Therapeutics using multiple injections of cells

Technical Field

[0001] The present invention relates to methods of treatment of various conditions including osteoarthritis, tendon injuries and pain using autologous adipose-derived cells suspensions in which the treatment comprises a course of multiple injections of a single preparation of autologous cells. The invention also relates to the preparation of compositions for use in such treatments.

Background of the Invention

[0002] Adipose tissue contains a cell population of large lipid filled adipocytes, and a non-adipocyte cell population, which comprises cells associated with various connective fibres and cells associated with capillaries and larger blood vessels. The non-adipocyte cell population is also thought to comprise a population of adipose-derived adult stem cells and consequently there has been interest in using adipose tissue as a source of isolated stem cells for various therapeutic applications.

[0003] In general, methods for obtaining adipose tissue-derived presumptive stem cells involves depleting adipocytes from adipose-derived non-adipocyte cells, which requires digesting adipose tissue with enzymes such as collagenase, and then separating the liberated cells by centrifuging the digested sample. During centrifugation, the adipose-derived non-adipocyte cells separate from the adipocytes to form a pellet, whereas the lipid containing adipocytes float. The non-adipocyte cell fraction is then used as a source of tissue stem cells.

[0004] The present inventors have earlier described the use of an adipose tissue-derived cell suspension which comprises adipocytes for the preparation of a pharmaceutical composition for use in the treatment of an inflammatory disorder or the alleviation of pain associated with an inflammatory disorder in a subject, and for the treatment and alleviation of pain of conditions such as a cartilage or bone disorders. This is described in Australian Patent Application No. 2009201915 and in International Publication No. WO2010/020005, the contents of which are incorporated herein by cross-reference.

[0005] There remains a need for improved methods for the treatment of conditions where regeneration of damaged cellular material, such as bone or cartilage, is desirable, such as osteoarthritis, tendon injuries. There remains a need also for improved methods for the treatment of pain, such as pain associated with osteoarthritis and tendon injuries as well as pain generally, including back pain.

Summary of the Invention

[0006] Methods previously described for the treatment of inflammatory disorders, such as osteoarthritis, as well as tendon injuries using adipose tissue-derived cell suspensions generally teach the administration of the composition or suspension to the subject as a single dose and that this can provide the subject with long term relief from the condition, for example in terms of greater mobility and reduced pain.

[0007] The inventors have surprisingly identified that an autologous adipose tissue-derived cell suspension may be frozen and retrieved for subsequent administration as a course of planned injections to provide an improved therapeutic outcome compared to a single dose. Surprisingly such frozen cells may be used without the need for culturing the cells after retrieval from frozen storage.

[0008] Accordingly, in a first aspect of the invention there is provided a method of treating osteoarthritis in a subject requiring said treatment, the method comprising administering to said subject a course of treatment comprising multiple doses over time of an autologous adipose tissue-derived cell suspension comprising adipose tissue-derived non-adipocyte cells, wherein a first dose comprises a portion of a freshly prepared cell suspension and a subsequent dose or doses comprise a portion of said cell suspension that has been stored frozen.

[0009] In a second aspect of the invention there is provided a method of treating a tendon injury in a subject requiring said treatment, the method comprising administering to said subject a course of treatment comprising multiple doses over time of an autologous adipose tissue-derived cell suspension comprising adipose tissue-derived non-adipocyte cells, wherein a first dose comprises a portion of a freshly prepared cell suspension and a subsequent dose or doses comprise a portion of said cell suspension that has been stored frozen.

[00010] In a third aspect of the invention there is provided a method of treating pain in a subject requiring said treatment, the method comprising administering to said subject a course of treatment comprising multiple doses over time of an autologous adipose tissue-derived cell suspension comprising adipose tissue-derived non-adipocyte cells, wherein a first dose comprises a portion of a freshly prepared cell suspension and a subsequent dose or doses comprise a portion of said cell suspension that has been stored frozen.

[00011] In a further aspect of the invention there is provided use of an autologous adipose tissue-derived cell suspension comprising adipose tissue-derived non-adipocyte cells for the manufacture of a medicament for treating osteoarthritis in a subject requiring said treatment, wherein the medicament comprises multiple doses suitable for administration to said subject over time, wherein a first dose comprises a portion of a freshly prepared cell suspension and a subsequent dose or doses comprise a portion of said cell suspension that has been stored frozen.

[00012] In a further aspect of the invention there is provided use of an autologous adipose tissue-derived cell suspension comprising adipose tissue-derived non-adipocyte cells for the manufacture of a medicament for treating a tendon injury in a subject requiring said treatment, wherein the medicament comprises multiple doses suitable for administration to said subject over time, wherein a first dose comprises a portion of a freshly prepared cell suspension and a subsequent dose or doses comprise a portion of said cell suspension that has been stored frozen.

[00013] In a further aspect of the invention there is provided use of an autologous adipose tissue-derived cell suspension comprising adipose tissue-derived non-adipocyte cells for the manufacture of a medicament for treating pain in a subject requiring said treatment, wherein the medicament comprises multiple doses suitable for administration to said subject over time, wherein a first dose comprises a portion of a freshly prepared cell suspension and a subsequent dose or doses comprise a portion of said cell suspension that has been stored frozen.

[00014] In a further aspect of the invention there is provided use of an autologous adipose tissue-derived cell suspension comprising adipose tissue-derived non-adipocyte cells for the manufacture of a medicament for treating osteoarthritis in a

subject requiring said treatment, wherein the subject is provided a course of treatment comprising multiple doses over time of said medicament, wherein a first medicament dose comprises a portion of a freshly prepared cell suspension and a subsequent medicament dose or doses comprise a portion of said cell suspension that has been stored frozen.

[00015] In a further aspect of the invention there is provided use of an autologous adipose tissue-derived cell suspension comprising adipose tissue-derived non-adipocyte cells for the manufacture of a medicament for treating a tendon injury in a subject requiring said treatment, wherein the subject is provided a course of treatment comprising multiple doses over time of said medicament, wherein a first medicament dose comprises a portion of a freshly prepared cell suspension and a subsequent medicament dose or doses comprise a portion of said cell suspension that has been stored frozen.

[00016] In a further aspect of the invention there is provided use of an autologous adipose tissue-derived cell suspension comprising adipose tissue-derived non-adipocyte cells for the manufacture of a medicament for treating pain in a subject requiring said treatment, wherein the subject is provided a course of treatment comprising multiple doses over time of said medicament, wherein a first medicament dose comprises a portion of a freshly prepared cell suspension and a subsequent medicament dose or doses comprise a portion of said cell suspension that has been stored frozen.

[00017] In a further aspect of the invention there is provided an autologous adipose tissue-derived cell suspension comprising adipose tissue-derived non-adipocyte cells for use in the treatment of osteoarthritis in a subject requiring said treatment, characterised in that said treatment comprises administration of multiple doses over time of said cell suspension, wherein a first dose comprises a portion of said cell suspension freshly prepared and a subsequent dose or doses comprise a portion of said cell suspension that has been stored frozen.

[00018] In a further aspect of the invention there is provided an autologous adipose tissue-derived cell suspension comprising adipose tissue-derived non-adipocyte cells for use in the treatment of a tendon injury in a subject requiring said treatment,

characterised in that said treatment comprises administration of multiple doses over time of said cell suspension, wherein a first dose comprises a portion of said cell suspension freshly prepared and a subsequent dose or doses comprise a portion of said cell suspension that has been stored frozen.

[00019] In a further aspect of the invention there is provided an autologous adipose tissue-derived cell suspension comprising adipose tissue-derived non-adipocyte cells for use in the treatment of pain in a subject requiring said treatment, characterised in that said treatment comprises administration of multiple doses over time of said cell suspension, wherein a first dose comprises a portion of said cell suspension freshly prepared and a subsequent dose or doses comprise a portion of said cell suspension that has been stored frozen.

[00020] In an embodiment the adipose tissue-derived cell suspension comprises aggregates of cells and or comprises pieces of adipose tissue. In an embodiment the adipose tissue-derived cell suspension further comprises adipocytes. In an embodiment the cell suspension is substantially free of adipocytes. In an embodiment the adipose tissue-derived cell suspension is prepared by a method that comprises removal of (i) part of the adipocyte content or (ii) substantially all of the adipocyte content during preparation of the adipose tissue-derived cell suspension.

[00021] In an embodiment the course of treatment comprises a first dose of a cell suspension comprising non-adipocyte cells and adipocytes and the cell suspension of one or more of the one or more subsequent dose or doses is substantially free of adipocytes. In an embodiment the course of treatment comprises a first dose of a cell suspension comprising non-adipocyte cells and adipocytes and the cell suspension of one or more of the one or more subsequent dose or doses comprises non-adipocyte cells and adipocytes.

[00022] In an embodiment the course of treatment comprises a first dose and a second dose. In an embodiment the course of treatment comprises a first dose, a second dose and a third dose. In an embodiment the course of treatment comprises a first dose, a second dose, a third dose and a fourth dose. In an embodiment the course of treatment comprises a first dose, second dose, a third dose, a fourth dose and a fifth dose.

[00023] In an embodiment the course of treatment comprises multiple doses each subsequent dose separated in time from the previous dose by between one week and ten weeks. In an embodiment the course of treatment comprises multiple doses each subsequent dose separated in time from the previous dose by between two weeks and eight weeks. In an embodiment the course of treatment comprises multiple doses each subsequent dose separated in time from the previous dose by between two weeks and six weeks.

[00024] In an embodiment the course of treatment comprises multiple doses administered over a total treatment period of between three and twelve months. In an embodiment the course of treatment comprises multiple doses administered over a total treatment period of between six and twelve months. In an embodiment the course of treatment comprises multiple doses administered over a total treatment period of between three and nine months. In an embodiment the course of treatment comprises multiple doses administered over a total treatment period of between six and nine months.

[00025] In an embodiment the subsequent dose or doses is administered to the subject soon after thawing, such as within about 10 minutes after thawing, or within about 20 minutes after thawing, or within about 30 minutes after thawing or within about one hour of thawing or within about two hours of thawing.

[00026] In an embodiment one or more of said doses comprises injection at multiple sites.

[00027] In an embodiment the pain is back pain. In an embodiment the pain is neuropathic pain. In an embodiment the pain is associated with osteoarthritis, stifle disease, wobblers, a tendon injury or a ligament injury. In an embodiment the pain is associated with atopic dermatitis. In an embodiment the pain is associated with rheumatoid arthritis, back pain, or multiple sclerosis. In an embodiment the pain is associated with a burn injury. In an embodiment the pain is neck and or shoulder pain, whiplash associated disorder, or complex regional pain syndrome. In an embodiment the pain is back pain. In an embodiment the pain is lower back pain. In an embodiment the pain is pain associated with a sciatic disorder.

[00028] In an embodiment the treatment is of pain for which there is no discernable causative clinical condition. In an embodiment the treatment is of pain for which there is no discernable causative clinical condition in the part or region of the body in which the subject experiences the pain. In an embodiment the pain is neuropathic pain. In an embodiment the neuropathic pain is pain for which there is no discernable causative clinical condition. In an embodiment the appropriate form of administration is injection. The neuropathic pain may be localised to one area of the body or it may be experienced in multiple sites of the subject's body. When experienced in multiple sites, the intensity of the pain may be similar at multiple sites or it may be different at multiple sites. In an embodiment the neuropathic pain is neuropathic facial pain. In an embodiment the pain is neuropathic facial pain and administration to said subject is by injection into the jaw or the gum. In an embodiment the injection into the jaw or the gum is at the original site of the pain. In an embodiment the pain is associated with a joint disease or joint disorder and the composition is administered to a site on said subject which is remote to said joint.

[00029] In an embodiment the administration is subcutaneous administration. In an embodiment the administration is intramuscular administration. In an embodiment the administration is in the rump, arm, or buttocks. In an embodiment the administration is into the neck of the subject, such as the nape of the subject, such as the scruff of the neck when the subject is a dog or cat.

[00030] In an embodiment the pain is back pain and the administration is by intramuscular injection. In an embodiment the method of treating osteoarthritis is by intra-articular injection into an affected joint. In an embodiment the method of treating a tendon injury is by injection directly into the tendon. In an embodiment the tendon injury is tendinopathy, tendinitis, or tendinosis. In an embodiment the tendon injury is chronic tendinosis.

[00031] In an embodiment the subject is a mammalian subject. In an embodiment the mammalian subject is an equine, feline, canine, bovine or porcine animal. In an embodiment the mammalian subject is a human.

[00032] In an embodiment the method further comprises obtaining adipose tissue from said subject. In an embodiment the adipose tissue is subcutaneous adipose tissue.

[00033] In an embodiment the method further comprises preparing an adipose tissue-derived cell suspension comprising adipose tissue-derived non-adipocyte cells and adipocytes, the method comprising:

dissociating a sample of adipose tissue from a subject to form a suspension of cells which comprises adipocytes and adipose-derived non-adipocyte cells;

centrifuging the suspension of cells to form a cell pellet, a floating cell layer which comprises adipocytes and an intermediate layer which is depleted of cells relative to the cell pellet and the floating cell layer; and

removing the intermediate layer and mixing the cell pellet and floating cell layer to form an adipose tissue-derived cell suspension which comprises adipocytes and adipose-derived non-adipocyte cells.

[00034] In an embodiment the method further comprises preparing an adipose tissue-derived cell suspension comprising adipose tissue-derived non-adipocyte cells, the method comprising:

dissociating a sample of adipose tissue to form a suspension of cells which comprises adipocytes and adipose-derived non-adipocyte cells;

centrifuging the suspension of cells to form a cell pellet, a floating cell layer which comprises adipocytes and an intermediate layer which is depleted of cells relative to the cell pellet and the floating cell layer; and

removing the intermediate layer and floating cell layer and mixing the cell pellet to form an adipose tissue-derived cell suspension which comprises adipose-derived non-adipocyte cells.

[00035] In an embodiment the method further comprises addition of one or more cryopreservatives to said adipose tissue-derived cell suspension. In an embodiment the method further comprises addition of autologous serum or autologous plasma to said adipose tissue-derived cell suspension.

[00036] In an embodiment the method further comprises dividing the adipose tissue-derived cell suspension comprising adipose tissue-derived non-adipocyte cells, optionally comprising adipocytes, into single dose volumes, a first dose of which is designated for same day administration to said subject and multiple additional single dose volumes are stored frozen.

[00037] In an embodiment the adipose tissue-derived cell suspension is divided into multiple aliquots, for example, into two, three, four, five, six, seven, eight, nine, ten, or up to about fifteen or twenty aliquots. In an embodiment the adipose tissue-derived cell suspension is divided into about five to about ten aliquots. Aliquots of a cell suspension may all be of approximately the same volume or may be of two or more different volumes. Aliquots of a cell suspension may all comprise approximately the same cell number or may be of two or more different cell numbers. In an embodiment the prepared adipose tissue-derived cell suspension comprises about 5 million to about 150 million cells. In an embodiment the prepared adipose tissue-derived cell suspension comprises about 5 million to about 100 million cells. In an embodiment an aliquot of an adipose tissue-derived cell suspension comprises between about 0.5 million to about 20 million cells. In an embodiment an aliquot of an adipose tissue-derived cell suspension comprises between about 0.5 million to about 5 million cells. In an embodiment an aliquot of an adipose tissue-derived cell suspension comprises less than about 5 million cells.

[00038] The summary of the invention described above is not limiting and other features and advantages of the invention will be apparent from the following detailed description of the preferred embodiments, as well as from the claims.

Brief description of the figures

Abbreviations

DMEM	Dulbecco's Modified Eagles Medium.
SVCs	stromal vascular cells.
SVF	stromal vascular fraction.
MSC	mesenchymal stem cell(s).

Definitions

[00039] The term "pharmaceutically acceptable" as used herein in the context of various components relevant to the invention, such as carriers, diluents, cryopreservatives, is intended to encompass not only such components which are suitable for administration to a human subject, but also those suitable for administration to a non-human mammalian subject. In particular embodiments, the pharmaceutically acceptable component is suitable for administration to a non-human mammalian subject. In particular embodiments the pharmaceutically acceptable component is suitable for administration to a human subject. In particular embodiments, the pharmaceutically acceptable component is suitable for administration to a non-human mammalian subject and to a human subject.

[00040] The terms "treating", "treatment", "therapy" and the like in the context of the present specification refer to the alleviation of the symptoms and/or the underlying cause of the condition or disease, such as osteoarthritis or tendon injury or pain. In certain embodiments a treatment will slow, delay or halt the progression of a disorder or the symptoms of the disorder or injury, or reverse the progression of the disorder or injury, at least temporarily. Hence, in the context of this invention the word "treatment" or derivations thereof such as "treating" when used in relation to a therapeutic application includes all aspects of a therapy, such as the alleviation of pain associated with the condition being treated, alleviation of the severity of the condition being treated, improvement in one or more symptoms of the condition being treated, etc. Use of the word "treatment" or derivatives thereof will be understood to mean that the subject being "treated" may experience any one or more of the aforementioned benefits.

[00041] Throughout this specification, reference to "a" or "one" element does not exclude the plural, unless context determines otherwise. Similarly, reference to "an embodiment" does not exclude the characteristic of that described embodiment applying in combination with one or more other embodiments described, unless the context determines otherwise.

[00042] The term "therapeutically effective amount" as used herein includes within its meaning a non-toxic but sufficient amount of a compound or composition for use in the invention to provide the desired therapeutic effect. The exact amount required

will vary from subject to subject depending on factors such as the species being treated, the age and general condition of the subject, co-morbidities, the severity of the condition being treated, the particular agent being administered and the mode of administration and so forth. Thus, for any given case, an appropriate "effective amount" may be determined by one of ordinary skill in the art using only routine methods.

[00043] In the context of this specification, the term "comprising" means including, but not necessarily solely including. Furthermore, variations of the word "comprising", such as "comprise" and "comprises", have correspondingly varied meanings. Hence, the term "comprising" and variations thereof is used in an inclusive rather than exclusive meaning such that additional integers or features may optionally be present in a composition, method, etc. that is described as comprising integer A, or comprising integer A and B, etc.

[00044] In the context of this specification the terms "about" and "approximately" will be understood as indicating the usual tolerances that a skilled addressee would associate with the given value.

[00045] In the context of this specification, where a range is stated for a parameter it will be understood that the parameter includes all values within the stated range, inclusive of the stated endpoints of the range. For example, a range of "5 to 10" will be understood to include the values 5, 6, 7, 8, 9, and 10 as well as any sub-range within the stated range, such as to include the sub-range of 6 to 10, 7 to 10, 6 to 9, 7 to 9, etc, and inclusive of any value and range between the integers which is reasonable in the context of the range stated, such as 5.5, 6.5, 7.5, 5.5 to 8.5 and 6.5 to 9, etc.

[00046] In the context of this specification, the terms "plurality" and "multiple" mean any number greater than one.

[00047] It is to be noted that reference herein to use of the inventive methods and compositions in treatment or therapy will be understood to be applicable to human and non-human, such as veterinary, applications. Hence it will be understood that, except where otherwise indicated, reference to a patient, subject or individual means a human or a non-human, such as an individual of any species of social, economic, agricultural or research importance including but not limited to members of the

classifications of ovine, bovine, equine, porcine, feline, canine, primates, rodents, especially domesticated or farmed members of those classifications, such as sheep, cattle, horses, pigs and dogs.

[00048] Where examples of various embodiments or aspects of the invention are described herein they will generally be prefaced by appropriate terms including "such as" or "for example", or "including". It will be understood that the examples are being described as inclusive possibilities, such as for the purpose of illustration or understanding and are not, unless the context indicates otherwise, being provided as limiting.

[00049] The pharmaceutical composition referred to herein may also be referred to as a medicament, such as when intended for therapeutic use. Hence, it will be understood that where the invention is described as including the use of a composition of described components for the preparation of a pharmaceutical composition for an intended therapeutic purpose, that description equally means use for the preparation of a medicament for that intended therapeutic purpose, unless the context indicates otherwise.

[00050] To the extent that it is permitted, all references cited herein are incorporated by reference in their entirety.

Detailed Description of the Preferred Embodiments

[00051] The present inventors have identified that surprisingly a course of treatment of various conditions using multiple administration of an autologous adipose tissue-derived cell suspension comprising adipose tissue-derived non-adipocyte cells administered over a period of time to the subject can provide improved outcomes for the subject. In this manner, the autologous adipose tissue-derived cell suspension comprising adipose tissue-derived non-adipocyte cells is typically one which has been prepared from a single adipose tissue extraction from the patient, divided into useful portions or aliquots, a first dose being administered soon after preparation of the cell suspension and other portions being stored under appropriate conditions until required for the second and, where appropriate, third, fourth, fifth, etc doses at which time the required dose is retrieved from storage and administered to the subject.

Adipose Tissue

[00052] The cell suspensions of the invention are autologous adipose tissue-derived cell suspensions. Adipose tissue may be human adipose tissue or mammalian animal adipose tissue, depending on the subject of the treatment. The adipose tissue may comprise "white" adipose tissue, or "brown" adipose tissue.

[00053] The adipose tissue may originate from any source in the subject's body which is accessible. Subcutaneous fat, for example, is readily accessible with only superficial wounding, or by using "keyhole surgery" techniques. For example adipose tissue may be tissue collected using liposuction techniques, or adipose tissue which is removed with reproductive tissue when de-sexing a male or female animal. The adipose tissue may be collected during a cosmetic procedure performed on the subject of the intended treatment. The adipose tissue may be collected specifically as part of the intended treatment of the subject for the indicated condition. The adipose tissue may be rinsed with a tissue culture medium or buffered isotonic solution to remove adherent blood cells, and may be trimmed or coarsely processed to remove large blood vessels or connective tissue elements prior to generating an adipose tissue-derived cell suspension.

[00054] The adipose tissue may be derived from a mature animal or from a juvenile animal.

[00055] In particular embodiments the subject is a companion animal, such as a canine or a feline domestic animal, or a working animal. In other particular embodiments the subject is a farm animal or racing animal selected from a horse, donkey, ass, cow, buffalo, sheep, goat, camel or pig. In other particular embodiments the subject is a human.

Adipose Tissue-Derived Cell Suspension

[00056] The term "adipose tissue-derived cell suspension" as used herein encompasses isolated cells from adipose tissue or small aggregates or pieces of adipose tissue, or a mixture of two or more of: isolated cells, small aggregates and pieces of adipose tissue. The adipose tissue-derived cell suspension comprises adipose tissue-derived non-adipocyte cells. The cell suspension may be obtained by mechanically dissociating adipose tissue using techniques which are readily available

in the art. Any suitable method for the mechanical dissociation of adipose tissue may be used, for example by mincing adipose tissue with blades, or with scissors, or by forcing adipose tissue through screens or meshes with a pore size sufficient to break the tissue into isolated cells or small pieces of adipose tissue, or a combination of these techniques. Small aggregates of adipose tissue may form when dissociated adipose-derived cells reassociate into larger assemblies, for example on standing in a medium. Small pieces or aggregates of adipose tissue may be less than ten millimetres in diameter, less than five millimetres in diameter, less than one millimetre in diameter, less than 500 μm in diameter or less than 250 μm in diameter.

[00057] The adipose tissue-derived cell suspension may be filtered through a mesh or screen to remove cell aggregates or tissue pieces which are greater than the mesh or screen pore size.

[00058] Proteolytic enzymes may be used to promote the dissociation of adipose tissue into an adipose tissue-derived cell suspension. Enzymes which are suitable for such a use are well known in the art, and include but are not limited to trypsin, and collagenase. It is usual to remove and/or otherwise inactivate the proteolytic enzymes before using the adipose-tissue-derived cell extract, as these enzymes may not be compatible with a desired *in vivo* use of the cells. The proteolytic enzymes may be used in combination with techniques for the mechanical dissociation of adipose tissue to generate an adipose tissue-derived cell suspension.

[00059] A mechanical dissociation technique may be used without using one or more proteolytic enzymes. The technique used in this manner may be used to rapidly generate an adipose tissue-derived cell suspension.

[00060] The cell suspension may be suspended in a liquid. The liquid may be added to the adipose tissue before, during or after the dissociation of the adipose tissue. The liquid may comprise a medium which is capable of maintaining adipose tissue cell survival for at least 24 hours under appropriate culture conditions. The liquid may comprise an isotonic buffered solution, such as a phosphate or a HEPES buffered saline, which is capable of maintaining adipose tissue cell survival for at least one hour. The liquid may comprise a tissue culture medium. The liquid may comprise serum or serum components which support or extend adipose tissue cell survival in

the cell suspension. The serum or serum components may be autologous serum or serum components.

[00061] In some embodiments the cell suspension may not have added liquid, but instead the cells are suspended in liquid which is formed during the dissociation of the tissue.

[00062] The preparation of an adipose tissue-derived cell suspension may comprise a centrifugation step. The centrifugation of isolated cells or small aggregates or pieces of adipose tissue suspended in a liquid, such as a medium, is at approximately 500 g for 10 minutes, or for sufficient time and at a sufficient g-force to generate a cell pellet which comprises adipose-derived non-adipocyte cells, above which is a layer of medium, floating above which in turn is a layer which comprises the viable adipocytes, and floating at the top is a layer of lipid which is derived from ruptured adipocytes. Following centrifugation, in certain embodiments the lipid layer and the medium layer will be discarded and the retained cells are mixed, leaving an adipose tissue-derived cell suspension which comprises viable adipocytes and adipose-derived non-adipocyte cells. In other embodiments, only the layer comprising the viable adipocytes will be retained. In other embodiments, the layer comprising adipocytes may be removed and hence not included in the adipose tissue-derived cell suspension. This will typically occur when preparing an adipose tissue-derived cell suspension which is substantially free of adipocytes. A cell suspension referred to herein as being substantially free of adipocytes means that the cell suspension has been significantly depleted of adipocytes compared to the starting material, such as by removal of the adipocyte fraction after centrifugation. It will be understood that substantially free of adipocytes when used in relation to a cell suspension includes complete absence of adipocytes and also includes the situation where minimal retention of adipocytes in the material has occurred. In other embodiments, only part of the adipocyte content of the adipose tissue may be removed in the preparation of the adipose tissue-derived cell suspension. In this case, the resultant cell suspension will comprise adipocytes, but at a reduced proportion relative to other retained components, such as the stem cells, compared to the proportion in the starting material. In an embodiment the adipose tissue-derived cell suspension comprises at

least 10% adipocytes by volume. In an embodiment the adipose tissue-derived cell suspension comprises between 10% and 30% adipocytes by volume.

[00063] One centrifugation step or multiple centrifugation steps may be used, for example to provide additional cell separation steps. In other embodiments, the preparation of an adipose tissue-derived cell suspension does not include a centrifugation step.

[00064] The adipose tissue-derived cell suspension may or may not comprise viable adipocytes. When present, the adipocytes may retain detectable quantities of lipid in their cytoplasm, and may be separated from adipose-derived non-adipocyte cells on the basis of the different density provided by the lipid. Lipid may be detectable using light microscopy techniques, including phase contrast microscopy, or by staining a sample of cells with a lipophilic dye such as Oil Red O. Adipocytes which retain lipid in their cytoplasm are considerably more fragile than other adipose-derived cells, and accordingly where viable adipocytes are desired techniques for dissociating tissue which damage or kill a large proportion of the adipocytes should be avoided. The ultrasonic dissociation of adipose tissue or techniques in which adipose tissue is vigorously shaken, for example, are unlikely to provide a cell suspension which contains large numbers of viable adipocytes. The viability of adipocytes may readily be determined using readily available techniques, such as the LIVE/DEAD cell viability assays (Life Technologies).

[00065] The adipose tissue-derived cell suspension may comprise both adipocytes and adipose-derived non-adipocyte cells. The adipose-derived non-adipocyte cells typically include cells of the stromal vascular fraction, including mesenchymal stem cells. Cells of the stromal vascular fraction typically pellet upon centrifugation conditions described herein of an adipose tissue-derived cell suspension.

[00066] In embodiments which comprise both adipocytes and adipose-derived non-adipocyte cells, the adipose tissue-derived cell suspension may be conveniently prepared by methods which comprise a centrifugation step, as described herein, in which both the adipocyte cell layer and the pelleted adipose-derived non-adipocyte cells are collected. Alternatively, in these embodiments the adipose tissue-derived

cell suspension may be prepared by dissociating adipose tissue as described herein without a centrifugation step.

[00067] The adipose tissue-derived cell suspension, optionally comprising adipocytes, may be stored under appropriate conditions. The storage conditions typically permit the retention of cell viability of some or all cells in the cell suspension, such as greater than 50%, greater than 60%, greater than 70%, greater than 80%, greater than 90%, or greater than 95%.

[00068] Where the adipose tissue-derived cell suspension is to be stored frozen it may be in any carrier liquid appropriate for freezing of cells. As an illustrative but not limiting example, the cells may be suspended in culture medium, which may be serum-containing or serum-free, such as DMEM, RPMI, minimal essential media, or in serum prior to freezing.

[00069] The adipose tissue-derived cell suspension typically also comprises autologous serum or plasma, which may be added during the preparation of the cell suspension or at a late stage of the preparation, such as when the cell pellet is separated from components not desired in the cell suspension being prepared.

[00070] Where the adipose tissue-derived cell suspension is to be stored frozen it typically also comprises a cryopreservative. It will be understood that any additives and method for storing the cell suspension without significant loss of cell viability over time may be used. For example, methods for the storage of mesenchymal stem cells are known in the art. As an example, the cell suspension may comprise dimethylsulfoxide (DMSO) or glycerol, at an appropriate concentration, such as 5% to 10%. As further examples, the cell suspension may comprise one or more cryopreservative sugars, such as trehalose, dextran, dextrose, sucrose at an appropriate concentration. For example, a cryopreservative sugar may be included at a concentration in the range of 1% w/v to 30% w/v. In a further example, a cryopreservative sugar may be included at a concentration in the range of 5% w/v to 10% w/v.

[00071] The constituents of the cell suspension, such as the liquid medium and the cryopreservative, are typically pharmaceutically acceptable at the concentrations used.

This has the advantage that the adipose tissue-derived cell suspension can be administered to the subject after thawing with minimal post-thaw processing.

[00072] The cell suspension is intended as part of a course of treatment for the condition afflicting the subject. In that manner the course comprises multiple doses of the cell suspension over a period of time to the subject from which the adipose tissue was obtained and from which the cell suspension was prepared. The time course of the treatment will typically be over weeks, to months and potentially a year or more. For ease of use the adipose tissue-derived cells suspension is typically divided into useful portions or aliquots soon after preparation and the material to be used for the second and, where appropriate, third, fourth, fifth, etc doses is then stored frozen for retrieval at an appropriate time. Typically, the stored material will be in portions or aliquots which comprise a single dose.

[00073] Typically, at least one aliquot or portion of the cell suspension for administration as a first dose of the course of treatment will be obtained from the prepared cell suspension prior to addition of the cryopreservative or other components intended to assist in the storage of the cell suspension.

[00074] The cell suspension may be referred to as a pharmaceutical composition as it typically also comprises one or more of a pharmaceutically acceptable carrier diluent, excipient or adjuvant.

[00075] The cell suspension is typically frozen under controlled conditions to minimize cell damage, for example by slow freezing, typically at a rate of about 1°C/min, such as by placing in a programmable freezing device, or in an insulated container in a -70°C to -90°C freezer. For storage, frozen cells are typically then transferred to liquid nitrogen storage.

[00076] A cell processing method and device which may be used for the preparation of adipose tissue-derived cell suspensions is described in co-pending application PCT/AU2012/000272, the contents of which are incorporated herein by reference.

Methods of treatment

[00077] The inventor has identified that an improved therapeutic outcome can be achieved by providing a course of treatment to a subject having osteoarthritis, a tendon injury or pain.

[00078] In the methods of the invention a first dose of the autologous adipose tissue-derived cell suspension comprising adipose tissue-derived non-adipocyte cells is administered to the subject soon after preparation of the cell suspension. In this context the first dose may be described as being administered to the subject as a freshly prepared cell suspension. In this context the term freshly prepared and similar terms used herein means that the cell suspension so administered is administered to the subject on the same day as it is prepared. As described herein the adipose tissue-derived cell suspension is prepared from adipose tissue obtained from the subject, hence the resultant cell suspension is an autologous cell suspension. Typically, the time taken from isolation of the adipose tissue from the subject through to the prepared cell suspension ready for administration is up to about two to three hours. A sample freshly prepared is therefore one which is ready for administration to the subject within about two to three hours of removal of the adipose tissue from the subject.

[00079] It will be understood that in the context of the methods of the invention a dose means the administration of the cell suspension to the subject at a given time, whether that dose be administered in a single application or in more than one application. As an illustrative example, a dose may consist of a single administration, such as a single injection into a targeted site on the subject's body. As a further illustrative example, a dose may consist of multiple administrations to one or more targeted sites on the subject's body, such as multiple injections. Any of the first, and or subsequent doses, such as any of the second, third, fourth, fifth, etc, doses may therefore be administered as a single application or as multiple applications.

[00080] The method may comprise a course of treatment comprising a first dose and a second dose, or a first dose, a second dose and a third dose, or a first dose, a second dose, a third dose and a fourth dose, or a first dose, second dose, a third dose, a fourth dose and a fifth dose.

[00081] Any appropriate time period between the first and each subsequent dose may be used. It is notable that the methods of the invention do not require that the subject be experiencing a relapse of the condition or an increase of symptoms of the condition, such as might occur if a dose was becoming less effective, to qualify for or to be given a subsequent dose or doses. Instead, it is the intended course of treatment in the methods of the invention that the subject be administered multiple doses of the cell suspension over a period of time for the treatment of the same condition in the individual over that time. As indicated herein, the condition being treated in the subject is typically selected from osteoarthritis, tendon injury and pain

[00082] In an embodiment the course of treatment comprises multiple doses in which each subsequent dose is separated in time from the previous dose by between one week and ten weeks. In an embodiment the course of treatment comprises multiple doses each subsequent dose separated in time from the previous dose by between two weeks and eight weeks. In an embodiment the course of treatment comprises multiple doses each subsequent dose separated in time from the previous dose by between two weeks and six weeks. For any given course of treatment the time period between each dose may or may not be a consistent period. As an illustrative example, the time period between the first and second dose may or may not be the same as the time period between the second and third dose.

[00083] In an embodiment the course of treatment comprises multiple doses administered over a total treatment period of between three and twelve months. In an embodiment the course of treatment comprises multiple doses administered over a total treatment period of between six and twelve months. In an embodiment the course of treatment comprises multiple doses administered over a total treatment period of between three and nine months. In an embodiment the course of treatment comprises multiple doses administered over a total treatment period of between six and nine months.

[00084] The pharmaceutical composition may be administered to the subject patient at a site remote from the afflicted area. In this context, "remote" means that the administration is not direct application of the cell suspension to the site of inflammation or other injury or disease being treated where such a site is identifiable. As an illustration, in the case of treatment of an arthritic joint, administration as

previously described in the art involved injection of adipose tissue-derived cell suspensions directly into the afflicted joint. Such administration requires a high degree of skill on the part of the treating physician or clinician to ensure appropriate precision. The handling of the affected limb or joint required in such administration also increases the distress experienced by the patient, be they human or non-human. By providing for the remote administration of adipose tissue-derived suspension the present invention offers improved methods, uses and compositions for the treatment of such diseases. For example, the remote administration may be by subcutaneous injection, such as in the scruff of the neck of an animal (for example a cat or dog) being treated, or by intramuscular injection. As a further example, administration to a dog by intramuscular injection may be in to thigh of the dog. As a further example, administration to a bovine by intramuscular injection may be in the caudal fold.

Treatment of pain

[00085] The inventor has identified that the methods of the invention are useful in the treatment of subjects having pain. Various specific types of pain are described herein, including the following.

Painful musculoskeletal conditions

[00086] Painful musculoskeletal conditions are common, with a prevalence of approximately 30%. For musculoskeletal conditions other than arthritis, the prognosis is generally good; most people recover within a few weeks following the onset of symptoms. However a significant minority do not recover and develop long lasting or chronic pain, defined as pain lasting longer than 3 months. For these patients prognosis is poor and recovery is slow. A major focus of contemporary research is the early identification of patients who are at a high risk of a poor outcome. A common finding from this research is that early high pain intensity is a risk factor for delayed recovery and the development of chronic pain.

[00087] The most frequent cause of chronic musculoskeletal pain is low back pain. Over 1 million Australians have a disability associated with their back problems and it is the leading reason for Australians leaving the workforce. Other common but less prevalent chronic pain conditions include: neck and shoulder pain, whiplash associated disorder (WAD) and complex regional pain syndrome (CRPS).

[00088] **Back pain** is an extremely common, difficult-to-manage and expensive health condition. In Australia back pain is associated with costs of around $9 billion/year. Over 85% of low back pain is 'non-specific' low back pain (NSLBP) in that a structural source of the pain cannot be reliably identified. Plausible therapeutic targets are innervated tissues and include the disc, the facet joint and the sacroiliac joint, however other tissues such as muscle and ligament may also be involved. Up to 15% of low back pain is radiculopathy where impingement of the nerve root causes symptoms including back and leg pain. Pain associated with radiculopathy is believed to be associated with a local inflammatory process. Treatments for radicular pain include advice to stay active, analgesia including NSAIDS, epidural corticosteroid injection and transfroaminal peri-radicular injections of corticosteroid.

Neck and shoulder pain

[00089] **Whiplash associated disorder (WAD)** are injuries to the neck caused by acceleration-deceleration energy transfer resulting most commonly from a motor vehicle accident. As with most painful musculoskeletal conditions, prognosis for new injuries is typically good in the first few weeks or months, but after 3 months recovery rates slow markedly and a significant proportion of patients develops chronic Whiplash Associated Disorder (WAD). High pain intensity is known to be a predictor or poor outcome. Whiplash is largely resistant to conservative treatments.

Complex Regional Pain Syndrome

[00090] Complex regional pain syndrome (CRPS) is a complication of minor trauma, usually to one limb, characterised by incapacitating pain, swelling, colour and temperature changes, and bone demineralisation in the limb. In Australia on average 5000 people are diagnosed with CRPS every year. The most common inciting minor trauma is wrist fracture. The incidence of CRPS following wrist fracture is 5-7%. Prognosis is poor and treatment options are often highly invasive, have significant side effect profiles and are only moderately effective. The major cause of CRPS is thought to be an aberrant inflammatory response to tissue injury and a more pro-inflammatory balance of inflammatory mediators is expressed by patients with CRPS compared to those without the condition.

Neuropathic pain

[00091] The inventor has identified that methods of the invention are useful in the treatment of subjects having pain for which there is no discernable clinical cause, such as some forms of neuropathic pain. Neuropathic pain refers to a group of painful disorders characterized by pain due to dysfunction or disease of the nervous system at a peripheral level, a central level, or both. It is a complex entity with many symptoms and signs that fluctuate in number and intensity over time. The three common components of neuropathic pain are steady and neuralgic pain; paroxysmal spontaneous attacks; and hypersensitivity.

[00092] Neuropathic pain can be very disabling, severe and intractable, causing distress and suffering for individuals, including dysaesthesia and paraesthesia. Sensory deficits, such as partial or complex loss of sensation, are also commonly seen. In addition, there are significant psychological and social consequences linked to chronic neuropathic pain, which contribute to a reduction in quality of life.

[00093] Neuropathic pain is quite common in general medical practice. In some forms, the neuropathic pain is not associated with any discernable clinical causative condition. As an example it is demonstrated herein that the compositions of the invention are effective in alleviating neuropathic facial pain. In some forms, the neuropathic pain is associated with a discernable clinical condition. The prevalence of trigeminal neuralgia is 2.1 to 4.7 persons per 100,000 of the population, and of painful diabetic neuropathy occurs in 11% to 16% of Type 1 diabetics as well as Type II diabetics and postherpetic neuralgia is found in approximately 34 persons per 100,000 of the population. Treatment of neuropathic pain is not easy. Patients with neuropathic pain do not always respond to standard analgesics such as non-steroidal anti-inflammatory drugs (NSAIDs) and to some extent neuropathic pain is resistant to opiates. The pharmacologic agents best studied and longest used for the treatment of neuropathic pain are antidepressants and anticonvulsants both of which may have serious side effects.

[00094] A composition of the invention may be administered to a subject for treatment of such pain at any appropriate site. Administration may typically be using an appropriate type of injection. For example, an injection may be subcutaneous, intramuscular, or directly into an accessible site at or near a site of the pain. As this

type of pain may manifest in multiple areas of the subject's body, for example jaw pain and limb or shoulder pain, the administration may be at or near to one site of the pain and remote from another site afflicted by pain. Typically, where multiple sites of the pain occur in a patient, the administration is at or near a site identified as an original or primary site of the pain. As an illustration of this treatment, the examples herein show treatment of neuropathic facial pain by injection into the subject's gum. A subject being treated may be administered a single application of a composition of the invention, such as a single injection or may be administered multiple applications, such as multiple injections.

[00095] The invention will now be described in more detail, by way of illustration only, with respect to the following examples. The examples are intended to serve to illustrate this invention and should not be construed as limiting the generality of the disclosure of the description throughout this specification.

Examples

Example 1. Xenogeneic treatment of an equine radio-carpal joint with human adipose-derived stromal vascular fraction cells

Preparation of adipose tissue

[00096] Liposuction was used to collect approximately 200 grams of adipose tissue from the abdomen and thigh of a human. The lipoaspirate was processed immediately after collection by washing with sterile saline and then digesting by adding sterile collagenase to a final concentration of 0.05% w/v. The sample was incubated at 37°C for 30 minutes, filtered through a 800 micron mesh and transferred to centrifuge tubes.

[00097] The centrifuge tubes were centrifuged at 1500g for 5 minutes. Four distinct layers were visible within the centrifuged sample: a small layer of free lipid on the surface, below which was a thick layer of adipose tissue and adipocytes and then a large clear layer of saline and a pellet of adipose tissue-derived non-adipocyte cells (SVF). The lipid layer, the layer of adipose tissue and adipocytes and the clear saline layer were aspirated and discarded. The SVF pellet was washed with 100 mL of saline and centrifuged at 1500g for 5 minutes. The SVF pellet was gently resuspended in

Dulbecco's Modified Eagle Medium (DMEM; Invitrogen) supplemented with 10% human serum. The sample was transported at room temperature for 4 hours. The cell suspension was centrifuged at 1500g for 5 minutes. The SVF pellet was resuspended in 5 mL of DMEM with 100 mg of gentamicin.

[00098] A 200 µL portion of the SVF cell suspension was added to 800 µL of 1x FACS Lysing Solution (Becton Dickinson) to fix the cells. A 300 µL portion of the fixed SVF cell suspension was filtered through a 35 micron nylon mesh topped tube. A 200 µL portion of this filtered sample was enumerated in TruCount tubes containing isoflow and propidium iodide (10 µg/mL) using a FacsScan flow cytometer. The total number of nucleated cells in the SVF cell was determined to be 11 million cells.

[00099] The cell suspension was injected into the right radio-carpal joint of a healthy mare. The joint space was confirmed by aspiration of synovial fluid. Samples of synovial fluid from this joint were aspirated at 12 hours, 36 hours, 6 days, 9 days and 19 days post-injection of the cell suspension. The pre-treatment and post-treatment synovial fluid samples were centrifuged at 3000g for 5 minutes. The synovial fluid was aliquoted and stored at -80°C.

Bio-Plex analysis of equine synovial fluid samples

[000100] The level of cytokines, chemokines and growth factors secreted by the human cells into the synovial fluid following introduction into the equine joint was assessed using a Bio-Plex Pro assay according to the manufacturers instructions (Bio-Rad).

Results

[000101] The results presented in Table 1 illustrate that within 2 and a half weeks following treatment of an equine joint with a human SVF cell suspension, the levels of human cytokines detectable in the synovial fluid decreased at each time-point. At the last follow-up time-point of 18 days, no human cytokines were detectable in the horse synovial fluid. This result indicates that the cells are no longer secreting factors into the synovial fluid and a subsequent treatment would be required.

Table 1. The level of human cytokines measured in the synovial fluid of an equine joint treated with human SVF cells. The values presented are in pg/mL.

Time point	Pre-treatment	Post-treatment time-point (hours; days)				
		12 h	36 h	6 d	9 d	18 d
PDGF	0	27	0	0	0	0
IL-1Ra	0	19	0	0	0	0
IL-2	0	1	0	0	0	0
IL-6	0	> 637	123	8	10	0
IL-7	0	2	0	0	0	0
IL-8	0	12	23	0	0	0
IL-9	0	5	0	0	0	0
IL-10	0	1	0	0	0	0
IL-12	0	2	0	0	0	0
IL-15	0	2	0	0	0	0
Eotaxin	0	108	0	0	0	0
FGF basic	0	4	0	0	0	0
G-CSF	0	85	2	0	0	0
GM-CSF	0	13	2	0	0	0
IFN-γ	0	20	2	0	0	0
IP-10	0	14	0	0	0	0
MCP-1	0	2	3	0	0	0
MIP-1α	0	5	0	0	0	0
MIP-1β	0	2	0	0	0	0
TNF-α	0	5	0	0	0	0
VEGF	0	14	0	0	0	0

Example 2. Xenogeneic treatment of an equine radio-carpal joint with passaged human adipose-derived cells

Collection and processing of adipose tissue

[000102] Adipose tissue was collected and processed as described in Example 1.

Expansion of cells

[000103] The SVF cell suspension was seeded into T175 cm^2 tissue culture flasks containing DMEM (Invitrogen) supplemented with 10% foetal bovine serum (FBS; Bovogen) and 1% penicillin-streptomycin solution (Invitrogen) and incubated at 37°C with 5% CO_2. A monolayer of cells was established and the media was changed on the flasks every 3 days. Once the monolayer reached confluency (approximately 7 days), the cells were passaged using TrypLE express (Invitrogen) and re-seeded at a ratio of 1:3 into fresh T175 cm^2 flasks. This process of expanding the cells was repeated until the cells reached passage 2.

[000104] The passage 2 cells were stripped from the flasks using TrypLE express (Invitrogen) and combined into 50 mL falcon tubes. The cell suspension was centrifuged at 1000g for 10 minutes. The pelleted cells were gently resuspended in Dulbecco's Modified Eagle Medium (DMEM; Invitrogen) supplemented with 10% human serum. The sample was transported at room temperature for 4 hours. The cell suspension was centrifuged at 1500g for 5 minutes. The SVF pellet was resuspended in 5 mL of DMEM with 100 mg of gentamicin.

[000105] A 200 µL portion of the cell suspension was added to 800 µL of 1x FACS Lysing Solution (Becton Dickinson) to fix the cells. A 300 µL portion of the fixed cell suspension was filtered through a 35 micron nylon mesh topped tube. A 200 µL portion of this filtered sample was enumerated in TruCount tubes containing isoflow and propidium iodide (10 µg/mL) using a FacsScan flow cytometer. The total number of nucleated cells in the cell suspension was determined to be 1 million cells.

[000106] The cell suspension was injected into the right radio-carpal joint of a healthy mare. The joint space was confirmed by aspiration of synovial fluid. Samples of synovial fluid from this joint were aspirated at 12-hours and 9 days post-injection of

the cell suspension. The pre-treatment and post-treatment synovial fluid samples were centrifuged at 3000g for 5 min. The synovial fluid was aliquoted and stored at -80°C.

Bio-Plex analysis of equine synovial fluid samples

[000107] The level of cytokines, chemokines and growth factors secreted by the human cells into the synovial fluid following introduction into the equine joint was assessed using the Bio-Plex Pro assay as described in Example 2.

Results

[000108] The results presented in Table 2 illustrate that human cytokines were detectable in the equine joint at 12 hours post-treatment. However, at the last follow-up time-point of 9 days, no human cytokines were detectable in the horse synovial fluid. This result indicates that the cells were no longer secreting factors into the synovial fluid and a subsequent treatment would be required.

Table 2. The level of human cytokines measured in the synovial fluid of an equine joint treated with human SVF cells. The values presented are in pg/mL

Time point	Pre-treatment	Post-treatment time-point	
		12 hours	9 days
PDGF	0	33	0
IL-1Ra	0	39	0
IL-2	0	0	0
IL-6	0	205	0
IL-7	0	0	0
IL-8	0	13	0
IL-9	0	26	0
IL-10	0	0	0
IL-12	0	0	0
IL-15	0	24	0

IL-17	0	0	0
Eotaxin	0	88	0
FGF basic	0	23	0
G-CSF	0	40	0
GM-CSF	0	0	0
IFN-γ	0	21	0
IP-10	0	0	0
MCP-1	0	50	0
MIP-1α	0	0	0
MIP-1β	0	0	0
TNF-α	0	0	0
VEGF	0	83	0

Example 3. Cryogenic storage of human adipose-derived stromal vascular fraction cells.

Collection and cryogenic storage of adipose derived cells

[000109] Liposuction was used to collect approximately 200 grams of adipose tissue from the abdomen and or thighs of each patient. The lipoaspirate was processed immediately after collection by washing with sterile saline and then digesting by adding sterile collagenase to a final concentration of 0.05%. The sample was incubated at 37°C for 20 minutes, filtered through a 800 micron mesh and transferred to centrifuge tubes.

[000110] The centrifuge tubes were centrifuged at 400g for 10 minutes, and the supernatant discarded. The cell pellet was resuspended in sterile saline and the samples were centrifuged again at 400g for 10 minutes. The supernatant was discarded and the pelleted cells resuspended in 20 mLs of autologous serum. The cell suspension was dispensed in 10 mL volumes in to cryogenic storage bags. A 300 µL portion of the cell suspension was removed and analysed for cell viability as

described below. DMSO plus 5% dextran was added to the cryogenic storage bags to a final concentration of 10% and the bags were frozen in a controlled rate freezer to -80°C. After 24 hours the bags were transferred to a liquid nitrogen dewar for one week.

Assessment of cell viability

[000111] The bags were thawed at 37°C and a 300 µL aliquot of the cell suspension was filtered through a 35 µm nylon mesh topped tube. The filtered sample was diluted in Isoflow (Beckman Coulter) and stained with propidium iodide (10 µg/mL) and Syto11 (1 µM) using a FacsScan flow cytometer. The percentage of viable and non-viable nucleated cells was determined.

Results

[000112] The cell viability was surprisingly high after cryogenic storage (Table 3). The drop in cell viability averaged just 12.9%. Such a small loss of cell viability is unlikely to affect the therapeutic effect from the cell suspension.

Table 3. Viability of cells pre and post freezing.

Pre-freezing	Post freezing
71.7	61.9
74.01	52.1
64.5	57.5
70.1	57.2

Example 4. Treatment of a horse with multiple intra-articular injections of frozen autologous adipose-derived cells.

Collection and processing of adipose tissue

[000113] A 6-year old horse with lameness in the right front limb was treated with multiple intra-articular injections of frozen autologous adipose-derived cells.

[000114] A 40 g sample of adipose tissue was collected by excision from the tail-base. The adipose tissue was rinsed with saline and then minced finely using scissors and mixed with 20 ml of Dulbecco's Modified Eagle's Medium (DMEM, Sigma). Collagenase (Sigma) was added to achieve a final concentration of 0.05% and the sample was incubated at 37°C for 60 minutes. During the incubation the sample was gently inverted by hand every 15 minutes.

[000115] Following collagenase treatment the sample was filtered through a mesh (300 micron pore size), transferred to a 50 ml centrifuge tube and centrifuged at 500g for 15 minutes.

[000116] Four distinct layers were visible within the centrifuged sample: a small (2 mm thick) layer of free lipid on the surface, below which was a white 10 mm thick layer of adipocytes and then a large clear layer of liquid which largely comprised DMEM and then a pellet of adipose-derived non-adipocyte cells. The small layer of lipid was carefully removed with a pasteur pipette. A fresh pasteur pipette was then carefully inserted through the adipocytes and the clear DMEM was removed without disturbing the floating adipocytes or the pelleted cells. This resulted in a sample that contained only the floating adipocytes and the pelleted cells. The floating adipocytes and the pelleted cells were gently mixed with a pasteur pipette and transferred to a 15 ml centrifuge tube.

[000117] The cells were then washed in DMEM to remove collagenase. DMEM was added to a final volume of 14 ml and the sample centrifuged at 500g for 10 minutes. This resulted in three distinct layers: floating adipocytes, DMEM and pelleted adipose-derived non-adipocyte cells. The DMEM was carefully removed by inserting a pasteur pipette through the adipocytes taking care not to disturb the adipocytes or the pelleted cells.

Enumeration of cells

[000118] A portion of the cell pellet was filtered through a 35 μm nylon mesh topped tube. The sample was enumerated and the viability determined in TruCount (Becton Dickinson, San Jose, USA) tubes containing isoflow Propidium iodide (10 μg/mL) and Syto11 (1 μM) using a FacsScan flow cytometer. The total number of viable nucleated cells in the SVF pellet was determined.

Freezing cells

[000119] The floating and the pelleted cells were gently resuspended in 20 mls of autologous serum. The cell suspension was dispensed in 2 mL volumes in to cryogenic vials. Each vial contained approximately 1 million cells. DMSO was added to the cryogenic vials to a final concentration of 10% and the vials were frozen in a controlled rate freezer to -80°C. After 24 hours the vials were transferred to a liquid nitrogen dewar.

Injection of cells

[000120] Vials were removed from liquid nitrogen storage and thawed at 37°C immediately prior to injection. Cells were injected in to the intercarpal joint. Injection of cells was repeated once a week for 4 weeks.

Assessment of the treatment effect

[000121] No negative effect was observed from the repeat injections. After the third injection an improvement in lameness was observed. Two weeks after the final injection, the horse was less lame. At ten weeks post-treatment the horse had improved further and was moving freely with no sign of lameness. The reduction in lameness of the subject was far greater than would have been expected from a single injection.

Example 5. Cartilage regeneration in a horse treated with multiple intra-articular injections of frozen autologous adipose-derived cells.

Arthroscopic examination

[000122] A 5-year-old mare presented with a subchondral bone cyst in the right medial femoral condyle, and, a cartilage defect over this site, which allowed communication to the underlying cyst. Radiographs were clearly demonstrative.

[000123] Arthroscopy was performed under general anaesthesia and visualisation of the cartilage defect showed a full thickness defect approximately 20 mm in diameter.

Collection and processing of adipose tissue

[000124] A 15 g sample of adipose tissue was collected by excision from the tail-base. The adipose tissue was rinsed with saline and then minced finely using scissors and mixed with 20 ml of Dulbecco's Modified Eagle's Medium (DMEM, Sigma).

Collagenase (Sigma) was added to achieve a final concentration of 0.05% and the sample was incubated at 37°C for 60 minutes. During the incubation the sample was gently inverted by hand every 15 minutes.

[000125] Following collagenase treatment the sample was filtered through a mesh (300 micron pore size), transferred to a 50 ml centrifuge tube and centrifuged at 500g for 15 minutes.

[000126] Four distinct layers were visible within the centrifuged sample: a small (2 mm thick) layer of free lipid on the surface, below which was a white 10 mm thick layer of adipocytes and then a large clear layer of liquid which largely comprised DMEM and then a pellet of adipose-derived non-adipocyte cells. The small layer of lipid was carefully removed with a pasteur pipette. A fresh pasteur pipette was then carefully inserted through the adipocytes and the clear DMEM was removed without disturbing the floating adipocytes or the pelleted cells. This resulted in a sample that contained only the floating adipocytes and the pelleted cells. The floating adipocytes and the pelleted cells were gently mixed with a pasteur pipette and transferred to a 15 ml centrifuge tube.

[000127] The cells were then washed in DMEM to remove collagenase. DMEM was added to a final volume of 14 ml and the sample centrifuged at 500g for 10 minutes. This resulted in three distinct layers: floating adipocytes, DMEM and pelleted adipose-derived non-adipocyte cells. The DMEM was carefully removed by inserting a pasteur pipette through the adipocytes taking care not to disturb the adipocytes or the pelleted cells.

Preparation of cells

[000128] All of the floating adipocyte layer plus approximately one quarter of the remaining cell suspension was loaded in to a syringe for immediate injection.

[000129] The remaining cell suspension was mixed with 15 mls of autologous serum and 1.5 mls of DMSO. The cells were enumerated by flow cytometry as described in Example 4.

[000130] The suspension was divided equally between 3 cryogenic vials and then frozen at a controlled rate of 1°C per minute to below -80° C and then transferred and stored in liquid nitrogen. Each vial contained approximately 2 million cells.

Injection of fresh cells

[000131] The cells were injected in to the cyst and freely in to the joint during the arthroscopy procedure.

Further injections using frozen cells

[000132] Vials were removed from liquid nitrogen storage and thawed at 37°C immediately prior to injection.

[000133] The first repeat injection was performed 6 weeks after the initial injection. One vial of cells were injected using ultrasound guidance into the cyst and free in the joint.

[000134] Two further injections of frozen cells in to the joint were performed at 12 weeks and 18 weeks after the first injection.

Post treatment examinations

[000135] Follow up examination at 6 weeks after the first injection revealed a marginal clinical improvement in terms of reduced pain and lameness. At 12 weeks after the first injection there was further improvement in terms of reduced pain and lameness. And at 18 weeks after the first injection there was a marked improvement in pain and lameness and serial ultrasound examinations revealed a reduction in the size of the cartilage defect.

[000136] Follow up radiographs were planned however, the mare died in the paddock possibly due to an episode of colic that was not detected. The owner graciously agreed to retrieval of the treated stifle joint and this was examined by open arthrotomy to reveal that the cartilage defect, in comparison to the presentation at arthroscopy 11 months previous, had reduced to less than 10 mm and subchondral bone could not be visualised.

[000137] The regeneration of cartilage in this clinical case would not have been expected with a single injection of adipose derived cells. In fact a number of horses with bone cysts had previously been treated with a single injection of autologous adipose derived cells and whilst these horses did show some clinical improvement in regards to lameness and mobility, there was no regeneration of the cartilage defect.

Example 6. Treatment of neuropathic facial pain with multiple injections of fresh and frozen autologous adipose derived cells.

[000138] A human patient suffering from neuropathic facial pain was treated initially with an injection of a fresh autologous adipose derived cell suspension and then received follow up injections of the same cell suspension that had been cryogenically stored.

Collection and processing of adipose tissue

[000139] Liposuction was used to collect approximately 200 grams of adipose tissue from the abdomen. The lipoaspirate was processed immediately after collection by washing with sterile saline and then digesting by adding sterile collagenase to a final concentration of 0.05%. The sample was incubated at 37°C for 20 minutes, filtered through a 800 micron mesh and transferred to centrifuge tubes.

[000140] The centrifuge tubes were centrifuged at 400g for 10 minutes, and the layer between the floating adipocytes and the pelleted cells was removed. The cell pellet and floating adipocytes were combined and filter sterilized saline was added until the tubes were full. The samples were centrifuged again at 400g for 10 minutes and the layer between the pelleted cells and the floating adipocytes was removed. The resulting cell preparation was diluted to a volume of 10 ml with autologous serum.

Injection of freshly isolated cells

[000141] A 2 ml aliquot of the cell suspension was drawn up into a syringe and injected in to the gum of the patient.

Cryopreservation of cells

[000142] The remaining 8 mls of cell suspension was mixed with 0.8 mls of DMSO and aliquoted in to 2 ml volumes in cryogenic vials. The vials were frozen at a controlled rate of 1°C per minute to below -80° C and then transferred and stored in liquid nitrogen.

Further injections using frozen cells

[000143] Further injections are planned for 3 months, 6 months and 12 months after the initial injection.

Example 7. Treatment of osteoarthritis and regeneration of cartilage in a human.

[000144] A human patient with osteoarthritis of both knees is treated with a series of injections of autologous cells in an attempt to achieve a long-term resolution of the condition and to achieve regeneration of the cartilage. Such an outcome would not be achieved with a single injection.

[000145] Adipose tissue is collected and processed as detailed in Example 6 to produce two syringes containing a freshly isolated adipose derived cell suspension and a series of vials that contained frozen adipose derived cell suspension.

[000146] The fresh cells are injected in to the knees of the patient immediately after the adipose tissue was collected.

[000147] The cryogenically stored cells are administered 6 weeks, 12 weeks and 18 weeks after the initial injection.

Example 8. Treatment with an injection of fresh autologous adipose derived cells during an arthroscopy procedure and subsequent injections with frozen cells.

[000148] A human patient with osteoarthritis of the left knee is treated with an injection of adipose derived cells during an arthroscopy procedure and then receives a series of injections of frozen autologous cells in an attempt to achieve a long-term resolution of the condition and to achieve regeneration of the cartilage. Such an outcome would not be achieved with a single injection.

[000149] Adipose tissue is collected and processed as detailed in Example 6 to produce a syringe containing a freshly isolated adipose derived cell suspension and a series of vials that contained frozen adipose derived cell suspension.

[000150] The fresh cells are injected in to the knee of the patient during the arthroscopy procedure.

[000151] The cryogenically stored cells are administered 6 weeks, 12 weeks and 18 weeks after the initial injection.

Example 9. Treatment of back pain with an injection of fresh autologous adipose derived cells and subsequent injections with frozen cells.

[000152] A human patient with lower back pain is treated with an injection of autologous adipose derived cells and then receives a series of injections of frozen autologous cells in an attempt to achieve a long-term resolution of the condition. Such an outcome would not be achieved with a single injection.

[000153] Adipose tissue is collected and processed as detailed in Example 6 to produce a syringe containing a freshly isolated adipose derived cell suspension and a series of vials that contained frozen adipose derived cell suspension.

[000154] The fresh cells are injected in to the lower back of the patient immediately after the cells were processed.

[000155] The cryogenically stored cells are administered 6 weeks, 12 weeks and 18 weeks after the initial injection.

Example 10. Treatment of a burn with an injection of fresh autologous adipose derived cells and subsequent injections with frozen cells.

[000156] A human patient with a third degree burn is treated with an injection of autologous adipose derived cells and then receives a series of injections of frozen autologous cells in an attempt to achieve complete healing of the burn with minimal scarring. Such an outcome would not be achieved with a single injection.

[000157] Adipose tissue is collected and processed as detailed in Example 6 to produce a syringe containing a freshly isolated adipose derived cell suspension and a series of vials that contained frozen adipose derived cell suspension.

[000158] The fresh cells are injected into and around the burn.

[000159] The cryogenically stored cells are administered 6 weeks, 12 weeks and 18 weeks after the initial injection.

Example 11. Treatment of an ulcer with an injection of fresh autologous adipose derived cells and subsequent injections with frozen cells.

[000160] A human patient with a leg ulcer is treated with an injection of autologous adipose derived cells and then receives a series of injections of frozen autologous

cells in an attempt to achieve complete healing of the ulcer with minimal scarring. Such an outcome would not be achieved with a single injection.

[000161] Adipose tissue is collected and processed as detailed in Example 6 to produce a syringe containing a freshly isolated adipose derived cell suspension and a series of vials that contained frozen adipose derived cell suspension.

[000162] The fresh cells are injected into and around the ulcer.

[000163] The cryogenically stored cells are administered 6 weeks, 12 weeks and 18 weeks after the initial injection.

Example 12. Treatment of rheumatoid arthritis with an injection of fresh autologous adipose derived cells and subsequent injections with frozen cells.

[000164] A human patient with rheumatoid arthritis is treated with an injection of autologous adipose derived cells and then receives a series of injections of frozen autologous cells in an attempt to achieve complete resolution of the condition. Such an outcome would not be achieved with a single injection.

[000165] Adipose tissue is collected and processed as detailed in Example 6 to produce a syringe containing a freshly isolated adipose derived cell suspension and a series of vials that contained frozen adipose derived cell suspension.

[000166] The fresh cells are injected IV.

[000167] The cryogenically stored cells are administered by IV injection 6 weeks, 12 weeks and 18 weeks after the initial injection.

Example 13. Multiple injections of autologous adipose derived cells as a course of treatment for osteoarthritis in humans.

Collection and processing of adipose tissue

[000168] Three human patients suffering from osteoarthritis of the knees or shoulders were treated with freshly isolated cells and subsequently with cryogenically stored cells.

[000169] Liposuction was used to collect approximately 200 grams of adipose tissue from the abdomen and or thighs of each patient. The lipoaspirate was processed immediately after collection by washing with sterile saline and then digesting by adding sterile collagenase to a final concentration of 0.05%. The sample was

incubated at 37°C for 20 minutes, filtered through a 800 micron mesh and transferred to four centrifuge tubes.

[000170] The centrifuge tubes were centrifuged at 400g for 10 minutes, and the layer between the floating adipocytes and the pelleted cells was removed. The cell pellet and floating adipocytes were combined and then split into equal amounts in to two centrifuge tubes. Filter sterilized saline was added until the tubes were full. The samples were centrifuged again at 400g for 10 minutes. One tube was used for preparing aliquots for cryopreservation and the other tube was processed to prepare a sample for immediate reinjection.

Injection of freshly isolated cells

[000171] The sample for reinjection was prepared by carefully removing and discarding the layer between the pelleted cells and the floating adipocytes. The cells were then resuspended in 8 mls of sterile saline, drawn up in to a syringe and injected in to the affected joint.

Cryopreservation

[000172] The sample for cryopreservation was resuspended in 13 mls of autologous filter sterilised serum plus 5 mls of sterile saline. A 4 mls volume of a commercial cryoprotectant (CryoSure) was added to the cell suspension before dispensing in to two cryopreservation bags. The bags were frozen in a controlled rate freezer and then transferred to a vapour phase liquid nitrogen dewar.

Injection of frozen cells

[000173] The patients discussed with their treating medical practitioner (TMP) as to the appropriate timing of their reinjection. The TMP recommended a reinjection taking into consideration the patients clinical symptoms and response to the initial injection.

Scoring pain

[000174] Patients were asked to score the pain in the treated joint at regular time points using the scale below.

No Pain 0 1 2 3 4 5 6 7 8 9 10 Extreme Pain

Results

[000175] Patients responded well to the initial treatment and all showed a decrease in pain scores. There was a further decrease in pain scores after the second injection (Table 4). Patients showed no ill effects from both injections.

Table 4: Patient scores on multiple injections.

Patient 1: Left shoulder		**Patient 2: Left knee**		**Patient 3: Left shoulder**	
Time-point	**Pain score**	**Time-point**	**Pain score**	**Time-point**	**Pain score**
Baseline	6	Baseline	8	Baseline	7
2 weeks	3.5	20 weeks*	2	2 weeks	3
4 months*	4	22 weeks	2	7 months*	2
6 months	3	6 months	2	8 months	2

Key: * At this time-point, the patient received a reinjection of their cryopreserved cells.

Claims

1. A method of treating osteoarthritis in a subject requiring said treatment, the method comprising administering to said subject a course of treatment comprising multiple doses over time of an autologous adipose tissue-derived cell suspension comprising adipose tissue-derived non-adipocyte cells, wherein a first dose comprises a portion of a freshly prepared cell suspension and a subsequent dose or doses comprise a portion of said cell suspension that has been stored frozen.

2. A method of treating a tendon injury in a subject requiring said treatment, the method comprising administering to said subject a course of treatment comprising multiple doses over time of an autologous adipose tissue-derived cell suspension comprising adipose tissue-derived non-adipocyte cells, wherein a first dose comprises a portion of a freshly prepared cell suspension and a subsequent dose or doses comprise a portion of said cell suspension that has been stored frozen.

3. A method of treating pain in a subject requiring said treatment, the method comprising administering to said subject a course of treatment comprising multiple doses over time of an autologous adipose tissue-derived cell suspension comprising adipose tissue-derived non-adipocyte cells, wherein a first dose comprises a portion of a freshly prepared cell suspension and a subsequent dose or doses comprise a portion of said cell suspension that has been stored frozen.

4. The method according to any one of claims 1 to 3, wherein the adipose tissue-derived cell suspension further comprises adipocytes.

5. The method according to any one of claims 1 to 3, wherein the course of treatment comprises a first dose, a second dose and a third dose.

6. The method according to any one of claims 1 to 3, wherein the course of treatment comprises a first dose, a second dose, a third dose and a fourth dose.

7. The method according to any one of claims 1 to 3, wherein the course of treatment comprises a first dose, second dose, a third dose, a fourth dose and a fifth dose.

8. The method according to any one of claims 1 to 3, wherein the course of treatment comprises multiple doses each subsequent dose separated in time from the previous dose by between one week and ten weeks.

9. The method according to any one of claims 1 to 3, wherein the course of treatment comprises multiple doses administered over a total treatment period of between three and twelve months.

10. The method according to any one of claims 1 to 3, wherein the subsequent dose or doses is administered to the subject within about two hours of thawing a frozen portion of said cell suspension.

11. The method according to any one of claims 1 to 3, wherein one or more of said doses comprises injection at multiple sites.

12. The method according to any one of claims 1 to 3, wherein the administration is selected from the group consisting of subcutaneous injection, intramuscular injection and intra-articular injection.

13. The method according to claim 1, wherein the method of treating osteoarthritis is by intra-articular injection into an affected joint.

14. The method according to claim 2, wherein the method of treating a tendon injury is by injection directly into the tendon.

15. The method according to claim 2, wherein the tendon injury is tendinopathy or tendinitis.

16. The method according to claim 3, wherein the pain is selected from the group consisting of back pain, neck and or shoulder pain, lower back pain, whiplash associated disorder, or complex regional pain syndrome, pain associated with a sciatic disorder, neuropathic pain, pain associated with osteoarthritis, stifle disease, wobblers, a tendon injury or a ligament injury, pain for which there is no discernable causative clinical condition in the part or region of the body in which the subject experiences the pain, neuropathic facial pain.

17. The method according to claim 3, wherein the pain is neuropathic facial or jaw pain and administration to said subject is by injection into the jaw or the gum.

18. The method according to claim 3, wherein the pain is back pain and the administration is by intramuscular injection.

19. The method according to any one of claims 1 to 18, wherein the subject is an equine, feline, canine, or a human.

20. The method according to any one of claims 1 to 19, wherein said first dose is of a cell suspension comprising non-adipocyte cells and adipocytes and one or more of the subsequent dose or doses is of a cell suspension substantially free of adipocytes.