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(54) **Title**: METHOD FOR ISOLATION, PURIFICATION AND INDUSTRIAL SCALE EXPANSION OF EQUINE ADIPOSE TISSUE DERIVED MENSENCHYMAL STEM CELLS

(57) **Abstract**: The invention relates to a method for isolation, purification and industrial scale expansion of equine adipose tissue derived mesenchymal stem cells (MSCs). The invention also relates to a method for treating and a therapeutic product for treating tendon injury, ligament injury, osteoarthritis and exercise induced pulmonary hemorrhage comprising MSCs.

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TITLE OF THE INVENTION

Method for isolation, purification and industrial scale expansion of equine adipose tissue derived mesenchymal stem cells

FIELD OF THE INVENTION

This invention relates to a method for isolation, purification and industrial scale expansion of clinical grade equine adipose tissue derived mesenchymal stem cells and to characterization of and uses for such cells.

BACKGROUND OF THE INVENTION

Stem cells are unspecialized cells that have two defining properties: the ability to differentiate into other cells and the ability to self-regenerate. The ability to differentiate is the potential to develop into other cell types.

Equine Mesenchymal Stem Cells (MSCs) are multipotent and can be obtained from various tissues in the equine body. MSCs isolated from equine adipose tissue possess different cytokines, proteins and express different genes as compared to MSCs derived from bone marrow, umbilical cord, and dental tissue. Equine adipose tissue-derived MSCs have a unique secretory profile, high pluripotency, high yield, ease of availability, and self-regenerating ability. These MSCs can be maintained and propagated in culture without them losing their characteristics, thereby yielding large numbers of MSCs in fewer population doublings keeping them safe, potent and stable as appropriate for various clinical applications in equines. Since these MSCs are known to be homogeneous populations, with stable and consistent phenotypic and genotypic characteristics which aid in homing to the site of injury, these MSCs have a potential in the treatment of several clinical conditions.

Race horses are prone to musculoskeletal injury of tendons and ligaments, which can be performance-limiting or career ending if not treated quickly and effectively. These tissues have a poor blood supply making it difficult for them to heal. Development of fibrous tissue at injury site increases the risk of re-injury. It is estimated that more than 30% of horses involved in racing and other forms of competition suffer from some sort of tendon and ligament injury. Where race horses compete on turf which is very soft and yielding, there is substantial stress on

their tendons and ligaments, whereas horses sustain joint problems if they race on harder dirt surfaces.

Medical management and non-steroidal anti-inflammatory drugs (NSAIDs) may not provide complete relief in many equines. Rest is a primary treatment in such cases. Normally, horses should not race for at least eight months after sustaining an injury but many are not ready for racing until 12 to 14 months in case of grade 2 injury.

Research has also been focused on orthopedic injuries in horses. Diseases of the musculoskeletal system such as osteoarthritis (OA) and tendinopathy are the major reasons behind early retirement and euthanasia of race horses. There have been numerous clinical trials of stem cell therapy in these animals and the results have been quite encouraging.

OA and tendon and ligament injuries are the most common ailments currently being treated with stem cells in clinical trials in performance horses -specifically, tendonitis of the superficial digital flexor tendon or "bowed tendon". Lesions of the deep digital flexor tendon that occur in the pastern region and within the hoof capsule are also common. Degenerative joint disease is a problem in performance horses and has a great economic impact on the equine industry. Although there are many therapies to support joint health, the majority of these treatments are to relieve the symptoms at best. Traditional approaches to tendon repair are based upon an initial period of rest to limit the inflammatory process followed by a controlled reloading program designed to promote the maturation and linear arrangement of scar tissue within the lesion. However, these treatment protocols are inefficient, resulting in prolonged recovery periods and frequent recurrence.

Fracture repair is an extremely challenging area of equine surgery. Many horses die of bone fractures due to the complexity of injury and inability to support the immense loads with current fixation equipment. One of the main challenges of bone repair in horses is the race between healing of the bone and either failure of implants (screws or plates) used for fixation or the development of laminitis in the opposite limb. Because horses cannot remain lying down for long periods of time, the horse will spend a good deal of time standing on three legs if the fourth is fractured. Unfortunately if a horse does not bear weight evenly on all four legs, the legs that are not injured can suffer terrible inflammation and changes in blood flow. Even with advances such as specialized bone plates being made in internal fixation methods, the repair of long bone

fractures in horses is extremely challenging. One of the main areas where stem cell therapy may be valuable for fracture treatment is to speed bone healing. The potential of stem cells to provide such an improved treatment represents a major breakthrough in veterinary medicine. In addition to fracture repair, because of their robust bone forming potential, stem cells may also be useful for regenerative therapies related to developmental bone disease. These conditions are commonly referred to as osteochondrosis (OCD) and have a tremendous economic impact on the equine industry.

An important issue in stem cell therapy is to attain consistent cell numbers, with phenotypically immune privileged actively replicating cells for administration and easy isolation. It is difficult to aspirate bone marrow from an animal whereas isolation of a small piece of adipose tissue from the animal is easy and advantageous. Adipose tissue derived MSCs may also be more competent than bone marrow derived MSCs in terms of proliferative ability and they are more responsive to bFGF according to some studies. Also, equine bone marrow mesenchymal stem cells (BM-MSCs) appear to senesce much earlier than adipose derived mesenchymal stem cells (AD-MSCs) and umbilical cord mesenchymal stem cells (UC-MSCs). The limited passage numbers of sub-cultured BM-MSCs available for use suggests that adipose tissue and umbilical cord tissue may be preferable for therapeutic purposes.

There are no reports in the literature for optimization of culture media so as to obtain higher yield of MSCs. However, several nutrient media have been tried for culturing stromal vascular fraction for obtaining a homogeneous population of equine adipose MSCs. Media like DMEM-Low Glucose, DMEM/F12, DMEM – high glucose, F12, Alpha-MEM, LP02, supplemented with fetal bovine serum, knockout serum replacement, and serum replacement media result in variable and often low yields of MSCs which display variable morphology. These media have not successfully been used in industrial scale expansion of equine adipose tissue derived MSCs and are also not cost effective for producing therapeutic doses of MSCs.

In many clinical trials, efficacy has been shown to be related to the dose of MSCs administered, highlighting the need for an industrial scale process giving a high yield of MSCs. There is a high demand for equine MSCs for numerous therapeutic applications but insufficient availability in the market. There is also a need for an efficient culturing system that gives an optimum yield at an affordable cost thereby reducing the demand-supply gap.

There is also a need for an optimal method of isolating, purifying and ultimately expanding equine adipose tissue derived MSCs in the least possible passages and minimum population doublings in order to obtain highly potent and young clinical grade MSCs having multi lineage differentiation capacity, showing consistency in cell numbers and amenable to off-the-shelf clinical use.

SUMMARY OF THE INVENTION

According to an embodiment of the invention there is provided a method for isolation, purification and industrial scale expansion of equine adipose tissue derived mesenchymal stem cells (MSCs) to obtain a yield of at least 215,000 cells per cm^2 pure clinical grade MSCs for allogenic use comprising over 95% cells which express positive markers CD44, CD90, and CD105, and less than 2% cells which express negative markers CD45, CD34 and HLA-DR, the method comprising the steps of:

a) extracting 10 to 20 grams of adipose tissue from multiple equine donors;
b) digesting the adipose tissue from individual equine donors with collagenase, centrifuging the digested tissue and collecting the stromal vascular fraction (SVF);
c) seeding the cells contained in the SVF into a culture medium; and
d) trypsinising and washing the cells once they reach confluence at passage 0;
e) pooling the cells obtained from the adipose tissue of multiple canine donors processed through steps (a) to (d);
f) seeding the pooled cells of passage 0 into a culture medium;
g) trypsinising and washing the cells once they reach confluence at passage 1;
h) seeding the cells of passage 1 into a culture medium; and
i) trypsinising and washing the cells once they reach confluence at passage 2,

wherein,

- step (a) is performed by biopsy assisted removal;
- cells from stromal vascular fraction are seeded in step (c) only if at least 60% cells are positive for CD 90;
- the SVF cells are seeded in to the culture medium in step (c) at a seeding density of at least 75000 cells per sq cm;

- prior to seeding in step (f), the mesenchymal stem cells are characterized based on the percentage of cells which express positive markers CD44, CD90, and CD105, and the percentage of cells which express negative markers CD45, CD34 and HLA-DR;
- the mesenchymal stem cells are seeded in to the culture medium in step (f) and step (h) at a seeding density of 1000 to 5000 cells per sq cm and express at least 95% of the positive markers CD44, CD90, and CD105 and at most 2% of the negative markers CD45, CD34 and HLA-DR;
- the culture medium comprises 25% to 75% Dulbecco's Modified Eagle's Medium-Knockout (DMEM-KO) and 25% to 75% alpha-Minimum Essential Medium (α-MEM) or upto 100% DMEM-KO or upto 100% α-MEM; and
- prior to the trypsinising at step (d), (g) and (i) 70 to 90% of the culture medium is changed at 3 to 6 day intervals after seeding until confluence is reached.

According to another embodiment of the invention there is provided a method for isolation, purification and industrial scale expansion of equine adipose tissue derived mesenchymal stem cells (MSCs) to obtain a yield of at least 215,000 cells per cm^2 pure clinical grade MSCs for autologous use comprising over 95% cells which express positive markers CD44, CD90, and CD105, and less than 2% cells which express negative markers CD45, CD34 and HLA-DR, the method comprising the steps of:

a) extracting 10 to 20 grams of adipose tissue from an equine donor;
b) digesting the adipose tissue with collagenase, centrifuging the digested tissue and collecting the stromal vascular fraction (SVF);
c) seeding the cells contained in the SVF into a culture medium; and
d) trypsinising and washing the cells once they reach confluence at passage 0;
e) seeding the washed cells of passage 0 into a culture medium;
f) trypsinising and washing the cells once they reach confluence at passage 1;
g) seeding the cells of passage 1 into a culture medium; and
h) trypsinising and washing the cells once they reach confluence at passage 2,

wherein,

- step (a) is performed by biopsy assisted removal;
- cells from stromal vascular fraction are seeded in step (c) only if at least 60% cells are positive for CD 90;

- the SVF cells are seeded in to the culture medium in step (c) at a seeding density of at least 75000 cells per sq cm;
- prior to seeding in step (e), the mesenchymal stem cells are characterized based on the percentage of cells which express positive markers CD44, CD90, and CD105, and the percentage of cells which express negative markers CD45, CD34 and HLA-DR;
- the mesenchymal stem cells are seeded in to the culture medium in step (e) and step (g) at a seeding density of 1000 to 5000 cells per sq cm and express at least 95% of the positive markers CD44, CD90, and CD105 and at most 2% of the negative markers CD45, CD34 and HLA-DR;
- the culture medium comprises 25% to 75% Dulbecco's Modified Eagle's Medium-Knockout (DMEM-KO) and 25% to 75% alpha-Minimum Essential Medium (α-MEM) or upto 100% DMEM-KO or upto 100% α-MEM; and
- prior to the trypsinising at step (d), (f) and (h) 70 to 90% of the culture medium is changed at 3 to 6 day intervals after seeding until confluence is reached.

According to another embodiment of the invention there is provided a therapeutic product for treating tendon injury, ligament injury, osteoarthritis and exercise induced pulmonary hemorrhage comprising MSCs suspended in multiple electrolyte solution supplemented with human serum albumin and dimethyl sulfoxide (DMSO) wherein, over 95% MSCs express positive markers CD44, CD90 and CD105, and less than 2% cells express negative markers CD45, CD34 and HLA-DR, and wherein the MSCs have undergone not more than 16 population doublings in vitro and are capable of at least 30 to 35 population doublings, the MSCs are capable of differentiating into adipocytes, osteocytes and chondrocytes, the MSCs express pluripotent markers like NANOG, SOX-2 and OCT-4, and secrete growth factors like TGF-β, VEGF and show immunomodulatory activity.

It is to be understood that both the foregoing general description and the following detailed description of the present embodiments of the invention are intended to provide an overview or framework for understanding the nature and character of the invention as it is claimed. The accompanying graphical representations are included to substantiate the invention and are incorporated into and constitute a part of this specification.

BRIEF DESCRIPTION OF THE DRAWINGS

In order that the disclosure may be readily understood and put into practical effect, reference will now be made to exemplary embodiments as illustrated with reference to the accompanying figures. The figures together with a detailed description below, are incorporated in and form part of the specification, and serve to further illustrate the embodiments and explain various principles and advantages, in accordance with the present disclosure where:

FIG. 1A (Passage 1) and FIG. 1B (Passage 2) respectively show the relation between yield of MSCs and seeding density of adipose derived MSCs in passage-1 (Working Cell Bank) and passage-2 (Final product) in various media conditions.

FIG. 2 shows morphological characteristics of adipose derived MSCs.

FIG. 3 shows immunophenotypic characterization of adipose tissues derived MSCs obtained according to an embodiment of the invention.

FIG. 4 demonstrates the differentiation capacity of equine adipose tissue derived MSCs obtained according to an embodiment of the invention to differentiate into osteocytes, adipocytes and chondrocytes.

FIG. 5 demonstrates expression of pluripotent markers like OCT-4, SOX-2 and NANOG on MSCs obtained according to an embodiment of the invention.

FIG. 6 shows secretion of growth factors by the equine adipose derived MSCs obtained according to an embodiment of the invention.

FIG. 7 demonstrates the therapeutic efficacy of equine adipose derived MSCs obtained according to an embodiment of the invention in treating tendon injury in a horse.

FIG. 8 shows the immunomodulatory effect of equine adipose derived MSCs obtained according to an embodiment of the invention

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

For simplicity and illustrative purposes, the present invention is described by referring mainly to exemplary embodiments thereof. In the following description, numerous specific details are set forth in order to provide a thorough understanding of the present invention. It will be apparent, however, to one of ordinary skill in the art that the present invention may be practiced without limitation to these specific details. In other instances, well known methods have not been described in detail so as not to unnecessarily obscure the present invention.

In the context of the invention, the term "combination media" as used in the specification refers to a culture media comprising 25% to 75% Dulbecco's Modified Eagle's Medium-Knockout (DMEM-KO) and 25% to 75% alpha-Minimum Essential Medium (α-MEM).

In the context of the invention, the term "clinical grade" as used in the specification refers to MSCs obtained according to an embodiment of the invention and having the same efficacy and safety after isolation, purification and expansion as their parent MSCs.

In the context of the invention, the term "confluence" as used in the specification means approximately 80% to 90% confluence of cells attained during cell culture.

In the context of the invention, the term "multiple electrolyte solution" as used in the specification includes normal saline, Plasmalyte-A and/or Ringer lactate.

The adipose tissue is preferably extracted from the dorsal gluteal muscle of the donor.

The media changes were seen to help in knocking out undesired cells and toxic wastes. Only up to 90% of the media was changed and at least 10% of the spent media was left behind for the purpose of conditioning. The media change also helped to eliminate cells which were not MSCs as cells which are not MSCs do not adhere to the culture flasks/chambers. Preferably, the culture medium comprises 50% DMEM-KO + 50% α-MEM; 75% DMEM-KO + 25% α-MEM or 25% DMEM-KO + 75% α-MEM. More preferably, the culture medium comprises 25% DMEM-KO + 75% α-MEM.

According to yet another embodiment of the invention there is provided a method of treating tendon injury, ligament injury, and osteoarthritis, comprising administering 1 to 2 doses of the therapeutic product, each dose comprising 10 to 100 million MSCs.

According to still another embodiment of the invention there is provided use of the therapeutic product for treating tendon injury, ligament injury, osteoarthritis and exercise induced pulmonary hemorrhage.

Optionally, the washed cells after trypsinisation at any step can be frozen in a freezing mixture comprising a cryoprotectant and stored under liquid nitrogen for subsequent use. The MSCs obtained in passage 0 can constitute a master cell bank (MCB), the MSCs obtained in passage 1 can constitute a working cell bank (WCB) and the MSCs obtained in passage 2 can constitute the clinical grade product. The cells obtained at the end of passage 0 can also be used for autologous clinical purposes. For allogenic product, multiple donors cells are to be mixed together at passage-0 and seeded for getting working cell bank and expanded to passage 1 and further to passage 2. The maximum population doublings of adipose tissue derived MSCs is approximately 30. The final product in the present invention is administered at a total population doubling of 17 i.e at a stage when the MSCs are highly potent. For autologous use, the MCB and/or the cells obtained at the end of passage 1 can be used as the product.

Clinical grade ready to use product is frozen in cryo vials or cryo bags containing 10 to 100 million cells suspended in multiple electrolyte solution supplemented with 10% injectable dimethyl sulfoxide (DMSO) as cryoprotectant and 5% injectable equine serum as a protein supplement.

The final product should be transported in liquid nitrogen charged dry shipper or dry ice for clinical use. Before transferring the cells to the dry shippers, the dry-shipper chamber should be saturated using liquid nitrogen. After saturation of the dry shipper with liquid nitrogen, excess liquid nitrogen is removed by decanting it from the dry shipper. The product which is to be transported is placed in a canister, which is provided in the dry shipper. The lid of the dry shipper is closed, the dry shipper is locked and sealed and transported to the site of administration within 7 to 9 days of charging. The dry shipper should not be exposed to direct sunlight, rain, or X-rays. After reaching the site of administration, the dry shipper should be placed at room temperature till the day of administration to the patient.

At the time of administration, the lid of the dry shipper is slowly opened. The cryo vial or cryo bags containing the product are removed from the canister and thawed in a 37°C water bath. The cryo vial or cryo bags should be held upright and swirled continuously till the last crystals melt. Immediately 3 ml to 50 of multiple electrolyte solution should be added to the cryo vials or cryo bag using a sterile syringe. The contents of the cryo vial or cryo bag should then be mixed thoroughly by swaying the vial or bag. The cell suspension is then ready to be administered at a dose of 10 million to 100 million cells intravenously or at affected site or intra-articularly.

In order that those skilled in the art will be better able to practice the present disclosure, the following examples are given by way of illustration and not by way of limitation.

Example 1: Determination of optimum basal medium for culture of equine adipose derived MSCs

The seeding density standardization was done to determine the optimum number of MSCs to be seeded to get maximum cell yield and a population doubling of approximately 5 to 7 per passage. Five different nutrient media were analyzed for culture of equine adipose derived MSCs. Dulbecco's Modified Eagle's Medium-knock out (DMEM-KO), Alpha modified minimum essential medium (α-MEM), 50: 50 DMEM-KO: α-MEM, 75:25 DMEM-KO: α-MEM, 25:75 DMEM-KO: α-MEM were used to see the growth rate, population doubling and Fold Expansion.

Seeding density was evaluated at the rate of 1000 to 5000 cells cm2 so as to identify optimal medium composition that gives higher yield. The process was performed for autologous clinical use and the yield of cells obtained at the end of passage 1 and passage 2 was determined. Results showed that seeding density of 5000 cells per cm^2 in 25: 75 DMEM-KO: α-MEM is the optimum seeding density and optimum basal medium that gives significantly higher yield i.e. 215,000 cells per cm^2 and found to be most optimum medium tested and significantly better than any other media and selected for large scale production as shown in Table 1 .

Table 1

Seeding Density (cells per cm^2)	Passage No	100% DMEM-KO	100% α-MEM	50:50 DMEM-KO:α-MEM	75:25 DMEM-KO:α-MEM	25:75 DMEM-KO:α-MEM
1000	1	100,000/cm^2	100,300/cm^2	90,000/cm^2	88,000/cm^2	128,000/cm^2
	2	100,000/cm^2	100,000/cm^2	80,000/cm^2	114,000/cm^2	118,000/cm^2
2000	1	100,000/cm^2	120,000/cm^2	99,000/cm^2	123,000/cm^2	139,000/cm^2
	2	100,000/cm^2	130,000/cm^2	100,000/cm^2	123,000/cm^2	124,000/cm^2
3000	1	110,000/cm^2	120,000/cm^2	100,000/cm^2	132,800/cm^2	145,000/cm^2
	2	70,000/cm^2	130,000/cm^2	104,000/cm^2	133,000/cm^2	126,000/cm^2
4000	1	120,000/cm^2	130,000/cm^2	123,200/cm^2	164,000/cm^2	157,000/cm^2
	2	90,000/cm^2	170,000/cm^2	115,000/cm^2	138,000/cm^2	134,000/cm^2
5000	1	130,000/cm^2	160,000/cm^2	136,000/cm^2	182,000/cm^2	189,200/cm^2
	2	140,000/cm^2	210,000/cm2	187,000/cm2	154,000/cm2	215,000/cm2

FIG. 1A (passage 1) and 1B (passage 2) (shows that a seeding density of 5000 cells per cm^2 is optimum in basal media consisting of 75% alpha MEM: 25% DMEM-KO as this gave the highest cell count of 215,000 cells per cm^2 that is equivalent to 1400 million cells in a 10-cell stack which will give 140 doses of 10 million cells for equine therapy.

Example 2: Isolation of equine adipose derived MSCs

Four thoroughbred horses were sedated and the paraxial caudo dorsal gluteal region was shaved and the skin was aseptically prepared. An inverted L pattern of local anesthetic infiltration was used for regional desensitization. A linear incision of 4-5 cm in length was made and approximately 10-20 grams of subcutaneous adipose tissue was dissected using curved scissors. The adipose tissue specimen was placed in 50 m l Polypropylene centrifuge tube containing sterile DPBS solution (without Calcium and magnesium) with antibiotics and sealed using a strip of Parafilm M®. The skin incisions made were closed with simple suture pattern. Equine adipose tissue was then transported to the lab for isolation and culturing of equine adipose derived MSCs.

The adipose tissue was transferred into a sterile tube/bottle using forceps. Approximately equal volume (w/v) of washing solution, i.e., a mixture of DPBS and antibiotic/ antimycotics was added to the tissue and washed extensively. The infranatant was removed and tissue was cleaned of visible blood clots and fibrous tissue. The washing step was repeated till the infranatant became clear. The tissue was then chopped into very small pieces using sterile scissors and was

then digested using approximately equal volume of 0.1-0.2 % pre warmed Type 1 Collagenase A solution. The collagenase action was neutralized by addition of Dulbecco's Modified Eagle's Media –Knock Out (DMEM-KO) containing 10 % Fetal Bovine Serum(FBS).

The neutralized cell suspension was centrifuged at 2200 -2500 rpm for 10 minutes to pellet out Stromal Vascular Fraction (SVF) cells. The Equine-SVF (ESVF) cell pellet was made into a cell suspension and filtered using 70 micron cell strainer. The filtered cells were then seeded in T 175 cm^2 tissue culture flasks and cell stacks at the rate atleast 75000cells per cm^2. The flasks / stacks were then transferred to humidified 5% CO_2 incubator at 37 °C. After 48 – 72 hours of seeding ESVF fraction, complete media change of the tissue culture flask /chamber was done. The cultures were maintained in 5% CO_2 incubator at 37 °C in combination media containing 10% FBS, 200 Mm L-glutamine and Antibiotic-Antimycotic w/v 10,000 U Penicillin, 10mg Streptomycin and 25 µg Amphotericin B per ml in 0.9% normal saline and bFGF 2ng/ml(Sigma Aldrich. Media changes were done once in three to six days till the culture attained 80% to 90% confluence. The cells were then trypsinized using 0.25 % Trypsin EDTA. These cells constituted Passage 0 cells. The trypsinized cells were cryopreserved in cryopreservation media comprising of 90% Fetal bovine Serum and 10% Dimethyl sulphoxide (DMSO), frozen to -80°C in programmable controlled rate freezer(PLANAR) and then stored in Vapour Phase of the Liquid Nitrogen Storage Tanks at -196°C.

The isolated nucleated cells obtained after processing of fat were counted using a heamocytometer and the obtained counts were found to be 2.5 to 3 million nucleated cells /ml of fat for 5 different samples. The nucleated cells seeded in culture flasks attached to the flask surface by day 1 and at day 3, spindle shaped morphology was seen, by day 12 -14 80% confluent cultures were obtained, which were then harvested and the SVF counts and MSC yields obtained in passage 0 are summarized in Table 2.

Table 2: Total cell counts obtained from fat and passage 0 cell counts from 5 different samples

Sample code	Weight of fat	SVF cell counts per gram of fat	Density of SVF seeding / cm^2	Passage 0 MSC count at 80 -85 % confluence
AD-EQ-E - 1	15 grams	3.33 x 10^6cells/ gram	0.08 – 0.1 x 10^6	95 x 10^6 cells
AD-EQ-E - 2	20 grams	2.9 x 10^6cells/ gram	0.08 – 0.1 x 10^6	99 x 10^6 cells
AD-EQ-E - 3	14 grams	3.35 x 10^6cells/ gram	0.08 – 0.1 x 10^6	60 x 10^6 cells
AD-EQ-E - 4	17 grams	3.70 x 10^6cells/ gram	0.08 – 0.1 x 10^6	120 x 10^6 cells
AD-EQ-E - 5	22 grams	2.5 x 10^6cells/ gram	0.08 – 0.1 x 10^6	111 x 10^6 cells

Example 3: Expansion of equine adipose derived MSCs to Passage 1and Passage 2 for autologous use:

For seeding of passage 1 cells, equine Passage 0 cells (MCB) from Example 2 were thawed and seeded at the rate of 1000 cells/cm^2 to 5000 cells/cm^2, , into 10-cell chamber stack having area of 6360 cm^2. The flasks / stacks were then transferred to humidified 5% CO_2 incubator at 37 °C. The cultures were maintained in 5% CO_2 incubator at 37°C in growth media comprising of combination media containing10% FBS , 200 Mm L-Glutamine and Antibiotic-Antimycotic w/v 10,000 U Penicillin, 10mg Streptomycin and 25 µg Amphotericin B per ml in 0.9% normal salineand bFGF 2ng/ml. Media changes were done once in four days till the culture attained 70-80% confluency. The cells were then trypsinized using 0.25 % Trypsin EDTA. These cells constituted Passage 1 cells. The trypsinized cells were cryopreserved in cryopreservation media comprising of 90% equine serum and10% Dimethyl sulphoxide, frozen to -80°C in programmable controlled rate freezer (PLANAR) and then stored in Liquid Nitrogen Storage Tanks at -196°C.

For seeding and maintenance of passage 2 cells, equine passage 1 cells were thawed and seeded at the rate of 1000-5000 cells/ cm^2 into Cell stacks. The stacks were then transferred to humidified 5% CO_2 incubator at 37 °C and maintained in 5% CO_2 incubator at 37 °C in growth media comprising of combination media containing 10% FBS 200 Mm L-Glutamine and

Antibiotic-Antimycotic w/v 10,000 U Penicillin, 10mg Streptomycin and 25 µg Amphotericin B per ml in 0.9% normal saline) and bFGF 2ng/mlMedia changes were done once in four days till the culture attained 80% to 90%confluence. The cells were then trypsinized using 0.25 % Trypsin EDTA. These cells constituted Passage 2 cells. The cells were cryopreserved in cryopreservation media comprising of 85% multiple electrolyte solution, 10% Inject able grade Dimethyl sulphoxide and 5% equine serum.

The spent media from all three passages were checked for sterility, endotoxin and pH and these were found to be within acceptable ranges.

MSC counts obtained at passage 1 and passage 2 are shown in Table 3 below:

Table 3

Sl no	Seeding density per cm^2 in passage 1 and passage 2	Passage 1 yields at 80 % confluence, counts /cm^2	Passage 2 yields at 80% confluence counts /cm^2
AD-EQ-E-W-P	1000 -5000	≥ 189,000 cells	≥ 215,000 cells

As is evident from Table 3, the cell count was found to be ≥ 215,000 cells per cm^2 i.e. almost 1000-1400- million cells in 6360 sq cm cells chamber. This effectively yields 140 doses of MSCs per chamber and thereby reduces the cost for production and bridges the demand- supply gap for MSCs in the market at present.

Example 4: Preparation of WCB from MCB

The cryovials of Example 2 were taken from the vapour phase of the liquid nitrogen storage tank and immediately placed in a water bath at 37°C.The vials were held straight and swirled in water bath till the last crystal dissolved out. The contents of the cryovials were then aspirated and resuspended in pre-thawed neutralization media. The tube containing cell suspension was centrifuged at 1400 to 1800rpm for 10 minutes. The supernatant was discarded and the pellet was re-suspended in a desired volume of complete media, mixed well and the viable cell count was taken.

Since the cells were to be seeded at the rate of 1000 cells/cm^2 to 5000 cell/cm^2 the cells of the four donors from the MCBs obtained in Example 2 were pooled in appropriate equal proportions

to make the required quantity of cells and then the cells were seeded into culture flasks/ chambers containing combination media.

Here, the first media change was done after 3 to 6 days from seeding. 70 to 90% of the spent media was aspirated and freshly prepared combination media was then added to cell culture flask or chamber. The second media change was similarly done after 5 to 6 days of the first media change. Once the culture attained 80%-90% confluence, the flasks or chambers were harvested.

The spent media was then removed from the flask or chamber and two aliquots were given for checking of sterility, endotoxin, mycoplasma and pH and these were found to be within acceptable ranges. The flasks or chambers were given two washes with DPBS. The wash was removed and 0.25% Trypsin EDTA was added and kept in 5% CO_2 incubator at 37°C for 2 to 3 minutes and then the flask was observed for detachment of cells. Trypsin activity was stopped by addition of Neutralization media and the neutralized cells were collected in centrifuge tubes. The tissue culture flask or chamber was given one more wash with neutralization media and the same was collected. The neutralized cells were then centrifuged at 1400-1800rpm for 5-10 minutes. The supernatant was then discarded, and the pellet was resuspended in complete media. The cell count was taken and 1 aliquot of cells was given for FACS Flow analysis, and Differentiation. The remaining cell suspension was centrifuged at 1000-1400rpm for 5-10 minutes. The supernatant was discarded and the pellet was resuspended in the desired volume of freezing mixture (cryoprotectant) such that the concentration of cells in the freezing mixture was three million cells per ml. The cells in freezing mixture were dispensed in to prelabelled cryovials at the rate of 1ml per cryovial. These vials constituted the working cell bank (WCB). The cryovials were then frozen in a controlled rate freezer to attain -80°C. The cryovials were then transferred to a vapour phase liquid nitrogen tank for further storage.

Example 5: Preparation of clinical grade product (Final product) from WCB

The cryovials of Example 4 were taken from the vapour phase of the liquid nitrogen storage tank and immediately placed in a water bath 37°C. The vials were held straight and swirled in water bath till the last crystal dissolved out. The contents of the cryovials were then aspirated and resuspended in pre-thawed neutralization media. The tube containing cell suspension was centrifuged at 1400 to 1800rpm for 5 to 10 minutes. The supernatant was discarded and the pellet was re-suspended in a desired volume of complete media, mixed well and the viable cell

count was taken. The cells were seeded at the rate of 1000 cells/cm^2 to 5000 cells/cm^2, into 10-cell chamber stack having area of 6360 cm^2.

Here, the first media change was done after 3 to 4 days from seeding. 70 to 90% of the spent media was aspirated and freshly prepared combination media was then added to cell culture flask or chamber. The second media change was similarly done after 5 to 6 days of the first media change i.e. at around the 12^{th} day from seeding. Once the culture attained 80%-90% confluence, the flasks or chambers were harvested.

The spent media was then removed from the flask or chamber and two aliquots were given for checking of sterility, endotoxin, mycoplasma and pH and these were found to be within acceptable ranges. The flasks or chambers were given two washes with DPBS. The wash was removed and 0.25% Trypsin EDTA was added and kept in 5% CO_2 incubator at 37°C for 2 to 3 minutes and then the flask is observed for detachment of cells. Trypsin activity was stopped by addition of Neutralization media and the neutralized cells were collected in centrifuge tubes. The tissue culture flask or chamber was given one more wash with neutralization media and the same was collected. The neutralized cells were then centrifuged at 1400-1800rpm for 5-10 minutes. The supernatant was then discarded, and the pellet was resuspended in multiple electrolyte solution. The cell count was taken and 1 aliquot of cells was given for FACS Flow analysis, and Differentiation. The cell count was found to be $\geq$ 215,000 cells per cm^2 i.e. almost 1400 million cells in 6360 sq cm cells chamber. This effectively yields 140 doses of MSCs per chamber and thereby reduces the cost for production and bridges the demand- supply gap for MSCs in the market at present.

The remaining cell suspension was centrifuged at 1000-1400rpm for 5-10 minutes. The supernatant was discarded and the pellet was resuspended in multiple electrolyte solution. The cell suspension was then filtered through a 20-40micron strainer and then centrifuged at 1000-1400rpm for 5-10minutes. The washing with multiple electrolyte solution was repeated twice and the supernantant was discarded and the pellet was resuspended in the desired volume of multiple electrolyte solution. The cell suspension was then filtered through a 40micron strainer and then centrifuged at 1400rpm for 10minutes. The washing with multiple electrolyte solution was repeated twice and the supernantant was discarded and the pellet was resuspended in the desired volume of multiple electrolyte solution containing 5% equine serum and 10%dimethyl sulfoxide (DMSO) which serves as a cryoprotectant such that the concentration of cells in the

freezing mixture was 10-100 million cells per 1-15 ml. The cells in freezing mixture were dispensed in to prelabelled cryovials at the rate of 1-15ml per cryobag. These bags constituted the Investigational Product (IP). The cryobags were then frozen in a controlled rate freezer to attain -80°C. The cryobags were then transferred to a vapour phase liquid nitrogen tank for further storage.

Example 6: Morphological analysis of adipose derived MSCs

Adipose derived MSCs when cultured in basal medium comprising 75% α-MEM: 25% KO-DMEM maintained the typical fibroblastic spindle-shaped morphology as shown in FIG. 2. Population doubling (PD) time of MSC on an average in nutrient media was 33± 1.12 hours.

Example 7: Analysis of Cell Surface Markers

The surface markers of adipose derived MSCs are analyzed by FACS antibodies after dissociation. Cells are stained with fluorescein or phycoerythrin coupled antibodies, including Cluster of Differentiation, CD34, CD44, CD45, CD90, CD105 (all antibodies purchased from Becton–Dickinson, San Jose, CA, USA). Stained samples and unstained control cells are analyzed with BD FACS Calibure. Immunophenotyping of the adipose derived MSCs shows high expression of stromal specific markers specific for MSCs and negligible expression of endothelial and hematopoietic markers as shown in FIG. 3, which is a characteristic of a pure MSCs population.

Example 8: Pluripotentent markers, Differentiation and Secretome analysis

The ability of equine adipose tissue derived MSCs isolated, purified and culturally expanded according to an embodiment of the present invention, to differentiate into the various lineages was investigated. Cells obtained as per Example 2, 3, 4 and 5 were plated and cultured in the specific differentiation media for adipogenic, chondrogenic and osteogenic differentiation, and an undifferentiated unstained control of equine adipose tissue derived MSCs was also maintained. Differentiation into adipocytes was confirmed by observing the lipid droplets after Oil red O staining as seen in FIG. 4, mineralization of the matrix / calcium deposition as assessed by Alizarin red S staining demonstrated the osteogenic differentiation potential of AD-MSCs as seen in FIG. 4 and a micromass culture, characteristic to chondrocytes was observed on staining with Alacian blue as seen in FIG. 4. Notably, differentiation into adipocytes, osteocytes and chondrocytes was more than 90%.

In order to determine the pluripotent capacity of the MSCs, the expression levels of pluripotent markers, NANOG, SOX-2 and OCT4 were determined. The expression levels of these pluripotent markers were found to be significantly high, as shown in FIG 5.

Spent media was collected at the end of passage 1 as per Example 4 and passage 2 as per Example 5.

FIG. 6 shows the levels of VEGF and TGF-β in the spent media. Levels of growth factors were more or less similar in MCB, WCB and Final product. This shows that culture method was consistent and uniform yielding uniform MSCs at the end of each passage.

Example 9: Preparation of Off-The-Shelf-Product for clinical use

As mentioned in Example 5, industrial scale production of adipose derived MSCs was done in cell stacks so as to get multiple dosages. MSCs were washed and trypsinized. Cells were washed minimum 3 to 4 times to remove all the trypsin. Cells were re-suspended in injectable media (freezing mixture) comprising multiple electrolyte solution supplemented with 10% DMSO, 5% equine serum 10 to 100 million cells were packed in cryo vial or cryobag in 1 to 15 ml of cells suspended in a freezing mixture and gradually frozen in a control rate freezer at the rate of 1°C per minute until -80°C. The cryobag was then stored in vapour phase liquid nitrogen container.

For clinical use, the cryo vial or cryobag was thawed at 37°C in a water bath for 2 minutes. 3 ml to 35 ml of either normal saline or Plasmalyte-A or Ringer lactate was added to make volume 5 to 50 ml. This dilution made DMSO 1.5 to 3.5% and equine serum from 0.75 to 1.25% which was within the allowable range for equine use without causing any toxicity, side effects or shock. The diluted solution could then be safely and efficaciously injected intravenously at the rate of 1 ml per minute.

Stability of MSC's was checked in normal saline for frozen equine MSCs as shown in Table 5

Table 5

Stability (Hours) for frozen sample	Normal Saline (% viability)
0	94.61%
24	93.42%
48	91.40%

It is clear from Table 5 that injectable normal saline gives more than 90% viability and stability up to 48 h during transport and after final dilution of the Final product.

Example 10: Efficacy of equine adipose derived MSCs in treating tendon and ligament injury by using a therapeutic product prepared according to the method of preparing the product for allogenic use according to an embodiment of the invention

Race horses are prone to musculoskeletal injury of tendon and ligament. The efficacy of equine adipose derived MSCs obtained according to an embodiment of the invention was studied by injecting these MSCs in horses suffering from tendon and ligament injury.

Eight horses with tendon and ligament injury were enrolled in the study. An Ultrasound was performed at the sight of injury and the degree of pain was recorded according to 3 pain scoring scales i.e. Lameness pain score, Pain during manipulation score and Stiff gait score. The scores were recorded before treatment i.e. basal score/screening score after which the horses were given 2 doses of 10 million MSCs at the site of injury. The pain scores for all the horses were recorded 1, 2 and 3 months post the injection. An ultrasound was also performed at the end of 3 months post injection (i.e. after the second injection). Table 6 illustrates the scores recorded before and after injection of MSCs at the site of injury. The pain was rated on a scale of 0-10 in which 0 determines least/ no pain and 10 determines maximum pain.

Table 6

DONOR	SCORE	Basal Score	1 Month follow up	2 months follow up	3 Months follow up
1	Lameness Score	3	1	0	0
	Pain during manipulation	3	2	0	0
	Stiff gait score	3	1	0	0
2	Lameness Score	2	1	1	1
	Pain during manipulation	2	0	0	0
	Stiff gait score	2	0	0	0
3	Lameness Score	1	1	1	1
	Pain during manipulation	3	1	0	0
	Stiff gait score	3	1	0	0
4	Lameness Score	2	1	1	1
	Pain during manipulation	2	1	0	0
	Stiff gait score	1	1	0	0
5	Lameness Score	3	1	1	1
	Pain during manipulation	3	2	0	0
	Stiff gait score	3	1	0	0
6	Lameness Score	2	1	1	1
	Pain during manipulation	3	1	0	0
	Stiff gait score	3	1	0	0
7	Lameness Score	3	1	1	1
	Pain during manipulation	2	0	0	0
	Stiff gait score	2	0	0	0
8	Lameness Score	1	0	0	0
	Pain during manipulation	2	0	0	0
	Stiff gait score	2	0	0	0

As is evident from the above results, reduction in pain was observed 1 month post the second injection. At the end of 3 months post the second injection, the pain was almost negligible. FIG. 7 demonstrates the tissue repair observed by performing an ultrasound at the site of injury before injection, 45 days after the second injection and 96 days after second injection.

Example 11: Immunomodulatory activity of equine adipose derived MSCs

To assess the immunomodulatory activity of the MSCs, immunological reactions were set up i.e. Phytoheamagglutinin (PHA) induced T-cell proliferation assay.

MSCs obtained at the end of Example 5 were harvested and seeded into 96 well plates at 1000 or 5000 cells/well densities and allowed to adhere for 24 hours. The next day, they were inactivated by incubation with Actinomycin-D (5µg/ml) for 15 minutes at 37°C.

To set up the PHA stimulation assay, individual populations of 3 native PBMCs were added to wells containing inactivated AD-MSCs. The positive control wells contained no AD-MSCs. PHA was added to each test and positive control well at a concentration of 10 µg/ml. The negative controls included inactivated MSCs and native PBMCs plated individually in the same concentrations, as in the test wells. No PHA was added to the negative control wells. The assay was allowed to proceed for 3 days before estimation of cell proliferation using the BrdU cell proliferation kit. The endpoint was calculated in terms of percentage T-cell proliferation relative to the positive control, which is assumed to show 100% proliferation. FIG.8 shows the dose dependent suppression of PHA induced T-cell proliferation by 1000 and 5000 equine MSCs (T-cell proliferation induced by PHA, in the absence of MSCs, serves as the positive control and is assumed to be 100%).

Example 12: Safety profile of equine MSC's

Acute Toxicity

In vivo acute toxicity study was conducted to evaluate the effect of a single dose of intravenous infusion of equine adipose derived mesenchymal stem cells on male and female Swiss Albino mice and Wistar rats. The mice and rats were divided into six groups of 10 animals (5 males and 5 females) each. The first three groups were the control groups. The first group received only Plasmalyte A, the second control group received Plasmalyte A + equine serum while the third control group received the vehicle i.e. Plasmalyte A + equine serum (5%) + DMSO (3%). The

other three test groups received IP (investigational product) i.e mesenchymal stem cells at concentrations of 2, 10 and 20 x 10^6 cells/kg body weight by intravenous route.

Following single dose of -intravenous injection, the animals were observed for a minimum period of 14 days. All animals were sacrificed at termination of the study and subjected to a complete necropsy. No clinical signs, mortality or morbidity was observed in the injected animals. The body weight gain by treated animals was not adversely affected during the 14 day observation period post-dosing. No gross pathological alterations were encountered at terminal necropsy in tissues / organs of any of the animals in this study. No adverse effects were observed in vehicle control group animals treated with the vehicle, with respect to survival, clinical signs, body weight gain and necropsy findings. No mortality or morbidity was encountered in the study groups.

Based on the findings of this acute intravenous toxicity study, in absence of any incidence of deaths and adverse effects among treated animals, Maximum tolerated dose (MTD) of Equine Adipose derived Mesenchymal Stem Cells in Swiss albino mice and Wistar rats was estimated to be greater than 20 X 10^6 cells/ kg body weight when administered intravenously. Equine adipose derived MSCs obtained according to an embodiment of the invention also showed a safety margin of greater than ten times (10X) the maximum therapeutic dose anticipated for use with equine subjects.

What has been described and illustrated herein are preferred embodiments of the invention along with some of their variations. The terms, descriptions and figures used herein are set forth by way of illustration only and are not meant as limitations. Those skilled in the art will recognize that many variations are possible within the spirit and scope of the invention, which is intended to be defined by the claims in the complete specification—and their equivalents—in which all terms are meant in their broadest reasonable sense unless otherwise indicated.

WE CLAIM:

1. A method for isolation, purification and industrial scale expansion of equine adipose tissue derived mesenchymal stem cells (MSCs) to obtain a yield of at least 215,000 cells per cm^2 pure clinical grade MSCs for allogenic use comprising over 95% cells which express positive markers CD44, CD90, and CD105, and less than 2% cells which express negative markers CD45, CD34 and HLA-DR, the method comprising the steps of:
    a) extracting 10 to 20 grams of adipose tissue from multiple equine donors;
    b) digesting the adipose tissue from individual equine donors with collagenase, centrifuging the digested tissue and collecting the stromal vascular fraction (SVF);
    c) seeding the cells contained in the SVF into a culture medium; and
    d) trypsinising and washing the cells once they reach confluence at passage 0;
    e) pooling the cells obtained from the adipose tissue of multiple canine donors processed through steps (a) to (d);
    f) seeding the pooled cells of passage 0 into a culture medium;
    g) trypsinising and washing the cells once they reach confluence at passage 1;
    h) seeding the cells of passage 1 into a culture medium; and
    i) trypsinising and washing the cells once they reach confluence at passage 2,

wherein,

- step (a) is performed by biopsy assisted removal;
- cells from stromal vascular fraction are seeded in step (c) only if at least 60% cells are positive for CD 90;
- the SVF cells are seeded in to the culture medium in step (c) at a seeding density of at least 75000 cells per sq cm;
- prior to seeding in step (f), the mesenchymal stem cells are characterized based on the percentage of cells which express positive markers CD44, CD90, and CD105, and the percentage of cells which express negative markers CD45, CD34 and HLA-DR;

- the mesenchymal stem cells are seeded in to the culture medium in step (f) and step (h) at a seeding density of 1000 to 5000 cells per sq cm and express at least 95% of the positive markers CD44, CD90, and CD105 and at most 2% of the negative markers CD45, CD34 and HLA-DR;
- the culture medium comprises 25% to 75% Dulbecco's Modified Eagle's Medium-Knockout (DMEM-KO) and 25% to 75% alpha-Minimum Essential Medium (α-MEM) or upto 100% DMEM-KO or upto 100% α-MEM; and
- prior to the trypsinising at step (d), (g) and (i) 70 to 90% of the culture medium is changed at 3 to 6 day intervals after seeding until confluence is reached.

2. A method for isolation, purification and industrial scale expansion of equine adipose tissue derived mesenchymal stem cells (MSCs) to obtain a yield of at least 215,000 cells per cm^2 pure clinical grade MSCs for autologous use comprising over 95% cells which express positive markers CD44, CD90, and CD105, and less than 2% cells which express negative markers CD45, CD34 and HLA-DR, the method comprising the steps of:
    a) extracting 10 to 20 grams of adipose tissue from an equine donor;
    b) digesting the adipose tissue with collagenase, centrifuging the digested tissue and collecting the stromal vascular fraction (SVF);
    c) seeding the cells contained in the SVF into a culture medium; and
    d) trypsinising and washing the cells once they reach confluence at passage 0;
    e) seeding the washed cells of passage 0 into a culture medium;
    f) trypsinising and washing the cells once they reach confluence at passage 1;
    g) seeding the cells of passage 1 into a culture medium; and
    h) trypsinising and washing the cells once they reach confluence at passage 2,

wherein,

- step (a) is performed by biopsy assisted removal;
- cells from stromal vascular fraction are seeded in step (c) only if at least 60% cells are positive for CD 90;
- the SVF cells are seeded in to the culture medium in step (c) at a seeding density of at least 75000 cells per sq cm;
- prior to seeding in step (e), the mesenchymal stem cells are characterized based on the percentage of cells which express positive markers CD44, CD90, and CD105, and the percentage of cells which express negative markers CD45, CD34 and HLA-DR;

- the mesenchymal stem cells are seeded in to the culture medium in step (e) and step (g) at a seeding density of 1000 to 5000 cells per sq cm and express at least 95% of the positive markers CD44, CD90, and CD105 and at most 2% of the negative markers CD45, CD34 and HLA-DR;
- the culture medium comprises 25% to 75% Dulbecco's Modified Eagle's Medium-Knockout (DMEM-KO) and 25% to 75% alpha-Minimum Essential Medium (α-MEM) or upto 100% DMEM-KO or upto 100% α-MEM; and
- prior to the trypsinising at step (d), (f) and (h) 70 to 90% of the culture medium is changed at 3 to 6 day intervals after seeding until confluence is reached.

3. The method as claimed in claim 1 or 2, wherein the adipose tissue is extracted from the dorsal gluteal muscle of the donor.

4. The method as claimed in claim 1 or 2, wherein the washed cells after trypsinisation are frozen in a freezing mixture comprising multiple electrolyte solution supplemented with 5% equine serum and 10% dimethyl sulfoxide (DMSO).

5. The method as claimed in claim 1 or 2, wherein the culture medium comprises 25% DMEM-KO and 75% α-MEM.

6. A therapeutic product prepared according to the method of claim 1 or 2 for clinical use.

7. A therapeutic product for treating tendon injury, ligament injury, osteoarthritis and exercise induced pulmonary hemorrhage comprising MSCs suspended in multiple electrolyte solution supplemented with human serum albumin and dimethyl sulfoxide (DMSO) wherein, over 95% MSCs express positive markers CD44, CD90 and CD105, and less than 2% cells express negative markers CD45, CD34 and HLA-DR, and wherein the MSCs have undergone not more than 16 population doublings in vitro and are capable of at least 30 to 35 more population doublings, the MSCs are capable of differentiating into adipocytes, osteocytes and chondrocytes, the MSCs express pluripotent markers like OCT-4, SOX-2 and NANOG, and secrete growth factors like TGF-β and show immunomodulatory activity.

8. A method for treating tendon injury, ligament injury, exercise induced pulmonary hemorrhage and osteoarthritis comprising administering two doses each of 10 million MSCs of the therapeutic product as claimed in claim 7, by intra-articular route.

9. Use of the therapeutic product as claimed in claim 7 for treating tendon injury, ligament injury, osteoarthritis and exercise induced pulmonary hemorrhage.

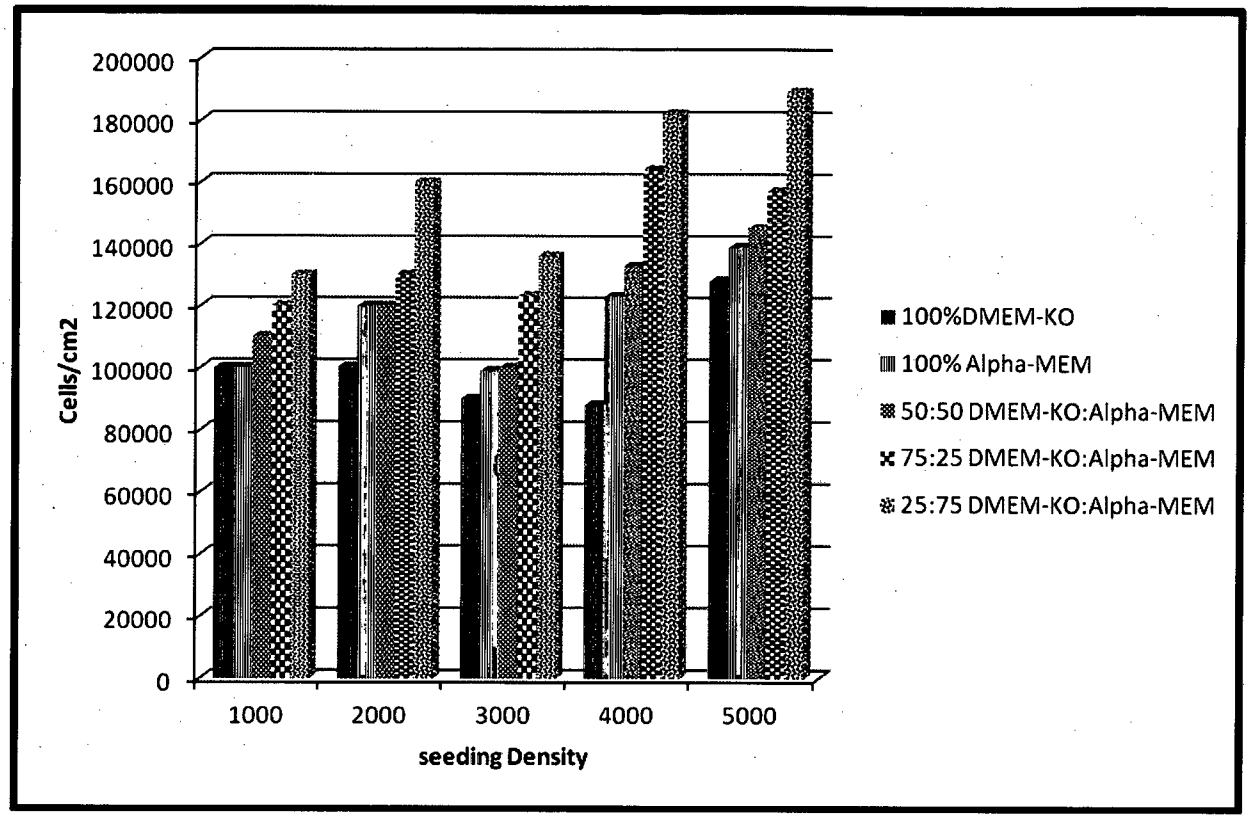

200000
180000
160000
140000
120000
100000
80000
60000
40000
20000
0
Cells/cm2
1000
2000
3000
4000
5000
seeding Density
100%DMEM-KO
100% Alpha-MEM
50:50 DMEM-KO:Alpha-MEM
75:25 DMEM-KO:Alpha-MEM
25:75 DMEM-KO:Alpha-MEM

FIG. 1A

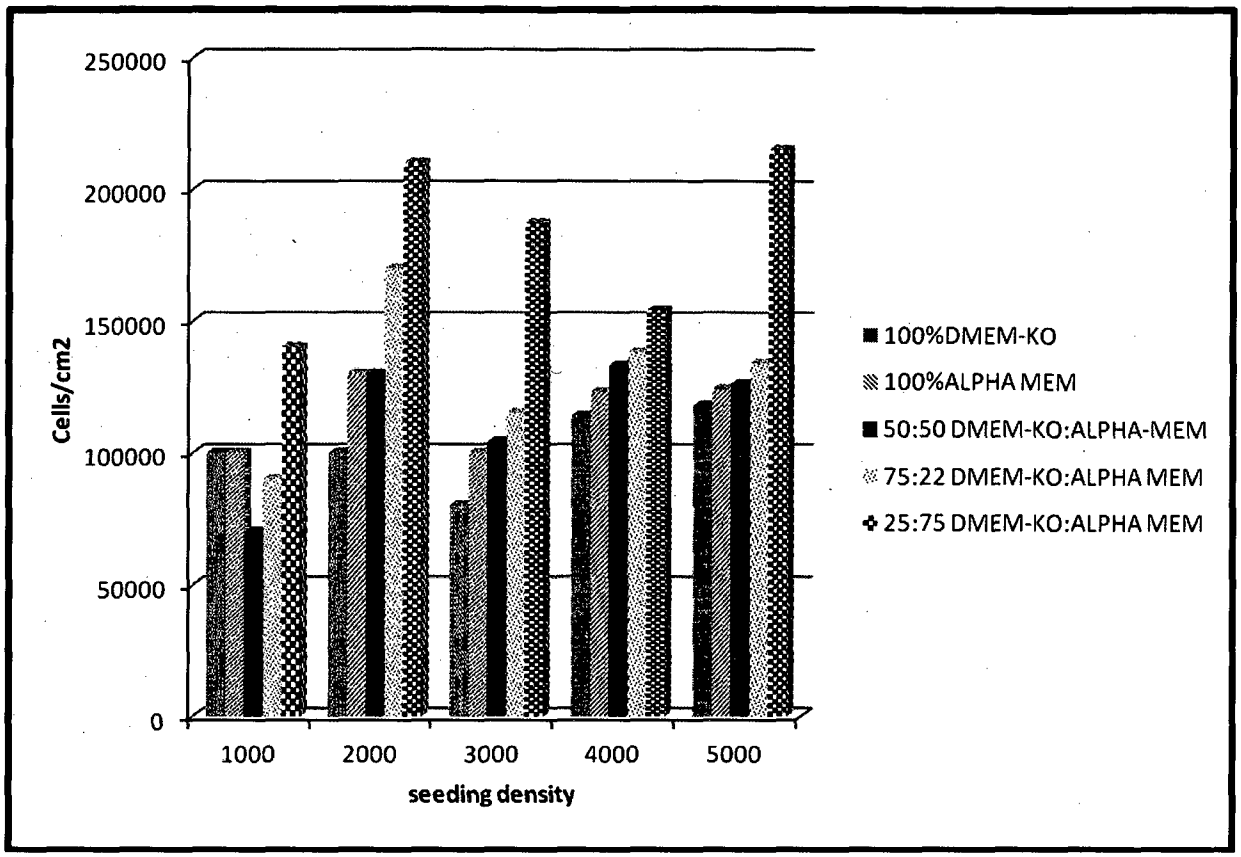

250000
200000
150000
100000
50000
0
Cells/cm2
1000
2000
3000
4000
5000
seeding density
100%DMEM-KO
100%ALPHA MEM
50:50 DMEM-KO:ALPHA-MEM
75:22 DMEM-KO:ALPHA MEM
25:75 DMEM-KO:ALPHA MEM

FIG. 1B

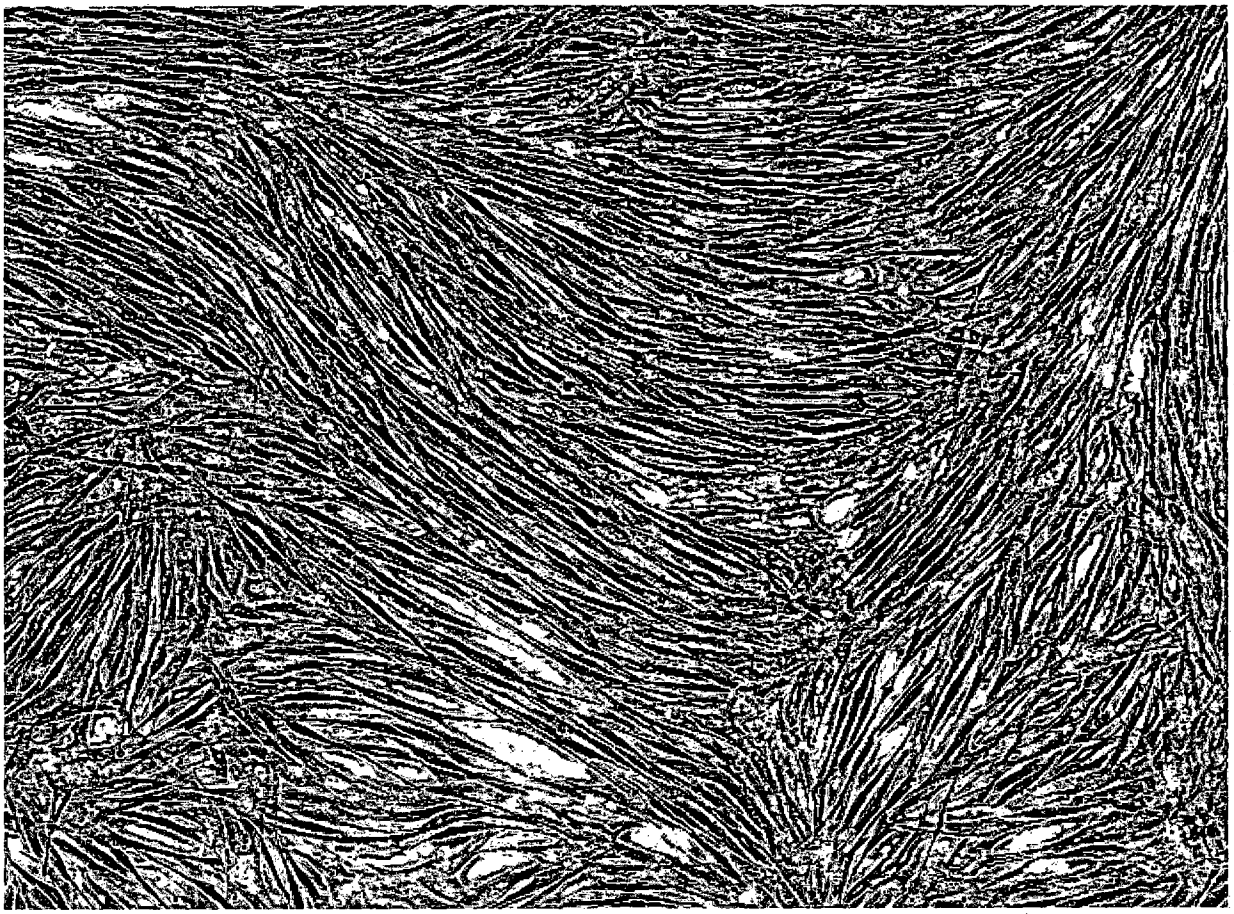


FIG. 2

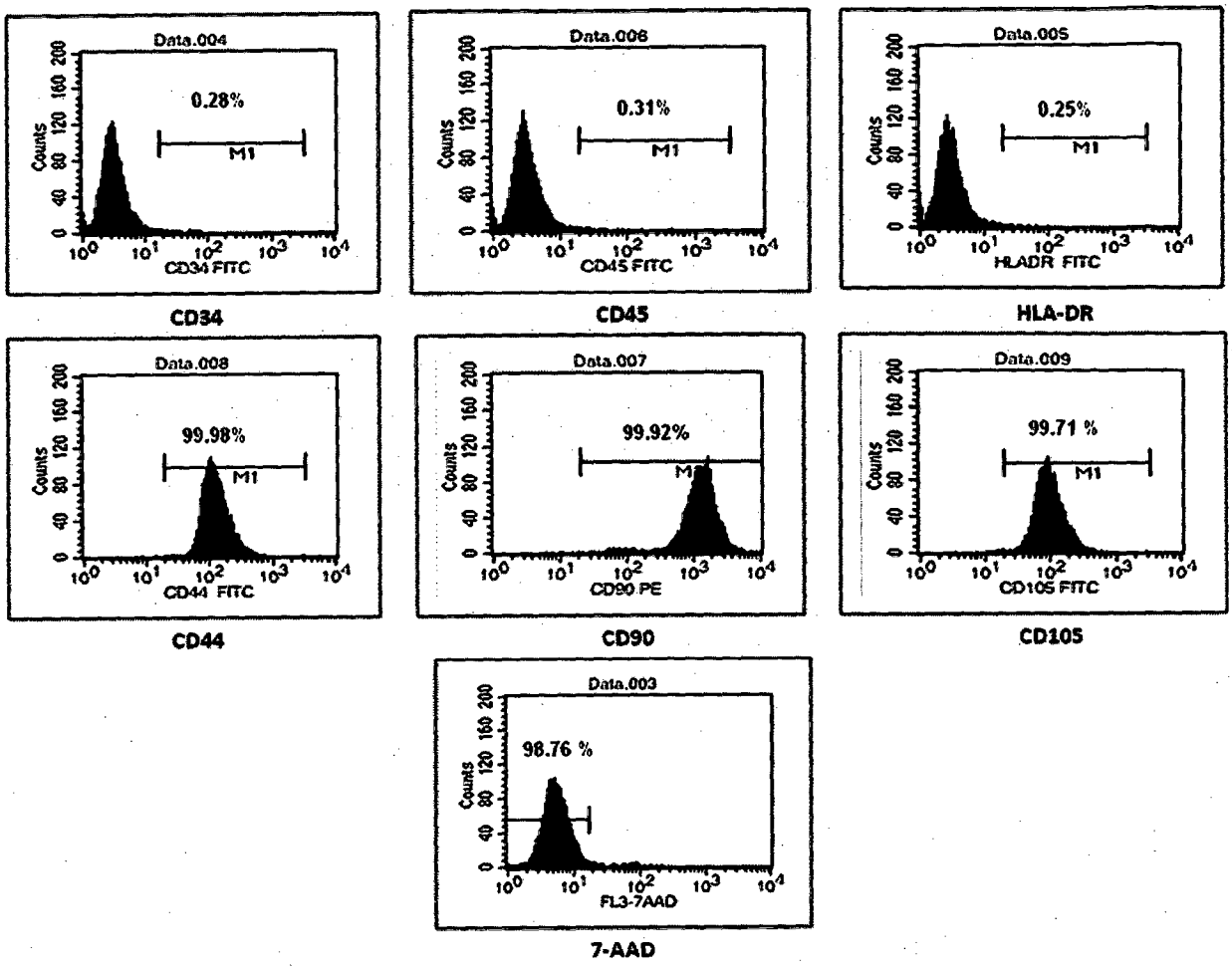

Data.004
0.28%
M1
Counts
CD34 FITC
CD34
Data.006
0.31%
M1
Counts
CD45 FITC
CD45
Data.005
0.25%
M1
Counts
HLADR FITC
HLA-DR
Data.008
99.98%
M1
Counts
CD44 FITC
CD44
Data.007
99.92%
M1
Counts
CD90 PE
CD90
Data.009
99.71 %
M1
Counts
CD105 FITC
CD105
Data.003
98.76 %
Counts
FL3-7AAD
7-AAD

FIG 3

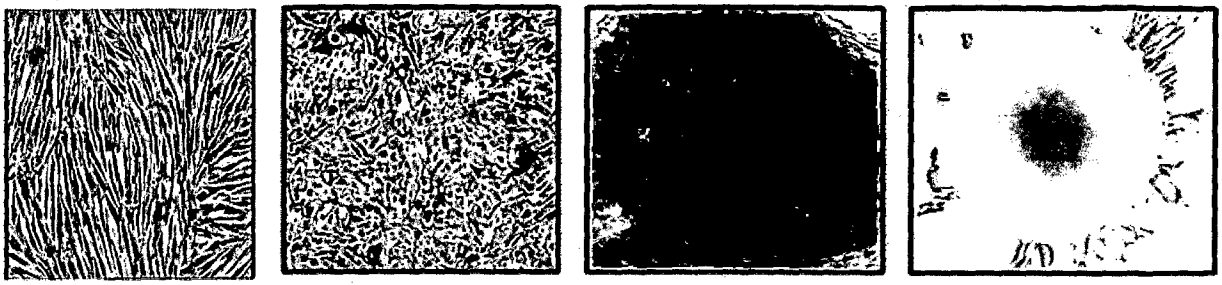


FIG 4

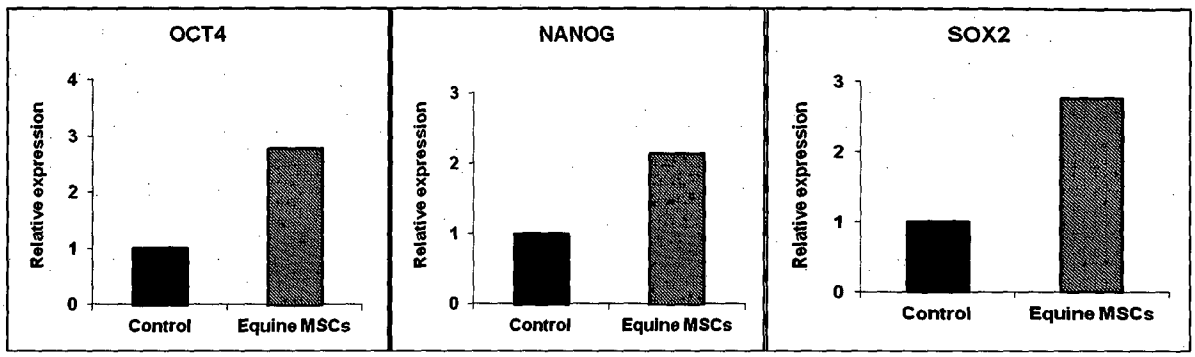


FIG 5

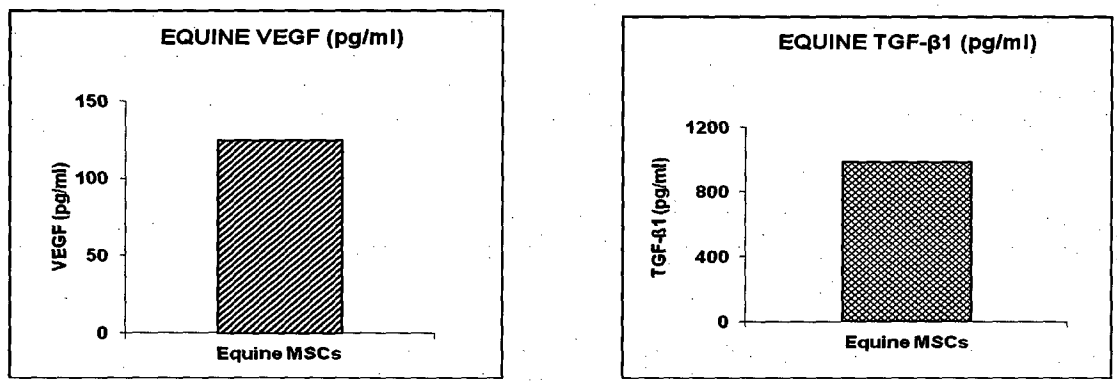
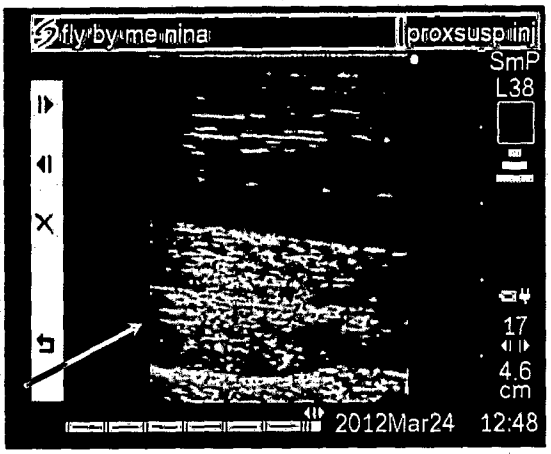
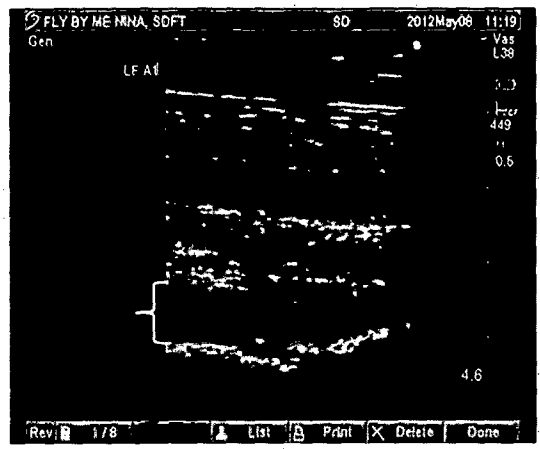


FIG 6

Pre Injection



Post injection 45 days



Post injection 45 days

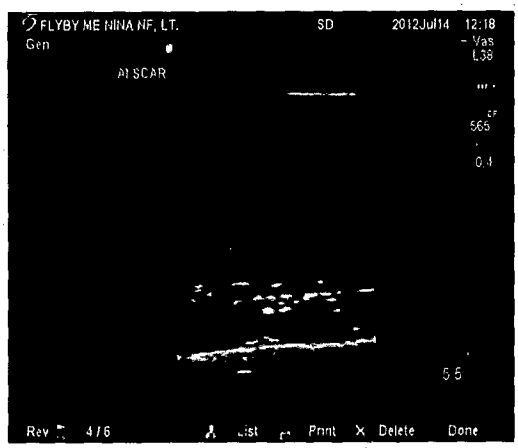


FIG 7

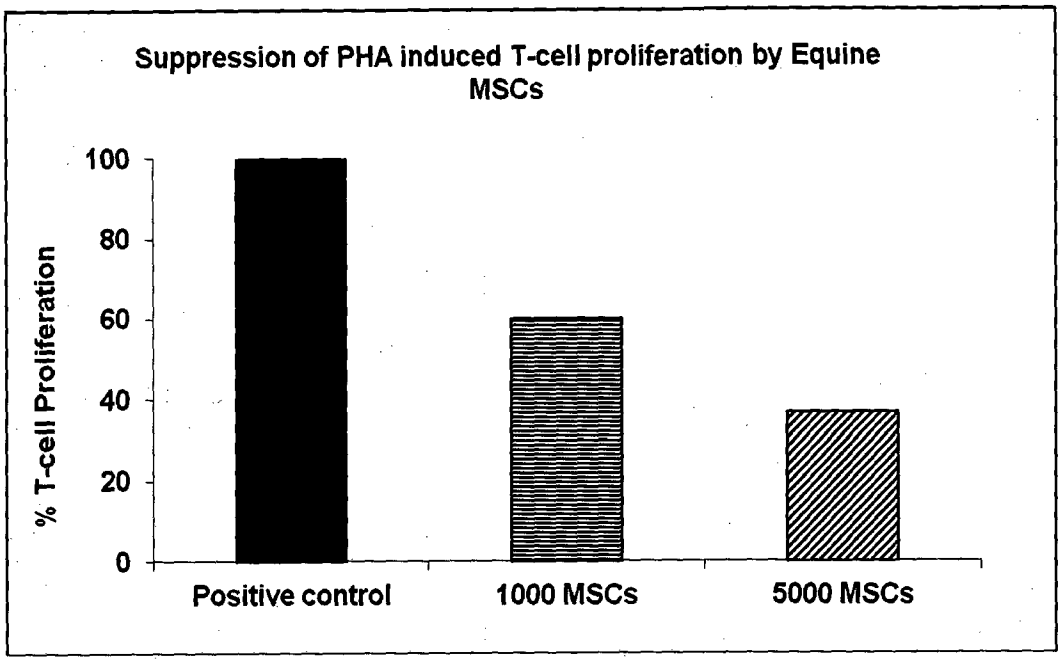

Suppression of PHA induced T-cell proliferation by Equine MSCs
% T-cell Proliferation
100
80
60
40
20
0
Positive control
1000 MSCs
5000 MSCs

FIG 8