuberculosis*.
- 15 31. The EV or pharmaceutical composition for use, method, or use according to claim 30, wherein the one or more species of bacteria are selected from the group comprising *Salmonella Typhimurium*, *Staphylococcus aureus* and *Pseudomonas aeruginosa*.
- 20 32. The EV or pharmaceutical composition for use, method, or use according to any one of claims 23 to 26, wherein the treatment is for biofilm disruption and/or planktonic bacteria killing.
- 25 33. The EV or pharmaceutical composition for use, method, or use according to any one of claims 23 to 32, wherein the patient is a human, such as a diabetic patient.
34. An EV or pharmaceutical composition according to any one of claims 1 to 22, for use in an aesthetic procedure, such as a skin aesthetic procedure, for example selected from the group comprising: the treatment of scars, skin laxity, wrinkles, liver spots, cellulite and skin discoloration.