Ratan Kumar Choudhary
Shanti Choudhary *Editors*

Stem Cells in Veterinary Science

Springer

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Preface

Stem cells have high hopes for regenerative medicine in human health and treating animal diseases. In recent years, it has gained significant momentum. Stem cells are unspecialized cells with profound self-renewal capability and differentiation ability into cells with specialized functions. Therefore, stem cell therapies are used to repair the body's inability to regenerate damaged tissues after acute or chronic ailments. Among the various stem cell types, mesenchymal stem cells hold promising therapeutic applications in treating many diseases because of the simplicity of isolation from various tissue sources and the lack of ethical concern regarding their usage. With the paucity of universal stem cell markers and the high plasticity of stem cells, biomarker-based identification may not precisely quantify stem cells. Administering incorrect doses of stem cells often results in unsuccessful outcomes both in human and veterinary medicine. This book provides the best and most updated information to students, clinicians, young budding veterinary scientists, professionals, and researchers. Reaching out current information to the young mind is critical for scientific research and teaching. Merits of stem cell therapy for the animal disease are naïve but poorly understood; therefore, they have not been practiced widely. The importance of stem cell research should not be underestimated. This book reviews the principle, practices, and up-to-date knowledge of animal stem cells therapeutic applications in veterinary medicine. We hope that our efforts will provide readers updated information about the application of stem cells in regenerative medicine of the animal diseases and techniques of characterizing them. It may provide a reference book of stem cell applications in veterinary and animal sciences.

Ludhiana, Punjab, India — Ratan Kumar Choudhary
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2021

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Editors and Contributors

About the Editors

Ratan Kumar Choudhary is an Assistant Professor at the College of Animal Biotechnology, Guru Angad Dev Veterinary and Animal Sciences University, Ludhiana, Punjab, India. He holds a master's degree in veterinary sciences and a doctoral degree in animal science with a specialization in bovine mammary stem cells and their characterization. His research chiefly focuses on the development of stem cell therapy for the dry period and mastitis in dairy animals. He worked at the United States Department of Agriculture (USDA) for six years (2007–2012), followed by two post-doctoral trainings at the University of Kentucky (2012–2013) and the University of Vermont (2018–2020), USA. He received many awards and fellowships during his Ph.D. including the Dean's Fellowship, R. F. Davis Scholarship and Jacob K. Goldhaber Travel Award, Beltsville Agricultural Research Center, USDA poster awards, first prize winner of the National Milk Producers Federation, Graduate Student Paper Presentation Award in Dairy Production, and Novus International Inc. Travel Award. He is currently serving as a reviewer of the international journals. He has published more than 40 research articles in peer-reviewed international journals, attended/presented research papers at more than 30 conferences, and authored one book. He is a member of various international scientific societies and organizations, e.g., the American Dairy Science Association, the European Association for Animal Production (EAAP), the Indian Society for the Study of Reproduction and Fertility, and the Veterinary Council of India.

Shanti Choudhary served at the University of Vermont, Burlington, the USA, as a Lab Manager and Lab Safety Officer for more than a year. She has extensive research experience in many aspects of molecular biology, including mammary stem cell physiology. She worked at the USDA in Beltsville, USA, as a visiting scientist for more than two years, where her focus was on parasitic diseases in animals. She has also worked as a Research Associate at Guru Angad Dev Veterinary and Animal Sciences University. She received a National Post-doctoral Fellowship (N-PDF; SERB-DST, India) for a project on developing an in vitro

model for the characterization and manipulation of buffalo mammary stem cells. She has published more than 30 research articles in peer-reviewed international journals and is a member of several scientific societies, e.g., the American Society for Biochemistry and Molecular Biology and the American Dairy Science Association. Currently, she is engaged in teaching at the Students College of Animal Biotechnology, GADVASU, Ludhiana.

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Part I

Overview and Introduction

Overview of Stem Cells and Their Applications in Veterinary Medicine

1

Ratan K. Choudhary

Abstract

Stem cell therapy has high hopes, and several veterinary diseases may be treated using stem cells. Recent advances in stem cell isolation, characterization, and studies parallel in human medicine have advanced this field. Stem cells possess self-renewal ability and infinite capacity to divide and differentiated into functional cells. The therapeutic potential of either autologous or allogeneic transplantation—the regeneration potential of stem cells gained through immunomodulatory functions by secreting growth factors and many cytokines. Many studies demonstrated in various animal models that stem cell transplantation is beneficial, especially in chronic and problematic diseases. This chapter describes different types of animal stem cells, mechanisms of their action, and some of the diseases in which treatment has shown promising effects in curing the ailments. The application of mesenchymal stem cells, derived from variety of tissues, in diseases of livestock species and pet animals is noteworthy.

Keywords

Stem cells · Types · Application · Mechanism · Veterinary medicine

1.1 History of Stem Cells

Stem cells (SCs) are blank or non-specialized cells capable of differentiating into various types of cells of the body. Self-replication, the capacity to differentiate into several cell types, and unlimited proliferation are the specialized properties of a stem

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cell. The existence of stem cells was shown long back in 1959 when DeOme and his colleagues characterized hyperplastic nodular lesion of mouse mammary tumour virus (MMTV) in mouse mammary gland (DeOme et al. 1959). By that time, investigators had developed the technology of removing endogenously developing epithelial cells form fat pad (called "cleared pad") under the mammary gland of mice and the ability to develop epithelium from exogenously infused cells in the cleared pad. There were many other investigators during 1960s who discovered similar regeneration properties of specialized cells in spleen and other organs. Later, slow proliferating clonogenic precursor cells of hemopoietic tissue were discovered where the successive passage of initial colonies of precursors cells retained the capacity to form stromal tissue like that of their hemopoietic organs (Friedenstein et al. 1974). Discovery of the pluripotent embryonic stem-like cells from inner cell mass (ICM) (Martin 1981; Evans and Kaufman 1981), where cells could form descents of all the tissue layers of the adult, has set a new promises of stem cell research. In the year 2007, the discovery of induced pluripotent stem cells (iPSCs) independently by two scientists namely, Shinya Yamanaka from Kyoto University, Japan (Takahashi and Yamanaka 2006) and James Thomson of University of Wisconsin-Madison, USA (Yu et al. 2007) led the revolution of stem cell research for therapeutic applications.

Though, it is hard to pinpoint when, where, and who discovered the foundation work of stem cells but the first blood transfusion attempted soon after the discovery of blood circulation system in 1628 by William Harvey. Key properties of stem cells defined by McCulloch and till in 1960s in mice spleen appears to be the one of the earliest discoveries of blood forming cells, hematopoietic stem cells or HSCs (Becker et al. 1963). Thus, the foundation of stem cell research lies with adult stem cells (HSCs) but not with the embryonic stem cells. Finally, the discovery of iPSCs has fuelled tremendous impetus to the regenerative medicine.

1.2 Types of Stem Cells

Stem cells, based on the source, has been classified into three types: (1) embryonic stem cells (ESCs), (2) adult stem cells (ASCs), and (3) artificially induced pluripotent stem cells (iPSCs). Another classification based on potency and differentiation capacity includes (1) totipotent, (2) pluripotent, (3) multipotent, (4) unipotent, and (5) progeny of stem cells called progenitor cells (Fig. 1.1). Cells derived from inner cell mass (ICM) of blastocysts are the source of ESCs. Somatic or adult stem cells are the undifferentiated cells present in various organs after its development. The key role of ASCs is the healing, growth, and repair of tissue in the event of damage. Among the various types of ASCs, the name of these cells depends on the tissue from where cells have been derived. They are mesenchymal stem cells (MSCs) of bone marrow, neural stem cell (NSCs), hematopoietic stem cells, skin stem cells, mammary stem cells, and so on. ESCs and ASCs are obtained from the body, but iPSCs are created or manmade stem cells that are induced from differentiated cells by rewiring various pluripotency transcription factors (OCT4, SOX2, c-Myc, and KLF4) of the genome of differentiated cells to behave like embryonic stem cells.

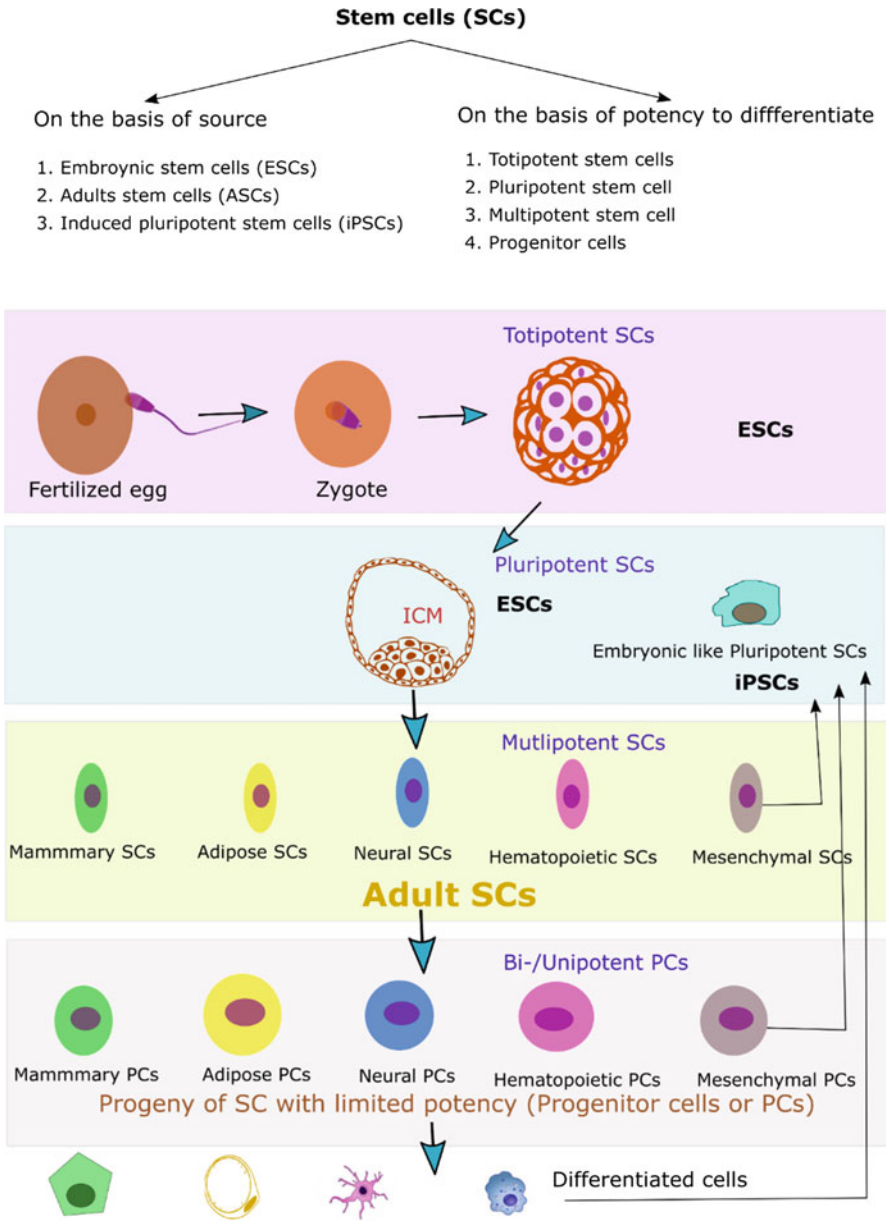

Fig. 1.1 Various types of stem cells and their differentiation

Capacity of totipotent stem cells includes the generation of embryonic (embryo proper) plus extra-embryonic tissues such as placenta. Cells of zygote and up to morula stage (solid balls of 16–32 celled stage) are totipotent. Cells derived from

inner cell mass (ICM) of blastocysts are pluripotent and are the source of ESCs. Pluripotent stem cells classified into naïve and primed ESC based on their in vitro growth characteristics and potency to give rise to all somatic cell linages. In mouse embryo proper, naive ESC are the cells' pre-implanted blastocysts whereas primed ESCs are of post-implanted blastocysts (Takahashi et al. 2018). In comparison to toti- and multi-potent stem cells, proliferative potential and differentiation ability of multi-potent stem cells are limited and produces only few varieties of cells. Generally, variety of cells are derived from closely related lineages. Adult stem cells (ASCs) are the good examples of multi-potent stem cells. Unipotent stem cells have very limited differentiation potential but have a unique repeated divisional capacity (Zakrzewski et al. 2019). Features of repeated divisional capacity make unipotent stem cell a good candidate for therapeutic application. Stem cell of skin that divides and produces dermatocytes is the good example of unipotent stem cell.

1.3 Stem Cells of Veterinary Importance

Embryonic Stem Cells Isolation of human ESCs in 1998 provided the first evidence of pluripotency and given rise to more than 200 cell types (Thomson 1998). Identification of pluripotency transcription factors namely OCT4, SOX2, NANOG, c-Myc, and KLF4 in ESCs provided the core internal circuit of pluripotency. Later, it was demonstrated how interplay between these pluripotency transcription factors determines differentiation of cells during transition phase in progenitor cell. For example, transcriptional circuit dynamic analysis showed OCT4 and SOX2 proteins maintain ESC identity by suppressing neural ectodermal differentiation while SOX2 promotes neural differentiation by suppressing mesodermal differentiation in progenitor cells (Thomson et al. 2011).

Use of ESCs in therapeutic applications is still controversial due to ethical concerns especially with human ESCs. In animals, in addition to ethical concern, the use of animal ESCs also involves religious issues. Therefore, the exploration of animal ESCs is yet to be explored fully. Moreover, lack of specific marker to identify ESCs in various animal species, the vulnerability of ESCs for teratoma formation (Solter 2006), and genomic instability of ESCs occurring during in vitro passaging (Tosca et al. 2015) caused to explore the alternate source of creating embryonic-like stem cells called induced pluripotent stem cells.

1.4 Adult Stem Cells

Mesenchymal Stem Cells (MSCs) MSCs are the alternative source of stem cells present in adult tissue. Though the origin of MSCs is not fully understood and hypothesized to derive from pericytes (Crisan et al. 2008), perivascular cells of microvasculature present in every organ and thus MSCs are harvested from bone marrow, adipose tissue, umbilical cords, dental pulp, and other organs including tonsils. MSCs are not only available in plenty but also lack ethical issues of tissue

harvest and least immunogenic upon autologous and heterologous application. MSCs are multipotent and give rise to bone, cartilage, tendon, ligaments, fat cells, skin, muscle, and connective tissues. Thus, the application of MSCs received the greatest attention in regenerative medicine. In companion animals, dogs, cats, and horse MSCs were harvested from (1) bone marrow, (2) adipose tissues, (3) placenta, (4) Wharton's jelly, (5) synovial fluids, (6) peripheral blood, (7) muscle and periosteum, (8) umbilical cord blood, and (9) muscle (reviewed in Voga et al. 2020). With the graded success of MSC therapy, various animals were depended on the source of stem cells and the species. Conventionally, MSCs are collected from either bone marrow or from adipose tissues. Cells from bone marrow are aspirated from femur of dogs and cats and from sternum of horse for the prospective isolation of MSCs (Marx et al. 2015). Bone marrow-derived MSCs are brought to the lab in special condition and proceed with either direct applications (after centrifugation to collect mononuclear cells) into the patients or culture for further purification and expansion and later applied to the patients. While the direct application of bone marrow-derived MSCs is not the pure population of MSCs but the mixtures of other cell types, growth factors and hormones and hence may provide quicker and better results. However, the culture and expansion of MSCs in the laboratory provides enriched population and abundant quantity for heterologous transplantations of cells into more patients. After fourth passages, MSCs are usually ready for clinical use and up to tenth passage cells can be harvested (Lee et al. 2014; Markoski 2016). In the later passage, MSCs loose in vivo characteristics and tends to differentiate, evidenced by the morphological characteristics and gene expression studies.

Applications of adipose-derived MSC for the treatment of inflammation of mammary gland, mastitis are noteworthy. Intra-mammary infusion of fetal adipose tissue-derived MSC into *S. aureus*-induced mastitis reduced somatic cell counts in bovine milk, indicating the possibility of allogenic transplantation of MSCs in the cow is safe for therapeutic application (Peralta et al. 2020). Fetal bovine MSC secretes antiproliferative factors and antibacterial peptides. In vitro studies indicated conditioned media of MSC decreased about 30% of bacterial population (Cahuascanco et al. 2019) and contained proangiogenic factors that supports neovascularization (Tao et al. 2016). One report on the application of adipose tissue-derived MSCs to treat goat mastitis indicated the efficacy of somatic tissue-derived stem cells in morpho-functional differentiation of cells into milk-producing epithelial cells (Costa et al. 2019). Such studies are indicating promising capabilities of adult stem cell's multipotentiality and immunomodulation properties as an alternative to the treatment of mastitis. Biotechnological and clinical potential of MSCs in livestock species and prospectus of its therapeutic applications has been described here (Hill et al. 2019).

Hematopoietic Stem Cells (HSCs) Similar to MSCs, HSCs are also available in plenty of amount from the bone mammary of developed (adult) tissue. Like that of MSCs, HSCs are multipotent stem cells which can give rise to different cells of immune systems, red and white blood corpuscles (RBCs and WBCs), and platelets.

Mammary Stem Cells (MaSCs) Application of MaSC for the use of treatment of mastitis is yet to be explored. Based on the work of secretome of MSC that enabled identification of healing processes by participating in inflammation, cell proliferation, remodelling of tissue repair and bacterial clearance (Krasnodembskaya et al. 2010; Cortés-Araya et al. 2018), MaSC-derived mammosphere has led the identification of factors and molecules that promoted angiogenesis, cell migration, and enhance cellular defence in the secretome (Ledet et al. 2018).

iPSCs The possibilities of use of iPSCs in veterinary science may bring great possibilities. In comparison to adult stem cells, use of iPSCs involves more safety challenges as all the induced cells are pluripotent and hence liable to uncontrolled division and tumour formation. Additionally, embryonic-like stem cells divide symmetrically (2 identical daughter cells) rather than asymmetrical (2 non-identical daughter cells, say one differentiated and the other stem cell). For the safety and tissue regeneration point of view, asymmetrical cells division is advantageous than the symmetrical division because it repairs the tissue while maintaining the stem cell pool. Moreover, upon receiving the differentiation signal, iPSCs tends to differentiate on all and none principle meaning all iPSCs will either terminally differentiated or none of them will differentiate. Adult stem cells have default asymmetrical divisional capacity in which they divide, differentiate, and maintain the stem cell pool in the tissues.

Remarkable works have been done on exploring the possibilities of ESCs and later iPSCs in pigs and other ungulates by Bhanu and his team. Difficulty in the identification of ICM (the source o ESCs) due to lack of ICM cell marker in ungulates hampered the research in those biomedically important species (Telugu et al. 2010). However, later the group successfully developed iPSCs from pig and other ungulates and showed capabilities of iPSCs to produce differentiated cells (Zhao et al. 2016). Potential of ESCs and iPSCs for committed progenitor cells and finally terminally differentiated cells for tissue or organ development is yet to come. In conclusions, a number of diseases can be treated with the applications of ESCs and ASCs (Fig. 1.2).

1.5 Mechanisms of Stem Cell Actions

Regeneration potentials of stem cells are achieved through re-epithelization, immunomodulations, remodelling of extracellular matrix and proangiogenic factors. Stem cells secrete growth factors, cytokines, growth regulators, anti-inflammatory factors, and some antibacterial peptides (Wangler et al. 2021). Initially, it was thought that stem cells, upon proliferation, provides (1) differentiated cells at the site of injection, or (2) migrate to distantly located tissue sites when injected into the circulation. Under the hypoxic condition, which is the microenvironment where stem cells maintain ‘stemness’ and the cell releases angiogenic growth factors like SDF-1, VEGF, FGF-2, angiopoietin-1, growth suppressor like TGF-alpha and

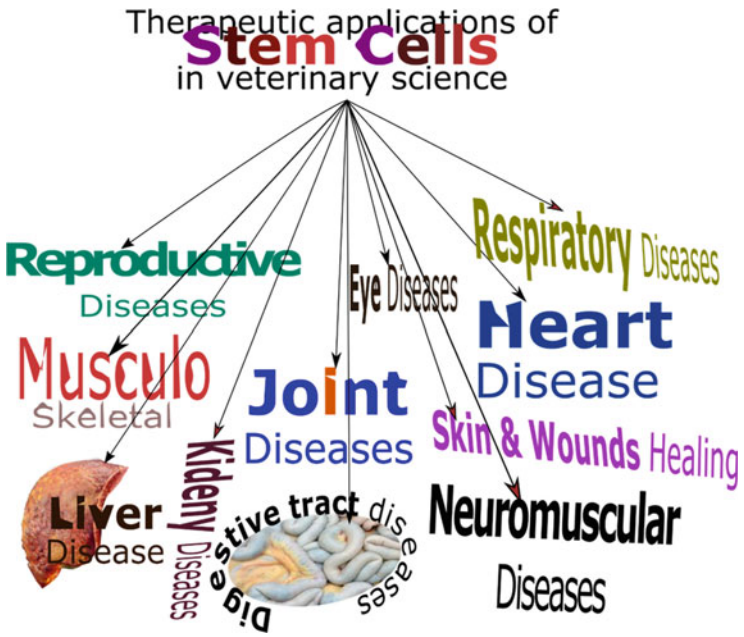

Fig. 1.2 Applications of stem cells for therapeutic purposes in various diseases of animals

TGF-beta and many cytokines like IL-1, IL-6, IL-10 (Spees et al. 2016) and even miRNAs loaded in extracellular vesicles (Asgarpour et al. 2020).

Transfer of Extracellular Vesicles (EVs) EVs include microvesicles, exosomes, and ectosomes that are generated either inward or outward budding of cellular plasma membrane. EVs are the lipid bilayer structures loaded with the cargos like proteins, fats, lipids, metabolites, nuclei acid (miRNA, mRNA), and other molecules has emerged as a significant cellular communicator (Raposo and Stoorvogel 2013). These EVs travel long distances inside the body and release from the healthy and diseased cells. Thus, stem cells also release EVs and transfer cargos to the other cell as a means of cellular communication. EVs was shown to increase the concentration of cytokine IL-10 (Park et al. 2019), IL-22, IL-23 (Hyvarinen et al. 2018), and TFG-beta (Crain et al. 2019). Communications between MSCs and cancer cells induces hybrid cell formation, indirectly evidenced by the presence of exosomes and micro-vesicles, which either promote or inhibit tumour (Melzer et al. 2018). Roles of EVs in stem cell biology has been discussed in detail elsewhere (Hur et al. 2020).

Paracrine Signalling Initial efforts to use stem cell therapy for the purpose of tissue regeneration was centred on the directed differentiation of these cells to the intended cell types. More recently, however, it is becoming apparent stem cells secrete factors which mediate action via paracrine fashion, rather than stem cell differentiation and repopulation. Paracrine mode of signalling is the cell-to-cell

communication method where, signalling molecule is released by one cell and the changes are induced in neighbouring cells. A resultant shift in research and the emergence of studies aiming to elucidate the paracrine mechanisms of stem cell action underlies tissue repair and regeneration mechanisms. Release of TGF-beta causes cell proliferation, differentiation, wound healing and angiogenesis. Appropriate regulation of TGFβ by the osteocytes in the tissue homeostasis has been emphasized for the management of bone diseases (Xu et al. 2018). This was based on the hypothesis that the eroded bone matrix of diseased patient releases TGFβ that has mitogenic and pro-survival activities, thus contributing to tumorigenesis (Portela et al. 2014). In addition, cardiac levels of IL1β and TNFα, factors implicated in angiogenesis, following bone marrow-MSC transplantation into ischemic myocardium showed optimal therapeuptic potential of stem cell (Kamihata et al. 2001).

Immunomodulation There are number of evidences showed that MSCs interact with innate and adaptive immune cells and bring immunomodulatory activities. MSCs inhibited CD^{4+} Th1 and Th2 cells, CD^{8+} T cells and NK cells proliferation and functions. Inhibition of T-cell was mediated by soluble cytokines and factors including TGFβ and human growth factors (Zhao et al. 2016). By understanding mechanisms by which MSCs modulate inflammation and contribute to healing will benefit the investigation of cellular therapy of animals. Animal models like pigs for cardiovascular diseases (Murphy et al. 2003) and goats for osteoarthritis (Shake et al. 2002) are currently used in understanding human diseases. It is a well-known fact that MSCs are hypoallergic due to low expression of HLA class I and no expression of HLA class II molecules and hence devoid of allogenic tissue rejection (Ryan et al. 2005). In addition to the release of soluble cytokines by MSCs, apoptotic and metabolically inactive MSCs also possess immunomodulation properties (Song et al. 2020), indicating cytokine independent mechanism. Apoptotic tissues-derived MSCs reduced rat mortality after sepsis induction, resulting in significantly low levels of circulating TNFα and T-cells (Chang et al. 2012). The current status of cellular and molecular mechanisms of MSC-mediated immunomodulation by live MSCs, dead MSCs and apoptotic MSCs has been discussed in the literature (Weiss and Dahlke 2019).

Mitochondrial Transfer Dysfunction of mitochondria has been associated with ageing and certain diseases like Alzheimer's disease, Parkison's disease, and type 2 diabetes. Mitochondrial dysfunction contributes to reactive oxygen species (ROS) production which ultimately disturbs homeostatic mechanism. Apart from the therapeutics mechanisms of stem cells, directly by cell differentiation, or indirectly *via* paracrine signalling, EVs, and immunomodulation, stem cells have also been shown to transfer mitochondria to the diseased cells. Mitochondrial transfer mediated in bone (Guo et al. 2020) and renal proximal tubular epithelial cells (Konari et al. 2019) enhanced cell proliferation, migration, and cellular differentiation in the tissue. Co-culture experiments of PC12 cells (neuronal cell line) with bone marrow-derived MSCs showed protective effects of neuronal cells under ischemic condition (Sarmah et al. 2020). Increasing evidences suggest that stem cells can directly donate

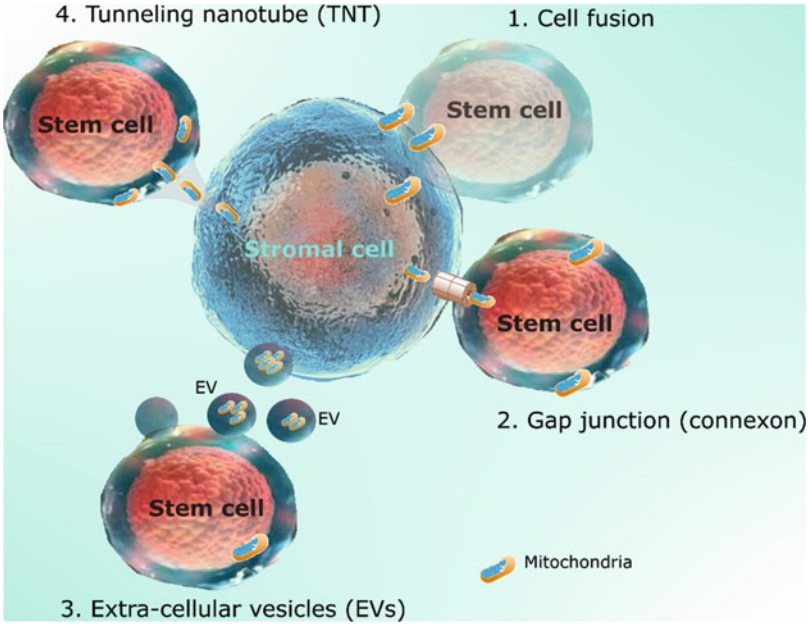

Fig. 1.3 Patterns of mitochondrial transfer from stem cell to stromal cell or diseased cell. These mechanisms include, (1) fusion of stem cell with the stromal cell, (2) transfer of mitochondria through gap junction (connexons, a transmembrane tunnel protein), (3) via extracellular vesicles (EVs) and (4) intracellular tunnelling nanotube (TNT) formation. Formation of TNT is regulated by TNFα/NFKβ and TNFαip2 signalling pathway (Jiang et al. 2016)

mitochondria in order to recover from cell injury, thus cells rescue mitochondrial damage-provoked tissue degeneration (Li et al. 2019). Mitochondrial transfer by stem cells thus could represent an emerging therapeutic approach for tissue regeneration of treating animal diseases. Various possible patterns of mitochondrial transfer from stem cell to somatic or diseased cells are presented (Fig. 1.3).

Homing Mechanisms of Stem Cells Homing is the process where stem cells migrate to specific tissue sites in response to chemoattractant gradients, released by the damaged tissue. Chemotactic factors like cytokines released from the damaged tissue. Understanding homing of stem cells is essential to enhance and refine its clinical significance in therapeutic settings. Chemo-attractive signals are perceived by the receptors present on the surface of stem cells. For example, stromal cell-derived factor 1 (SDF-1) and osteopontin (OPN) are the chemokine released by the damaged tissue sends chemoattractive signals to the cells expressing CXCR4 (Wynn et al. 2004) and integrin-β1 (Chen et al. 2014), respectively. There are many other factors like hepatocyte growth factor, insulin-like growth factor, TGF-beta1, dimers of vascular cell adhesion molecule 1 and very late antigen 4 (VCAM 1-CLA 4), matrix metalloproteinases 2 (MMP-2) and tissue inhibitor of metalloproteinases 3 (TIMP-1), PDGFR, and others (Becker et al. 1963).

Homing of stem cells depends on the route of administration of the cell. Local injection of stem cells is preferred but an invasive method like intraperitoneal injection. Intravenous injection of stem cells is the least invasive but suffers with many limitations. The limitations of intravenous injections of stem cells are (Voga et al. 2020): (1) stem cells have to exit circulation in order to reach the site of injury, (2) cells are squeezed in dimension to the size of narrowest capillary, particularly in lungs where the average diameter of capillary is 14 μm in comparison to the cell's diameter (25–30 μm), (3) high expression of integrin-beta 1 by lung epithelium attracts more number MSCs which blocks the availability of MSCs by cell entrapment, (4) systemic injection of stem cells may require high dose of stem cells to have a sufficient number of stem cells reaching to the site of injury, and (5) circulation of stem cells in the blood for a longer period (more than 24 h) may not provide viable MSCs to the damaged tissue. However, long-term effects of MSCs have been reported which likely to be mediated through non-viable and apoptotic MSCs.

1.6 Clinical Applications of Stem Cells in Regenerative Veterinary Medicine

Worldwide, investigators and veterinary clinicians are working on applications of stem cell for therapeutic potential in autologous and allogenic fashions either freshly isolated or cultured and expanded stem cell in the laboratory for the treatment of various diseases of animals. This indicates that the stem cell therapy in veterinary science is a reality. Existing literatures support that the stem cell transplantation is safe and beneficial to the animal health.

Diseases of Reproductive System Reproductive diseases in animals causes great loss to farmer's profit. Inflammation of uterus (endometritis), poorly developed ovarian follicles in female, and testicular degenerative disease in males are some of the reproductive disorders. In mares, endometritis is the main cause of reproductive failure and an incurable disease. Application of bone marrow-derived MSC did not restore ovarian functions in aged mares (Grady et al. 2019). However, in human MSCs therapy appears to restore ovarian functions (Yoon 2019). One may argue that the failure of MSC therapy in mare could be (1) due to old age of mare as fertility is naturally reduced in aged animals, (2) source of stem cells as MSCs can be derived from various tissue namely adipose, bone marrow, umbilical cord, menstrual blood in human, amniotic fluid, and (3) insufficient dose of MSCs. Study showed that, in human, multiple doses of MSCs ($1–5 \times 10^6$ cells/kg body weight) improved ovarian functions in primary ovarian insufficiency (Gadkari et al. 2014), whereas in mice 1×10^6 to 5×10^8 cells (Kalhori et al. 2018) were used for clinical applications. Mechanisms of stem cell action in restoring ovarian functions have been shown to help in folliculogenesis, prevention of granulosa cell apoptosis, regulation of reproductive hormones and vasculogenesis.

Stem cells treatment could offer solutions to infertility related disease. Testicular degeneration in dogs often leads to the production of defective sperms and affects fertility. Viability and fertility of frozen-thawed canine sperm were improved using adipose tissue-derived MSCs (Qamar et al. 2020) and exosomes derived from conditioned media of MSCs (Qamar et al. 2019). These results showed that like the availability of frozen semen from cattle, we may use freeze pure breed canine sperm for artificial insemination of female dogs. It is well known that during cryopreservation, cellular injury to organelles like plasma membrane, mitochondria, and nucleus occurs that affects post-thaw sperm motility and hence impaired fertility. The possible mechanisms of improved fertility of canine were due to the secretion of proteins like annexin, dysferlin, and fibronectin by MSCs (Qamar et al. 2020). These proteins are involved in the repairing process at a cellular level at many ways like prevention of cells from apoptosis and improvement of membrane integrity. Other possible mechanisms of restoring canine fertility are also possible like regulating glucose metabolism of sperm (Hsiao et al. 2019) that provides more energy to the cell for enhanced motility.

Musculoskeletal Disease Treatment of horse musculoskeletal disease using MSCs has been fairy a good success. Many reports with varying successful results have been reported since more than a decade (Wilke et al. 2007; Broeckx et al. 2014, 2019). Intra-articular administration of adipose tissue-derived MSCs in horse resulted no lameness after 3 months of treatment (Nicpon et al. 2013). Properties, cultural characteristic, and potential applications of MSCs in the management of equine diseases have been described (Gugjoo et al. 2019a, b). Ready-to-use stem cell containing products (like Arti-Cell Forte from Boehringer Ingehlheim and Investigational Veterinary Product or IVP from Dechra Limited, UK) are also available for the clinically applications in treating horse's degenerative disease.

Autologous stem cell therapy using adipose tissue-derived MSCs treated chronic osteoarthritis in canine (Black et al. 2007). MSCs derived from umbilical cord (UMSCs) have also shown promising results in treating osteoarthritis in dogs. No adverse reactions were reported in using UMSCs (Kim et al. 2019). Interestingly, allogenic transplantation of MSCs appears to be safe in dogs for osteoarthritis (Shah et al. 2018). In vivo studies summarizing the clinical effects of MSCs in canine osteoarthritis are given in Table 1.1. Majority of the results of stem cell applications for canine disease therapy are positive; however, investigators consider the results inconclusive (Gugjoo et al. 2019a, b), suggesting more basic research are needed to understand the biology of MSCs or other stem cell types for positive and conclusive results.

Liver Diseases Liver diseases (acute and chronic) are common in canines. Viral or bacterial infection, cancer, trauma, ingestion of toxic substances, and endocrine disturbances can cause liver disease in dogs. Many studies have tried the administration of autologous and allogeneic MSCs treatment with varied success. Mechanisms by which MSCs repair liver tissue is the generation of different cell

Table 1.1 Various studies and their results of canine mesenchymal stem cell therapy for osteoarthritis (table adapted from Sasaki et al. 2019)

Source	Injection/trans-plantation	Combination use	Cell number (million)	Model	Evaluation method
Bone marrow	Single intraarticular, autologous	–	7–8	Partial thickness articular cartilage defect	Histology
Adipose tissue	Single intraarticular, autologous	–	7–8	Partial thickness articular cartilage defect	Morphology, histology, fluorescence analysis
Adipose tissue	Four times intraarticular, allogeneic	–	5 (three times), 66 (once)	Intact	Pain and lameness scoring, immunohistochemistry
Bone marrow	Transplantation with scaffold, allogeneic	Scaffold	0.01	Full-thickness cartilage defect	Histology, immunohistochemistry, micro-CT
Synovium	Single intraarticular, autologous	Hyaluronic acid	0.05, 5 or 50	Partial thickness articular cartilage defect	Histology
Adipose tissue	Single intraarticular, allogeneic	Platelet-rich plasma	10	Cranial cruciate ligament transection	Lameness scoring, focal compression strength, extracellular matrix composition, histopathology, real-time PCR
Bone marrow	Single intraarticular, allogeneic	Hyaluronic acid	10	Partial thickness articular cartilage defect	Gross appearance, magnetic resonance imaging, histology, immunohistochemistry
Umbilical cord	Single intraarticular, allogeneic	–	1	Surgical manipulation of articular cartilage	Magnetic resonance imaging, radiography, ultrasonography, blood test, scanning electron microscope

types of the liver (hepatocytes, Kuffer cells, and hepatic stellate cells) and the secretion of various cytokines that modulates the immune system, apoptosis, and vasculogenesis. In canines, the liver fibrosis model to establish with autologous transplantation of bone-marrow-derived MSCs and documented improved liver fibrosis without adverse effect (Matsuda et al. 2017). However, caution should be kept in mind that prolongs immunomodulation and anti-inflammatory conditions to treat liver fibrosis may have adverse effects and thus, alternative strategies must be taken to improve outcomes of stem cell therapy for prolong use in treating chronic conditions. Strategies to improve liver stem cell success may include modification of stem cell niche, pretreatment or co-treatment of the recipient and gene modifications (Hu et al. 2019). Intravenous injection of adipose tissue-derived MSCs experimentally induced liver injury and caused restoration of liver function (Teshima et al. 2017), indicating the possibility of stem cell therapy for hepatic disease in dogs.

Kidney Diseases Chronic diseases of kidney is one of the common issues of old aged cats and dogs. The disease is manifested by fibrosis, nephritis, and tubular atopy. Currently, only the renal transplantation is the therapeutic solution to the chronic kidney disease (Voga et al. 2020). Very few studies conducted the same investigator (Quimby et al. 2016; Quimby and Dow 2015) and reported various strategies to look critically for MSCs-based therapy for the feline chronic kidney diseases. More research is required to know about the best source of MSCs (bone-marrow vs adipose tissue-derived cells), route of cell administration, dose of cells, stages of MSCs including the impact of age/health/status of tissue donor animals (SAGE 2018).

Joint Diseases To study the effects of stem cells therapy in bovine, caprine could be the alternative model animal. Bovines are heavy and especially grazing animals have locomotor issues. One such study aiming to test the efficacy of autologous MSCs for superior cartilage repair, bone marrow harvested cells were injected intra-articularly and demonstrated hyaline like cartilage regeneration in goats (Nam et al. 2013), suggesting potential applicability of stem cell therapy in livestock animals. In horse, bone spavin is a degenerative joint disease of horse and cattle evidence by bony outgrowth within the lower hock joint caused by osteoarthritis. Result of a study conducted in horses with intraarticular autologous transplantation of adipose tissue-derived MSCs suggested positive and long-lasting effect of stem cell therapy and had no signs of lameness after 180 days of the treatment (Nicpon et al. 2013), indicating long-lasting effects of stem cell therapy. There are many other studies in pet animals showing improved and promising results of stem cell treatment in joint diseases.

Digestive Tract Diseases Application of stem cell therapy has gained importance in treating inflammatory conditions of the digestive disorders especially inflammatory bowel diseases (IBD). In canine, a single dose (at two million/kg body weight) of adipose tissue-derived MSCs has reduced gastrointestinal inflammation (Pérez-Merino et al. 2015). Not much work has been done on this aspect in other animals.

Robust stem cell isolation and in vitro propagations protocol, characterization of different subsets of stem cells for various diseases of digestive system and understanding of digestive disorders are the keys to establish stem cell therapy.

Eye Diseases Dry eye disease or keratoconjunctivitis sicca (KCS) is one of the prevalent eye diseases affecting up 35% of human and 4–20% of dogs. The mechanisms of the development of the disease are not really understood. However, the most common cause of KCS immune-mediated inflammatory response targeted against the production of tear by affecting lachrymal glands. It causes discomforts, visual disturbances, and damage to the ocular surface. Treatment of KCS is difficult and effective and safe treatment of this disease is lacking (Villatoro et al. 2015). However, in 2016 Bittencourt et al. reported that even a single dose of allogenic administration of MSCs in canine has positive effects on KCS over the long period (up to a year).

Heart Diseases Cardiac diseases in animal include myocardial infarction, dilated cardiomyopathy, degenerative valvular disease, congestive heart failure, and other heart problems. Research has indicated the ability of cardiomyocytes (cells of the heart) to regenerate after myocardial injury, indicating the possibility of therapeutic interventions. In an interesting study, one study claims for improved heart functions in dogs suffered with chronic valvular disease when administered with puppy's deciduous teeth stem cells (Petchdee and Sompeewond 2016). In a study conducted on Dobermans with retrograde coronary venous, allogeneic administration of adipose tissue-derived MSCs did not increase days of survival but the method of stem cell treatment was safe (Pogue et al. 2013). In addition to intracoronary infusion and intramyocardial injection of stem cells, retrograde coronary venous infusion (RCVI) is one of the methods of stem cell administration aimed for improved cardiac functions (Gathier et al. 2018). A combination of two stem cells—mesenchymal (MSCs) and cardiac stem cells (CSC)—administered intra-cardially to immunosuppressed swine (model animal for human research) showed (Quijada et al. 2015) twofold reduction in scar tissue formation improved left ventricular compliance and contractibility in comparison to placebo animals.

Skin and Wound Healing Skin wound healing is a complex process and well-orchestration of inflammation, matrix formation and tissue remodelling. Cell therapies bring combined actions of immune modulation, growth factors, angiogenesis, extracellular matrix production, and cell differentiation (Giles et al. 2015). Chronic wounds are difficult to heal, as they are associated with other underlying diseases. Generally, in a normal wound healing process, skin infection is prevented and tissue integrity and functions are restored. Faster rate of healing to prevent infection, but compromised healing process resulting in scar formation. Cell-based therapy has actively been used for the treatment of dermal wounds in dogs. Multipotent stem cells and progenitor cells have high proliferative potential, ability to differentiate and secrete different types of growth factors and cytokines that improves wound healing. Cells like endothelial progenitor cells, bone marrow-

derived mesenchymal stem cells (BM-MSCs), and adipose-derived stem cells (ASCs) are being used for cellular therapy. Interestingly, allogenic transplantation of ASCs in canine is possible and results in improved wound healing (Enciso et al. 2020). In a recent study conducted using BM-MSCs, both fresh and frozen cells were capable of promoting wound healing (Bharti et al. 2020), suggesting stem cells have promising potential to treat the cutaneous wound in clinical cases.

Mastitis Economic loss due to mastitis is huge. In the United States alone, mastitis causes approximately $2 billion dollars annual loss to the dairy industry (Donovan et al. 2005). The current treatment of mastitis is to use broad-spectrum antibiotics to control bacterial infection but do not address the tissue regeneration. In vitro study suggests antibiotics induce hypoxia inducing factor 1 alpha (HIF1A) and causes oxidative damage (Elliott and Jiang 2019) that may even slow down the growth of mammary epithelial cells and tissue regeneration. Although antibiotic is the best available method to control mastitis, but has been associated with antibiotic resistance and milk residue. Recent study showed the potential application of stem cells in treating bovine mastitis. *S. aureus* induced mastitis in Holstein Friesian cows treated intramammarily with antibiotics (control group: days 4 and 5) or a suspension of adipose tissue-derived stem cells (2.5×10^7 cells: day 4 and 5) showed reduced colony formation unit (CFU/ml of milk) of bacterial in stem cells quarters (Peralta et al. 2020). Interestedly inoculation of repeated doses of allogenic stem cells was safe to use in healthy cows demonstrating possible cell-based therapy for mastitis treatment. In another study, conditioned DPBS from amniotic membrane stem cells injected into the induced mastitis mammary gland of cow showed improved milk quality of milk of the treated cow in comparison to the antibiotic-treated cow. Upon injection of conditioned media into the teat canal, pH value and titratable acidity of milk were significantly different in the experimental group (Ting et al. 2020), indicating another possibility of using AMSC-derived secretome as an alternative therapy in replacing antibiotics in treating bovine mastitis.

Neuromuscular Diseases Spinal cord injury due to trauma is one of the common injuries in dogs. Sometimes, naturally occurring intervertebral disc disease in larger breeds of dogs like German Shepherd has also been observed whose conventional treatment of the disease has very limited success. Injury to the spinal cord is because neuromuscular disease is manifested by the altered gait, pain, and sometimes permanent locomotor disability. Autologous and allogenic bone marrow MSCs therapy alone or in combination with conventional therapy showed some locomotor recovery in dogs. However, naturally occurring degenerative disc disease in German Shepherd dogs did not show positive clinical outcome after 1, 6, and 12 months of MSCs treatment (Steffen et al. 2017).

1.7 Conclusions

With advancement in understanding biology of stem cells and their applications in animal diseases and trail results, it seems possible to use healing properties, immunomodulatory activities, and low immunogenicity of adult stem cells, in particular mesenchymal stem cells, for therapeutic applications in various diseases of livestock species and pet animals.

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