

Research Article

Endogenous Adult Telomerase Positive Stem Cells Increase in Equine Peripheral Blood Following Exercise

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Abstract

Stout, et al., 2007 reported a base-line level of primitive endogenous adult Telomerase Positive Stem Cells (aTPSCs) circulating within adult porcine peripheral blood which increased dramatically, i.e., 23.5-fold, after just 90 minutes of trauma. Ongoing studies from our laboratory have shown the presence of similar primitive stem cells in adult equine blood and that the base-line levels of stem cells varied according to the particular breed of horse. The current study was undertaken to test the hypothesis that aTPSCs would increase in number in the peripheral blood following stress in the same horse. Stress in this study was defined as moderate exercise, i.e., 10 minutes of cantering. Blood withdrawal followed the guidelines of Fort Valley State University's IACUC. Adult horses had their blood withdrawn immediately prior to and immediately after exercising. The blood was obtained by venipuncture. Isolated aTPSCs were assayed by trypan blue staining, CEA-CAM-1 for totipotent stem cells and SSEA-4 for pluripotent stem cells. This study demonstrates that moderate exercise will increase the level of these primitive pluripotent stem cells in the blood of adult equines. Studies are ongoing to address their functional significance during injury and repair.

Keywords: Wound Healing; Adult-Derived Stem Cells; Blood; Equines; Autologous

Introduction

Young, et al., identified a very rare population of endogenous adult Telomerase Positive Stem Cells (aTPSCs) in 15 species of adult animals, including horses [1]. This population was composed of a range of cells, by size, cell surface markers, expressed genes and differentiation

potential (Fig. 1).

The identified cells were all telomerase positive. Totipotent Stem Cells (TSCs) form all somatic cells of the body, gametes and nucleus pulposus of the intervertebral disc. Halo-Like Stem Cells (HLSCs), Corona-Like Stem Cells (CLSCs), Pluripotent Stem Cells (PSCs) and Germ Layer Lineage Stem Cells (GLSCs) form all somatic cells of the body from all three embryonic germ layer lineages, e.g., ectoderm, mesoderm and endoderm. Ectodermal Stem Cells (EctoSCs) will form all progenitor cells of the ectodermal lineage, but will not transdifferentiate into mesodermal-based cells or endodermal-based cells. Mesodermal Stem Cells (MesoSCs) will form all progenitor cells of the mesodermal lineage, but will not transdifferentiate into ectodermal-based cells or endodermal-based cells. And Endodermal Stem Cells (EndoSCs) will form all progenitor cells of the endodermal lineage, but will not transdifferentiate into ectodermal-based cells or mesodermal-based cells (Fig. 1).

Young, et al., demonstrated a very small number of adult Telomerase Positive Stem Cells (aTPSCs) in human peripheral blood [2]. The number of these cells increased slightly in the presence of various co-morbidities [1]. Stout, et al., reported the presence of aTPSCs circulating within adult porcine peripheral blood and that the number of circulating aTPSCs increased dramatically (23.5-fold) after 90 minutes of trauma [3]. Preliminary studies from Young, et al., have shown the presence of aTPSCs in adult equine blood [4]. Base-line levels of these stem cells varied according to the breed of the horse (submitted). The current study was undertaken to test the hypothesis that the equine aTPSCs increase in number in the peripheral blood following exercise (mild stress).

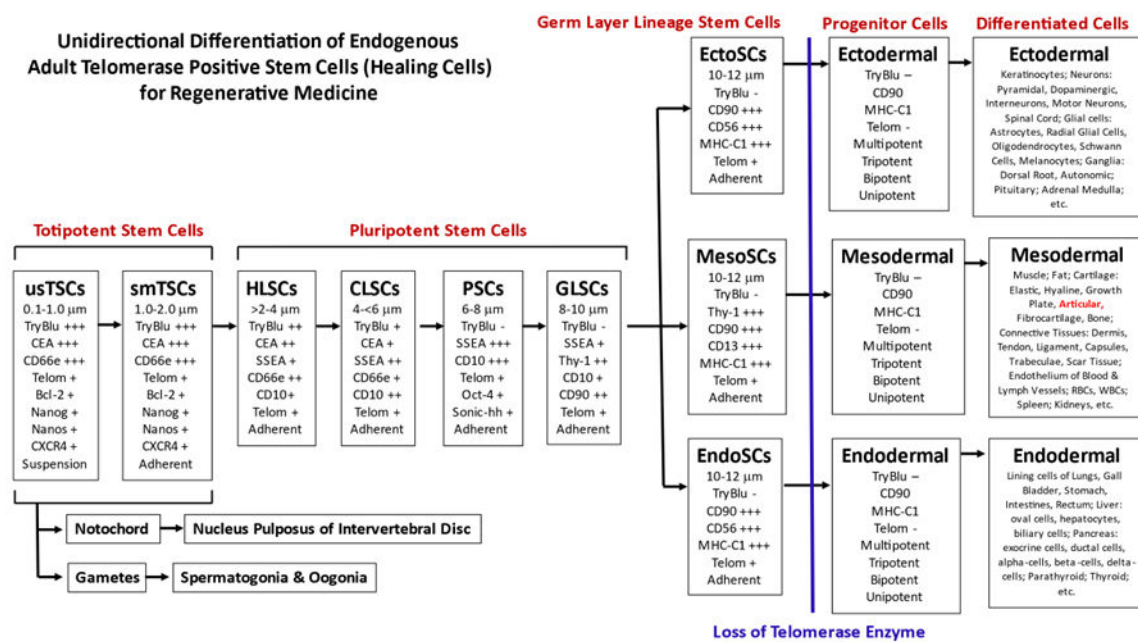


Figure 1: Summary of aTPSCs with respect to subcategories, size, Trypan blue staining, cell surface markers, expressed genes, growth in culture and differentiation potential [1]. Reprinted with permission from Young HE. Adult telomerase positive stem cells and combinatorial nutraceutical supplement pill, 50 years in the making. American Journal of Medical and Clinical Research & Reviews. 2024;3(8): 1-106.

Method and Materials

The use of animals in this study complied with the guidelines of Fort Valley State University Institutional Animal Care and Use Committee. Their use also complied with the criteria of the National Research Council for the humane care of laboratory animals as outlined in the "Guide for the Care and Use of Laboratory Animals" prepared by the Institute of Laboratory Animal Resources and published by the National Institutes of Health [4].

Tissue Harvest

Four horses were used as test subjects. Blood was obtained by venipuncture following standard acceptable veterinary practice. Blood was withdrawn prior to and following moderate exercise from the same horse using sterile procedures. Each subject was used as their own control. Four ml of blood were placed into tubes containing 15% (w/v) EDTA. The tubes were inverted three to four times to mix the blood with the EDTA and then stored at 4°C for 48 hours [1].

Moderate Exercise

Moderate exercise in this study was defined as cantering for ten minutes.

Stem Cell Isolation

After 48 hours of gravity separation, the blood had separated into a floating serum fraction and a sedimented cellular pellet (hematocrit). The cellular pellet contained red blood cells, white blood cells, platelets and hematopoietic stem cells [1]. The serum fraction, containing aTPSCs, was withdrawn by a sterile pipet, placed into a second sterile tube and stored at 4°C [1].

Stem Cell Counting

Fifteen microliters of the serum from each horse was mixed with 15 microliters of sterile 0.4% Trypan blue. The resulting solution was placed onto a hemocytometer and the isolated cells counted and photographed (Fig. 2).

Stem Cell Identification

Cells within the serum fraction were stained with an antibody to carcinoembryonic antigen-Cell Adhesion Molecule-1 (CEA) to identify CEA-CAM-1 positive cell types in the serum, potentially TSCs, HLSCs and CLSCs (Table 1).

Char ¹	TSC ²	HLSC ³	CLSC ⁴	PSC ⁵	GLSC ⁶	EctoSC ⁷	MesoSC ⁸	EndoSC ⁹
Size in microns	0.1-2.0	>2-<4	4-<6	6-8	>8-10	>10-12	>10-12	>10-12
0.4% Trypan Blue	Entire Cell Positive	Halo Positive Center-Negative	Corona Positive Center-Negative	Entire Cell Neg ¹⁰	Entire Cell Neg	Entire Cell Neg	Entire Cell Neg	Entire Cell Neg
Viability	100%	100%	100%	100%	100%	100%	100%	100%
Telomerase	Pos ¹¹	Pos	Pos	Pos	Pos	Pos	Pos	Pos
Cell Surf Markers	CEA ^{Pos12}	CEA ^{high} SSEA ^{low}	CEA ^{low} SSEA ^{high}	CEA ^{Neg} SSEA ^{Pos13}	CEA ^{Neg} SSEA ^{high} Thy1 ^{low14}	CEA ^{Neg} SSEA ^{Neg} Thy-1 ^{Pos} MHC-I ¹⁵	CEA ^{Neg} SSEA ^{Neg} Thy-1 MHC-I	CEA ^{Neg} SSEA ^{Neg} Thy-1 MHC-I
Cell Types Formed	HLSCs, CLSCs, PSCs, GLSCs, EctoSCs, MesoSCs, EndoSCs, All PCs ¹⁶ , All DCs ¹⁷ , Gametes ¹⁸ , NP of IVD ¹⁹	CLSCs, PSCs, GLSCs, EctoSCs, MesoSCs, EndoSCs, All PCs, All DCs	PSCs, GLSCs, EctoSCs, MesoSCs, EndoSCs, All PCs, All DCs	GLSCs, EctoSCs, MesoSCs, EndoSCs, All PCs, All DCs	EctoSCs, MesoSCs, EndoSCs, All PCs, All DCs	EctoPCs ²⁰ , EctoDCs ²¹	MesoPCs ²² , MesoDCs ²³	EndoPCs ²⁴ , EndoDCs ²⁵

Table 1: Adult telomerase positive stem cell characteristics. Char1, characteristics; TSCs2, totipotent stem cells, HLSCs3, halo-like stem cells; CLSCs4, corona-like stem cells; PSCs5, pluripotent stem cells, GLSCs6, germ layer lineage stem cells; EctoSCs7, ectodermal stem cells; MesoSCs8, mesodermal stem cells; EndoSCs9, endodermal stem cells; Neg10, negative; CEAPos11, positive; CEA, carcino-embryonic antigen-cell adhesion molecule-1 positive; SSEAPos13, stage specific embryonic antigen-4 positive; Thy-1Low14, low; MHC-I15, Major Histocompatibility Complex Class-I (self-recognition cell surface molecule); All PCs16, all somatic progenitor cells; All DCs17, all somatic differentiated cells; Gametes18, oogonia and spermatogonia; NP of IVD19, nucleus pulposus of intervertebral disc; EctoPCs20, ectodermal-derived somatic progenitor cells; EctoDCs21, ectodermal-derived somatic differentiated cells; MesoPCs22, mesodermal-derived somatic progenitor cells; MesoDCs23, mesodermal-derived somatic differentiated cells; EndoPCs24, endodermal-derived somatic progenitor cells; EndoDCs25, endodermal-derived somatic differentiated cells. Reprinted in part with permission from Young HE, Speight MO. Characterization of endogenous telomerase-positive stem cells for regenerative medicine, a review. *Stem Cell Regen Med* 2020;4(2):1-14.

In brief, the serum fraction was placed in 15-ml polypropylene tubes (Falcon, Becton Dickinson Labware, Franklin Lakes, NJ) and mixed with an equal volume of ELICA fixative at ambient temperature (Dragonfly Foundation for Research and Development, DFRD, Macon, GA) from 5 minutes to 7 days [1,5]. The resulting mixture was centrifuged at 2,000 x RCF (relative centrifugation force) and the supernatant decanted to bleach. The cell pellet was re-suspended and mixed with 14-ml of Dulbecco's Phosphate Buffered Saline, pH 7.4 (DPBS) (Invitrogen, GIBCO, Grand Island, NY) at ambient temperature to wash

the fixative from the cells. The mixture was centrifuged at 2,000 × RCF. The supernatant was decanted into bleach and the cells re-suspended. The washing process was repeated a second time to ensure the removal of the fixative from the cells. To ensure consistency of results, the cells underwent the same immunocytochemical staining procedure as out tissue sections, albeit in the polypropylene tubes and with DPBS washing and centrifugation.

Immunocytochemistry

Fixed cells from the serum fraction were incubated with 95% ethanol and then washed with 14 ml of DPBS, as described above. The cells were incubated with 5 ml of 5.0% (w/v) Sodium Azide (Sigma, St. Louis, MO) in DPBS for 60 minutes. They were washed in DPBS as described above and incubated with 5 ml of 30% hydrogen peroxide (Sigma, St. Louis, MO) for 60 minutes to irreversibly inhibit endogenous peroxidases [1,5]. The cells were rinsed with DPBS as described above and incubated for 60 minutes with blocking agent (Vecstatin ABC Reagent Kit, Vector Laboratories Inc., Burlingame, CA) in DPBS [5]. The blocking agent was removed by centrifugation. The supernatant was decanted, the cells re-suspended and washed with DPBS. The cells were incubated with the primary antibody for 60 minutes. The primary antibody consisted of 0.005% (v/v) Carcinoembryonic Antigen-Cell Adhesion Molecule-1 (CEA-CAM-1) in DPBS [1,5]. The primary antibody was removed by centrifugation and the supernatant decanted. The cells were re-suspended and washed with DPBS. The cells were incubated with the secondary antibody for 60 minutes. The secondary antibody consisted of 0.005% (v/v) biotinylated affinity purified, rat absorbed anti-mouse immunoglobulin G (H + L) (BA-2001, Vector Laboratories) in DPBS [6]. The secondary antibody was removed by centrifugation and the supernatant decanted. The cells were re-suspended and washed with DPBS. The cells were incubated with avidin-HRP for 60 minutes. The avidin-HRP consisted of 10-ml of 0.1% (v/v) Tween-20 (ChemPure, Curtin Matheson Scientific, Houston, TX) containing 2 drops reagent-A and 2 drops reagent-B (Peroxidase Standard PK-4000 Vecstatin ABC Reagent Kit, Vector Laboratories) in DPBS [1,5]. The avidin-HRP was removed by centrifugation, supernatant decanted and washed with DPBS. The cells were incubated with AEC peroxidase substrate (Sigma) for 60 minutes [6]. The AEC substrate was prepared as directed by the manufacturer. The substrate solution was removed by centrifugation and the supernatant was decanted. The cells were re-suspended and washed with DPBS. Fifteen microliters of re-suspended cells were placed on a hemocytometer and analyzed.

Visual Analysis

Stained cells were visualized using a Nikon TMS phase contrast microscope with bright field microscopy at 40x, 100x and 200x. Photographs were taken with a Nikon CoolPix 995 digital camera.

Results

Three cell types were visualized following staining with Trypan blue (Figure 2). Staining revealed the presence of numerous very small spherical structures stained that completely with the Trypan blue dye. The Trypan blue positive small spherical structures were designated as TSCs (Table 1) [1]. A second group of cells were observed. The cells were slightly larger than the TSCs and had a peculiar staining pattern with Trypan blue. The outer peripheral rim of the cells stained with Trypan blue dye, while the inner central area was void of staining. These cells were designated as HLSCs (aka, Tr-TSC/PSC) (Table 1) [1]. The third category of cells was about twice the size of the HLSCs and did not stain with the Trypan blue dye. These cells appeared as white (glowing) spheres on a blue background of Trypan blue dye and were designated as PSCs (Fig. 2) (Table 1) [1]. The putative TSCs and HLSCs exhibited positive staining for CEA-CAM-1 (Fig. 2). The entities designated as TSCs were very small spherical structures that were positive for the CEA-CAM-1 antibody. Larger circular structures with a rim of CEA-CAM-1 positive staining and clear centers were designated as HLSCs. Clear cells that were void of CEA-CAM-1 staining could not be visualized with bright field antibody staining.

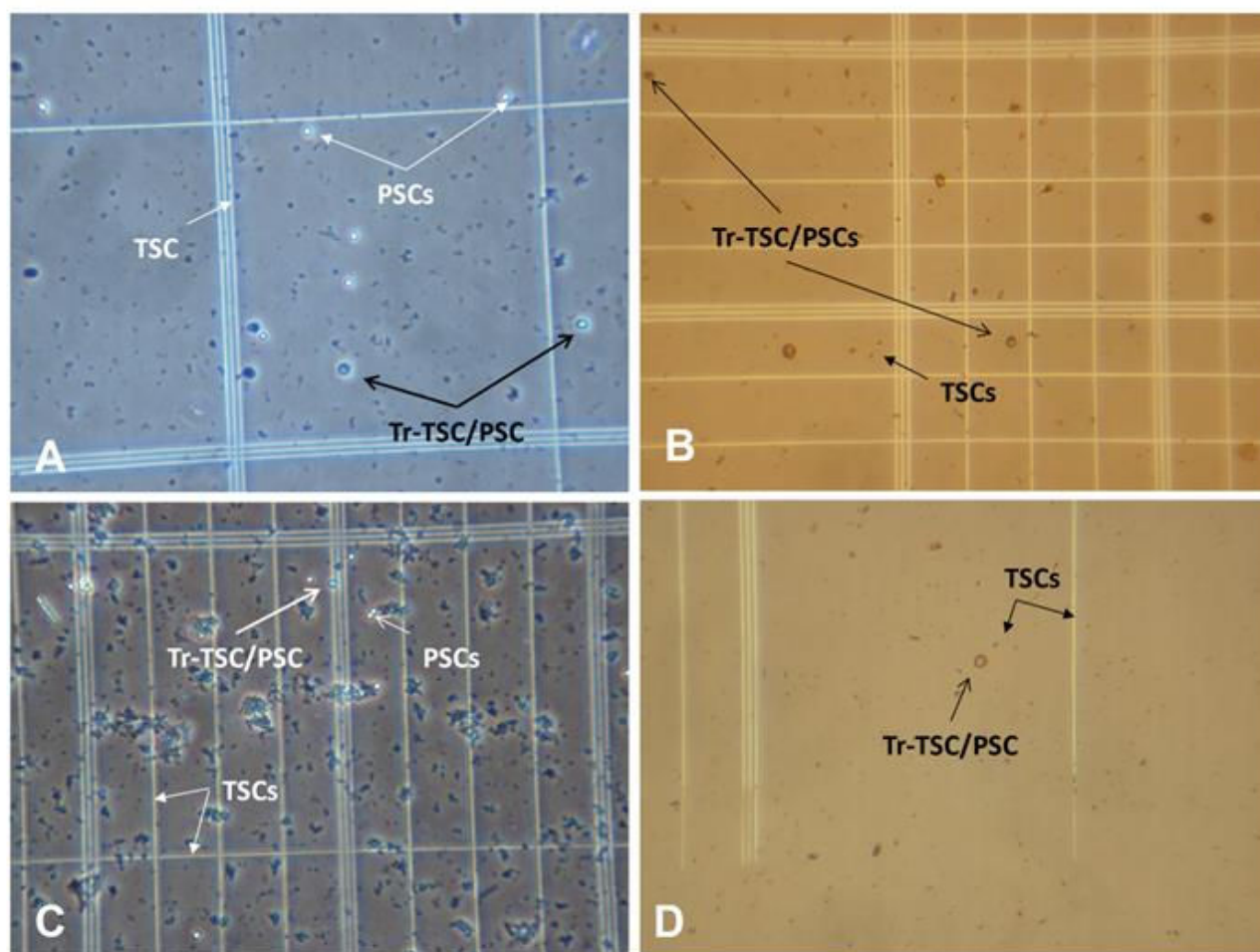


Figure 2: Bovine(A,B) and Equine(C,D) stained serum fractions. A: Bovine serum fraction stained with 0.4% Trypan blue. TSCs are small dark round circles, HLSCs (Tr-TSC/PSC) are cells with a peripheral rim of Trypan blue staining and PSCs have white glowing nuclei with phase contrast microscopy, 100x mag. B: Bovine serum fraction stained with CEA-CAM-1. TSCs are small dark-red round circles, HLSCs (Tr-TSC/PSCs) have a peripheral rim of CEA-CAM-1 positive staining, 100x mag. C: Equine serum fraction stained with 0.4% Trypan blue. TSCs are small dark round circles, HLSCs (Tr-TSC/PSC) are cells with a peripheral rim of Trypan blue staining and PSCs have white glowing nuclei with phase contrast microscopy, 100x mag. D: Equine serum fraction stained with CEA-CAM-1. TSCs are small dark-red round circles, HLSCs (Tr-TSC/PSC) have a peripheral rim of CEA-CAM-1 positive staining, 100x mag. Reprinted with permission from Young HE, Lochner F, Lochner D, Lochner D, et al. Primitive Stem Cells in Adult Feline, Canine, Ovine, Caprine, Bovine and Equine Peripheral Blood. J Stem Cell Res. 2017; 1(1) 004: 1-6.

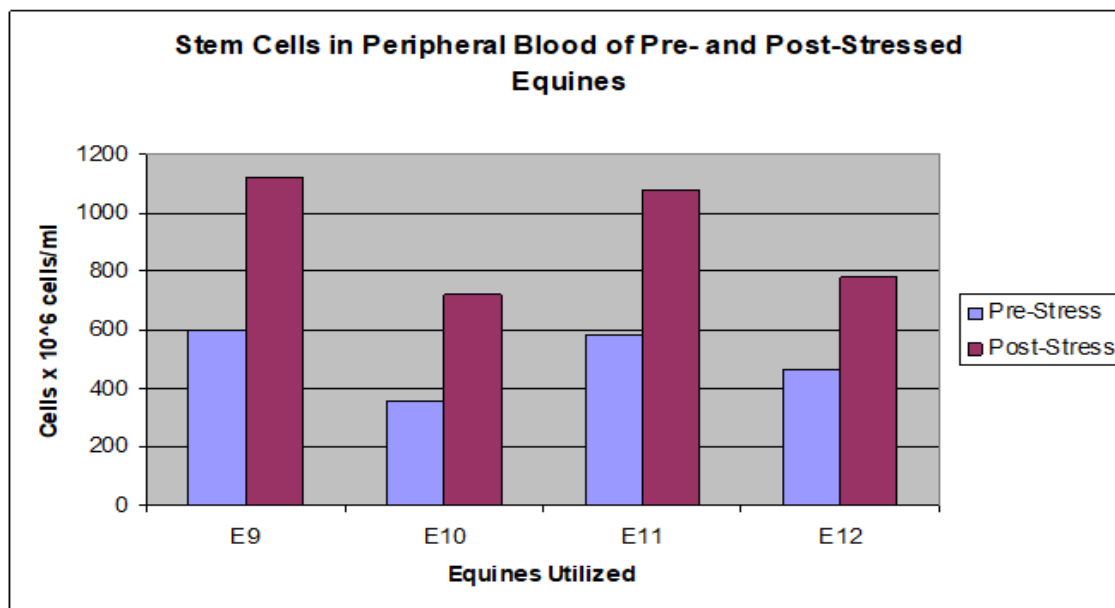


Figure 3: Stem Cells in Peripheral Blood of Pre- and Post-Stressed Equines. Four equines were utilized, E9, E10, E11 and E12. Blood samples were taken before and after exercise. Exercise consisted of 10 minutes of cantering. Blood samples were processed for isolation, visualization and counting of TSCs and HLSCs (Trypan Blue positive and CEA-CAM-1 positive staining) and PSCs (Trypan Blue negative staining only). Cell counts were standardized to number of aTPSCs times 10⁶ cells per milliliter of serum.

As shown in Fig. 3, there was an increase in circulating TSCs, HLSCs and PSCs in the peripheral blood following exercising, e.g., 10 minutes of cantering. And, on average, stem cell numbers after exercising were approximately twice what they were at rest for each one of the four horses examined.

Discussion

The results show that TSCs, HLSCs and PSCs circulate within the peripheral equine blood of these horses. The results demonstrate that moderate exercising (cantering) approximately doubles the concentration of the circulating stem cells. Furthermore, these results demonstrate the ease with which adult endogenous aTPSCs can be harvested from adult equine blood. Autologous adult Mesenchymal Stem Cells (MSCs) are being used in clinical veterinary practice to treat a variety of musculoskeletal disorders in horses [10,11]. In these treatments autologous stem cells are isolated from various tissues, predominantly adipose tissue (fat). The adult MSCs are then injected into the site of injury or pathology [9-11]. However, harvesting of stem cells from fat is cumbersome and extremely painful in the animal. Moreover, the process leaves a site that may be slow to heal and prone to infection [9-11].

Stem cells and progenitor cells have been isolated from a myriad of adult tissues, such as brain, bone marrow, bone, periosteum, perichondrium, pancreas, skeletal muscle, cementum, dental pulp, olfactory mucosa, blood vessels, heart, kidney, dermis and fat in 15 species of animals, including horses [1,12]. Young and Speight classified the aTPSCs into eight categories based on cellular size and the presence of cell surface markers, as well as other unique characteristics (Fig. 1,4) [6].

This study demonstrated that aTPSCs increase in the adult peripheral equine blood following moderate exercise. Furthermore, it demonstrated that the aTPSCs can be obtained from the peripheral blood, rather than obtaining MSCs from fat or bone marrow. A recent study demonstrated that aTPSCs could be isolated from the peripheral blood of a Komodo Dragon, Wedel Crane, Spectacled Bear, German Shepard and six humans and used to treat decreased ambulation and pain, due to osteoarthritis of their respective joints [13]. The individuals (n=10) noted that it was 100% safe to accept the stem cells for transplant and 100% efficacious at reversing systemic pain and decreased ambulation.

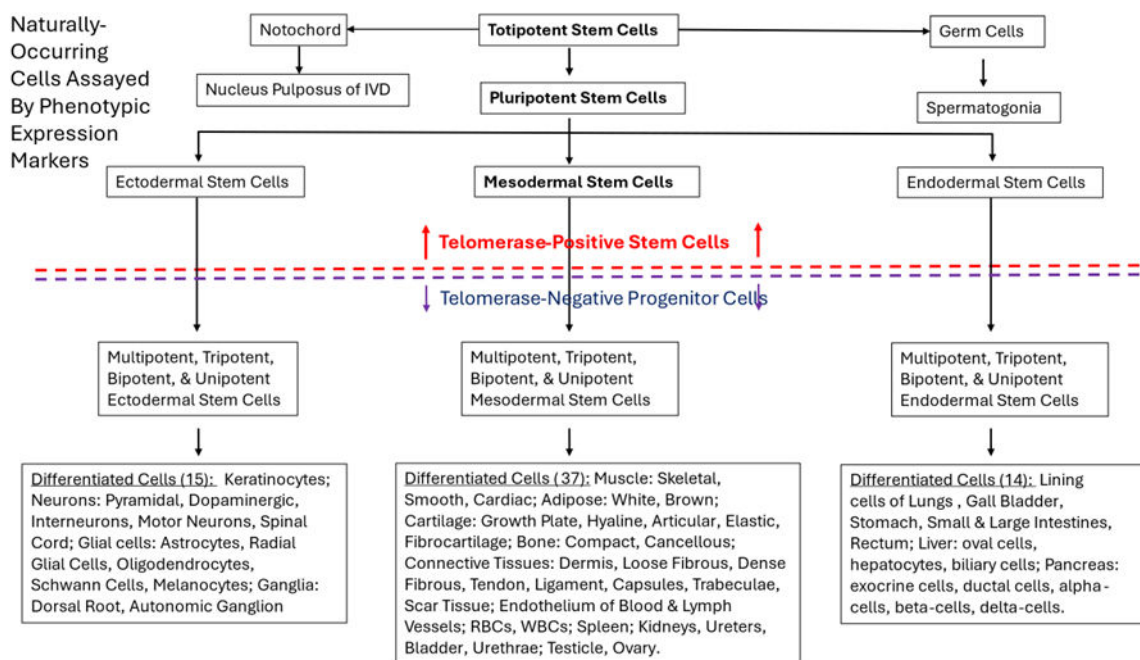


Figure 4: Unidirectional differentiation of primitive undifferentiated telomerase positive totipotent stem cells, through subsequent differentiation steps, i.e., telomerase positive pluripotent stem cells, telomerase positive ectodermal stem cells, telomerase positive mesodermal stem cells and telomerase positive endodermal stem cells, to lose the telomerase enzyme and become telomerase negative multipotent, tripotent, bipotent and unipotent progenitor cells, which then differentiate into terminally differentiated adult cells Reprinted with permission from Young HE, Speight MO. Characterization of endogenous telomerase-positive stem cells for regenerative medicine, a review. *Stem Cell Regen Med* 2020; 4(2):1-14.

Conclusion

The peripheral blood of horses, obtained by simple venipuncture, could be used as a source of aTPSCs for musculoskeletal injuries, avoiding the pain and morbidity often associated with the MSC being obtained from fat or bone marrow, to allow a better healing of the joint tissues.

Conflict of Interests

The authors have no conflict of interest to declare.

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