The Role of Exosomes in Regenerative Medicine

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1.0 INTRODUCTION

Regenerative therapy (and those who practice in this space) is a microcosm of medicine focused on recapitulating the conditions of our youth in order to restore, rather than repair, morphological and physiological aberrancies as they appear. As a medical society, it is far more attractive to offer our patients the hope of regeneration in order to afford them the ability to restore a younger version of themselves rather than just repairing their injuries. Whether rehabilitating a disc, a joint, a nerve or the entire individual the dream has always been to live longer and better.

Mesenchymal Stem Cells (MSCs), named by Arnold Caplan nearly 30 years ago, were originally termed MSCs secondary to their innate ability to replicate while maintaining the attribute of multi-potential lineage. In short, these simple cells maintain the ability to form numerous types of mesodermal tissues (1). Their capacity to form bone, cartilage, fat, skin and other tissues in vitro led to the fallacious belief that they could be transplanted and would engraft in vivo to form these same elements.

Over the past few decades, we have seen thousands of clinics throughout the US, set up shop, with the hope of utilizing these same cells in directed protocols to tackle a myriad of medical conditions ranging from aesthetics to neurological diseases. Zealots of bone marrow, fat and allogeneic cells from umbilical cord and Wharton's jelly, continue to argue over the benefits of their respective cell-based tissue products (Table 1). But what is the mechanism of action?

Table 1. *Stem cell-based choices in regenerative medicine.*

Autologous	**Age**	**Dose**	**Immune Tolerance**	**Ease of Use**
SVF/Fat	Advanced	Limited	Tolerant	Difficult
Bone Marrow	Advanced	Limited	Tolerant	Difficult
Allogenic (Cellular)				
Umbilical Cord	Young	Limited	Incomplete	Easy
Wharton's Jelly	Young	Limited	Incomplete	Easy
Allogenic (Acellular)				
MSC Exosomes	Young	Large	Tolerant	Easy

Dr. Caplan has released a paper, "Mesenchymal stem cells; time to change the name!" outlining the research of many other prominent scientists. In this treatise, Dr. Caplan eloquently points to these cells as "medicinal signaling cells", capable of releasing paracrine effectors which thereby influence the body via immunomodulatory and trophic mechanisms (2). These bioactive factors essentially upregulate resident stem cells, which reside throughout all the tissues of our bodies, and affect the phenotypic and physiological expression of our immune system. So if these bioactive factors are what will be the honey, then why is so much attention paid to the bees? There are a few simple and some not so simple answers tied to this question. The first and most important answer lies in the fact that the old textbooks remain on the shelves and those who have read them continue to preach their teachings. Equally important is the fact that once we are trained as physicians we hold our initial teachings as dictum. Additionally, most of us reading this book are interventional pain physicians. We enjoy doing interventional cases. Bone marrow aspirations and lipoaspirations are within the scopes of our training and are part of our armamentarium. Finally,

and not to be minimized is the fact that alternative therapeutic options have only recently become available on the marketplace.

2.0 WHAT ARE EXTRACELLULAR VESICLES ?

So what are exosomes and microvesicles? As paracrine effectors, they have a role in signaling by transferring their contents from one cell to another. We have discussed cells, specifically mesenchymal stem cells. Once we are born, all of our MSCs are termed adult. As adult MSCs these cells are too far down the cell cycle to directly transdifferentiate into other cell types. Only fetal or embryonal stem cells along with induced pluripotent stem cells retain the level of stemness to form different tissues. Currently in the US, ethical and moral considerations "along with cancer risks" preclude the use of these cells in clinical medicine. Adult MSCs cause changes via paracrine messengers (2). These messengers are termed exosomes and microvesicles, collectively known as extracellular vesicles (EVs).

Exosomes, the smaller of these two vesicles, measure 40 to 100 nm and are lipid membrane packets formed by a two-step budding process. Formed by inward budding of membranous vesicles in a multi-vesicular body, they fuse with the plasma membrane to release these ultra-tiny vesicles. Microvesicles refer to somewhat larger packets of a few hundred nanometers formed by budding directly from the plasma membrane. Both exosomes and microvesicles contain transmembrane proteins from their parent cells, which are important in regulating uptake by other cells (3). By conserving these transmembrane proteins it has been shown that uptake is facilitated by other cells to a much greater degree than if the cargo was simply released into the extracellular environment. Exosomes and microvesicles are not exclusive to stem cells and are released by many cells throughout the body. Immune cells, cancer cells and aging cells all secrete different vesicles which contain vastly different cargos of information. This information includes messenger RNAs, micro RNAs, and various proteins. The intrinsic durability of the extracellular vesicle membranes makes them uniquely durable and naturally biocompatible. Additionally, the wide spectrum of

proteins and messenger RNA contained within these EVs allows for a vastly greater capacity of information compared with single molecule messengers like hormones , growth factors and cytokines. Finally, the transmembrane protein receptors allow EVs to traffic or home to areas of injury and inflammation while facilitating uptake by numerous cells (4).

EVs are important in autocrine signaling (local between same cells), paracrine signaling (local between different cells) and endocrine signaling (between distant cells). EVs have been found in all bodily fluids. EV cargos are specific to each type of cell, while cells grown in different environments will also modify their production of EV contents. Commercially, at present, research grade purified EV solutions are only available from placental tissues whose MSCs secrete a cargo rich in growth and immunomodulatory substances. Of course, any resident stem cells who traffic to the areas of concern, will then secrete EVs specific to themselves and will be modified by their own local extracellular microenvironment. Much of the difficulty in bringing purified EVs to market is related to the scalability and standardization of the product. Similarly, concentrating the product to a physiologic level necessary to effect change has also proven to be a large obstacle for many companies and continues to be a significant barrier of entry into the marketplace. Variances of 0.1 to 2 mg of exosomes are isolated from cell numbers of up to 60 million MSCs (4). The current commercial product quotes 15 mg per standard five mL vial and also produces a higher concentration product containing 8 mg in a one mL dosing. A handful of companies are currently developing exosome products – some very specialized – and no doubt others will soon join the race. The Bioinformant, in their commercial publication, *The Market for Stem Cell Exosomes* lists multiple companies pursuing production of EVs (5, 6).

3.0 BENEFITS OF EXTRACELLULAR VESICLES

Similar to MSCs, EVs as secretory products have been shown to travel via local diffusion; deliver proteins, micro RNA and messenger RNA; and home (7, 8, 9). Unlike MSCs, EVs demonstrate a number of advantages distinct from their parent cells. They can travel systemically without the risk of clumping (as is seen with large peripheral intravascular doses of MSCs). As much smaller particles, EVs do not demonstrate a first pass effect into the lungs when administered intravascularly. EVs can cross the blood-brain barrier easily without utilizing mannitol (3). While allogeneic MSCs may be perceived as foreign by the innate and adaptive immune system and quickly whisked away, EVs are able to evade the immune response. EVs from healthy stromal cells do not contain DNA, so that there is no risk of malignant transformation. Alternatively, autologous MSCs are of the same age as the donor patient and are therefore limited by the inherent age of the individual. Older cells are less robust in the production of growth factors, micro RNA and messenger RNA and are frequently limited in total number. Finally, stem cell harvesting requires time and expertise whereas EVs provide an out of the freezer solution via easy storage, administration, and controllable dosing.

4.0 KEY THERAPEUTIC EFFECTS OF MSC EVS

Microvesicles and exosomes of mesenchymal stem cells provide very attractive therapeutic benefits. At the very core of these are their trophic (regenerative) and immunomodulatory capabilities which dictate their indications. A discussion of the trophic effects of EVs requires an understanding of the resident stem cells they act upon. Tissue-resident stem cells lie quiescently within niches throughout our bodies. This population of cells are partially undifferentiated and once activated can proliferate and migrate to sites of injury where they acquire a mature phenotype in order to facilitate repair and remodeling. The balance of progenitor cell quiescence and activation is a hallmark of a functional niche and is regulated by internal and external signals. Known niches are seen in the central nervous system, skeletal muscles, liver, skin, kidney, heart, lung, and joints (10, 11). In the joints alone, a myriad of cells have been described and include: chondrocyte progenitor cells (CPCs), cartilage-derived stem/progenitor cells (CSPCs), synovium resident multipotent progenitor cells, osteoblast/osteoclast resident MSCs within the subchondral bone, and chondrogenic cells within the infrapatellar fat pad (12).

Many of the immunomodulatory effects of MSC EVs are related to the influences upon phenotypic expression of certain cells. Macrophages and microglia (macrophages of the central nervous system) demonstrate two distinct appearances. M1 macrophages are pro-inflammatory and secrete inflammatory cytokines whereas M2 macrophages secrete an anti-inflammatory milieu regarding their secretions. MSC EVs were shown to influence the conversion of M1 macrophages into M2 macrophages (13, 14, 15, 16). Similarly, T-cells are described as predominantly T Helper (T_H) or T Regulatory (T_{Reg}) cells with T_H cells further subdivided into T_H1 cells which stimulate cytotoxic T cells and T_H2 cells which stimulate B-cells. T_H1 cells are markedly more inflammatory than T_H2 cells. T_{Reg} cells also downregulate inflammation. MSC EVs are also known to convert

T_H1 to T_H2 cells and increase the amount of T_{Reg} cells (13, 14). These phenotypic changes along with the internal production of significant anti-inflammatory cytokines like IL-10, TGF-ß3, TIMP, TNFαRA and IL-1RA provide for the immunomodulatory and anti-inflammatory effects seen after MSC EV administration.

Many of the anti-fibrotic benefits of MSC EVs are attributable to several factors. They produce large amounts of TGFß3 which regulates cell adhesion and extracellular matrix formation. In scar repair they increased the ratio of Collagen Type III to Type I. Additionally, MSC EVs displayed inhibition of granulation tissue leading to fine reticular collagen with fewer fibroblasts (17). Finally, MSC exosomes prevent apoptosis (cell death) through numerous techniques, including the promotion of redox homeostasis and appropriate autophagy/mitophagy.

During inflammatory and ischemic conditions (e.g., cerebrovascular accident, myocardial infarction) cells lose ATP/NADH, experience oxidative stress (e.g., increased production of reactive oxygen and nitrogen species [ROS/RNS]) and subsequently die. Assays have shown that MSC exosomes contain all five enzymes in the ATP-generating stage of glycolysis: GAPDH, PGK, PGM, ENO, and PKM2 (18).

This anti-apoptotic effect is perhaps the most important beneficial effect of MSC EVs because it relates to the powerhouse of the cell – the mitochondria. Mitochondrial dysfunction with subsequent death is a leading cause of endothelial injury and a cytoprotective effect here could lead to eventual decreases in cardiovascular disease, stroke, and myocardial infarction. By improving mitochondrial fitness, restoring a normal morphology, and removing damaged mitochondria appropriately (mitophagy), MSC EVs are able to ameliorate the effects of oxidative stress imposed by severe inflammation and ischemia (19).

5.0 BIODISTRIBUTION AND TARGETING OF EXTRACELLULAR VESICLES

Few studies have evaluated the biodistribution of extracellular vesicles in murine models. A short biodistribution phase is followed by a longer elimination phase (20). Route of administration has been shown to influence EV biodistribution. Intravenously delivered EVs show rapid uptake by macrophages of the mononuclear phagocyte system, accumulating predominantly within the liver, spleen, and lungs. In the liver, significant uptake is accomplished by Kupffer cells, while alveolar macrophages predominate in the lungs. High splenic levels are attributed to circulating lymphocytes and macrophages which bind EVs and then traffic there. Most EVs are subsequently eliminated from organs and biofluids within 360 minutes indicating active uptake and degradation by different cell type with some elimination via hepatic and renal processing (21).

The route of administration certainly influences EV biodistribution. Comparing intraperitoneal (IP), subcutaneous (SQ), and intravenous (IV) routes of delivery - certain salient differences are worth noting as they may play a role in establishing tailored protocols. In murine studies, EV administration by IV had the highest accumulations in the liver and spleen when compared to IP and SQ routes. The pancreas had a higher portion of accumulated EVs via IP followed closely by SQ injection, however overall rates of EV accumulation was highest in IP and IV administration when compared to SQ (22). Footpad administration resulted in highest localization into the lymph nodes while intranasal delivery to the cribriform plate yielded the highest brain delivery via peripheral injection. Finally, it has been noted that periocular injections of EVs reached the neurosensory retina, while intrathecal delivery allows for optimal CNS penetration (21). This is significant because when whole cells are delivered, either via autologous or allogenic sources, the first pass effect leaves the majority of the cells lodged within the lungs.

Different cell sources also play an important role in EV migration. While it has been proven that immune cells preferentially traffic to the spleen, so too do dendritic cell EVs likewise end up there. It is likely that the EVs maintain many of the surface receptor ligands and binding proteins of the parent cells. So important are these ligands that even species origin does not affect homing qualities (22); however, it is possible to affect targeting by changing certain membrane-bound protein ligands. RVG, a ligand that binds acetylcholine receptors, allows for a twofold greater accumulation in brain with increased levels in muscle and heart as well when attached to EVs (22). As for tumors, their leaky vasculature allows for permeation and retention, so that nanoparticles like exosomes and microvesicles will deposit there within sixty minutes if given intravenously. Additional modifications would no doubt provide even more exceptional targeting into tumors and other tissues (20).

6.0 PARABIOSIS – WHAT CAN BE LEARNED?

Parabiosis is defined as the procedure of joining two animals so that they share each other's blood circulations. Heterochronic parabiosis occurs when two different aged animals are connected. Over the years it has been shown that factors from young animals were able to elegantly activate molecular signaling in the older counterparts to increase tissue regeneration within hepatic, muscle, and neural elements (23). Since the hallmark of aging is the decline of regenerative properties linked to impaired function of stem and progenitor cells this has sparked the launch of a number of companies to try to reproduce these results in humans. Alkahest in California has set up clinics to treat Alzheimer's with plasma from 20-year-old human donors while Ambrosia, also based in California, sells plasma to all comers. But what is in the plasma? There are no appreciable stem cells present, but we should realize that because most stem cells are pericytes (cells sitting atop capillaries)(24) we may assume that plasma is a dilute fluid of extracellular vesicles. So, over time there is a relative rejuvenation of the older paired animal as the trophic and immunomodulatory mediators bathe the tissues and resident stem cells. But what of the younger animal? Much less attention has been paid in the literature to the less lucky of the two animals. In fact, new emerging evidence points to a senescence-associated secretory phenotype (SASP) which has a negative effect on stem cell niches. Hayflick defined a term in the 1960's as "replicative senescence." This was attributed to repeated cell divisions ultimately decreasing telomere length so that cells eventually could not divide. A newer term "premature senescence" relates to exogenous stressors affecting cells with normal telomere length. Senescence associated extracellular vesicles cause migration of phagocytic cells, induce inflammation, disrupt tissue architecture, and enhance malignant transformation and fibrosis. It has also been hypothesized that SASPs lead to impaired autophagy (i.e., appropriate death of senescent cells)

contributing to the pathogenesis of age-related diseases. Inflammatory cytokines seen within these SASPs are also associated with inflammatory and metabolic disorders (Table 2). And while "young blood" rejuvenated the older stem cell niches, SASPs decrease functionality of the niches thereby impairing tissue maintenance and repair, and they may even spread premature senescence to bystander cells (25).

Since the 1500's when Ponce de Leon travelled the world looking for the fountain of youth, scientists have arduously sought for the singular factor responsible to stop the aging process. For those who have studied parabiosis, the answer may be attributed to a protein, GDF-11 (25). Many have asserted that this protein is responsible for the rejuvenation process. Perhaps it serves as an interesting footnote that the mRNA that codes for GDF-11 has been assayed within the exosomes of neonatal placental MSCs (Table 3).

Table 2. *Key immune and growth factors present in MSC exosomes.*

BMP5	Stimulates Bone Growth
GDF15	Regulates inflammation, apoptosis, cell repair, and growth
OPG	Stimulates Bone Growth/Blocks Osteoclast Precursor Formation
G-CSF	Stimulates Bone Marrow to Procedure Granulocytes and Stem Cells
SCF	Responsible for Stem Cell and Melanocyte Growth
TGFß3	Most Important Anti-Inflammatory Protein. Converts Inflammatory T Cells into Anti-Inflammatory Regulatory T Cells.
VEGF	Stimulates Formation of Blood Vessels
ICAM-1	Binds Inflammatory Ligands on White Cells
IL-1RA	Binds and Sequesters the Inflammatory Cytokine IL-1
IL-6	Responsible for Macrophage Activation
IL-10	Anti-Inflammatory Cytokine responsible for Immunomodulation and Regulatory T Cell Conversion
MCP-1	Recruits Mononuclear Cells to Treatment Area
MIP-1	Also known as CC1-4, Recruits Mononuclear Cells to the Treatment Area
PDGF-BB	Growth Factor Used to Stimulate Healing in Soft and Hard Tissues
TIMP1 & TIMP2	Blocks Cartilage and Extracellular Matrix Degradation, Important for Cartilage Repair
HGF	Involved in Organ Regeneration and Wound Healing
GDNF	Promotes Survival of Neurons
BDNF	Supports Survival of Neurons and Encourage Growth
FGF	Potent Growth Factors Affecting Many Cells
TNFR1	Binds and Inactivates the Inflammatory cytokine TNF-α

Table 3. *Some key mRNA present in MSC exosomes.*

IL-1RA
TIMP1 & TIMP2
TNFR1 and TNFR2
Numerous Histone Deacetylase mRNAs
GDF11 - Potent anti-aging agent
GDF15 - Regulates inflammation
IGFBP2 - One of six IGF binding proteins that bind IGF-1 and IGF-2
IGFBP3
IGFBP4 - Reportedly anti-tumorigenic effects against prostate cancer, colon cancer, and glioblastoma
IGFBP6
OPG
SCFR
TGF-ß1 & TGF-ß3
VEGF
VEGFR-2
BMP4 - Involved in bone and cartilage development, fracture repair, and muscle development
BMP7 - Important in bone homeostasis
PTEN - A potent tumor suppressor gene
Numerous Key miRNA

7.0 HOW LONG SHOULD MSC EXOSOMES PERSIST *IN VIVO*?

In a study in 2016, forty patients with stage 3 and 4 chronic kidney disease were randomized into 2 groups. One group was given intravenous and intra-arterial exosomes, while the second group served as a control. Although renal functions improved significantly in the treatment arm as seen by increases in GFR and decreases in serum creatinine and BUN, what was most striking was the chronicity of benefit seen in anti-inflammatory markers TGF-ß and IL-10, and the pro-inflammatory TNF-α. The anti-inflammatory TGF-ß1 and IL-10 peaked at 12 weeks and persisted above baseline throughout the 52 week follow up while TNF-α showed a trough level at 12 weeks and persisted below baseline at 52 weeks. A rather remarkable persistence given the fact that only two injections were given initially one week apart. Given the rather transient traceability of EVs in vivo, and the limited half-life of proteins, the chronicity of these findings reinforces the importance of the mRNA they bear (26).

8.0 COULD MSC EXOSOMES HELP FIGHT TYPE II DIABETES MELLITUS?

By the age of 65 it is estimated that 50% of the US population will suffer from Type II Diabetes Mellitus or impaired glucose tolerance (27). This disease is intimately associated with many of the severe afflictions suffered by seniors during their final decades of life. Cardiovascular disease, stroke, and myocardial infarction share Diabetes Mellitus as a prognosticating factor. Could MSC exosomes help fight or even prevent Type II Diabetes Mellitus and decrease the risk of these and other serious age-related diseases?

To this end, it has been proven that MSC exosomes promote a systemic anti-inflammatory milieu which persists for many months. Decreasing inflammation also enables insulin to bind its receptors with greater affinity thereby increasing its relative action. Additionally, the anti-apoptotic effects of exosomes decrease the death rate of B-Cells in the pancreas and increase production of insulin (28). Finally, the peripheral effects are a little more intricate and must be appreciated in steps. As we age, we develop sarcopenia. Sarcopenia is defined as loss of muscle tissue. Sarcopenia is marked by capillary rarefaction (27), which is a systemic loss of capillary volume. There two synchronous processes cause a relative decrease in our metabolism secondary to reduced muscle mass along with a concomitant difficulty for insulin and glucose to traffic to the muscle cells. Exosomes promote capillarization through VEGF and pro-angiogenic miRNAs (27, 29, 30). By increasing capillary surface area and through proliferating factors from the “young blood,” satellite cells (muscle resident stem cells) are activated and more muscle tissue is formed (30). This serves to increase the overall metabolism as well as enable delivery of insulin and glucose intracellularly – thereby effectively lowering postprandial glucose levels along with HgbA1C over time.

9.0 WHAT TYPES OF MEDICAL CONDITIONS MIGHT BE AIDED BY MSC EXOSOMES?

- Musculoskeletal – Joints, discs, muscles, bones, ligaments, tendons
- Neurodegenerative – MS, Parkinson's, Alzheimer's, Huntington's, ALS, Cerebellar Ataxia
- CNS Injury/Trauma – CVA, CTE, TBI, SCI, Transverse Myelitis, Cerebellar Ataxia
- Burns/Scars/Ulcers
- Heart Disease – MI, Angina, CHF
- Lung Disease – COPD, Pulmonary Fibrosis, Interstitial Lung Disease
- Liver Disease
- Kidney Disease
- Inflammatory Bowel Disease – UC, Crohn's
- Alopecia
- Neuropathy/CIDP
- Erectile Dysfunction
- Urinary Incontinence
- Peripheral Vascular Disease
- Cerebral Palsy/Seizure Disorders/Autism
- Numerous Aesthetic Applications
- Depression/Bipolar Disorder
- Drug Addiction
- Type II Diabetes Mellitus
- Infertility
- Aging

10.0 HOW CAN MSC EXTRACELLULAR VESICLES BE DELIVERED?

The tiny size of EVs allows for easy injection based therapies. Alone, these miniature powerhouses can be delivered through needles as diminutive as 30 gauge. Direct delivery is recommended intravenously, intrathecally, and intranasally. When injecting into other areas of the body, it is often prudent to utilize a scaffold to limit traffic out of the injection site. Common autologous scaffolds like PRP or PRFM serve the dual purpose of cell retention and cell migration as well, without significantly elevating the cost of the procedure. Combining EVs with PRP/PRFM utilizing a 22 gauge needle will allow time to inject without compromising safety. Some procedures do not require image guidance and can be accomplished in office. More invasive procedures like intradiscal injections, cervical intrathecal injections (31), and deep perispinal injections are best performed utilizing X-Ray guidance – while joint, tendinous, perineural, and other musculoskeletal indications are well suited for ultrasound guidance. Many courses around the country are available in order to help hone the skills necessary for image-guided therapy, and additional books will serve the injectionist well for easy on-site reference. Unlike bone marrow aspiration/concentration and mini-lipoaspiration with concentration (with or without enzyme degradation) an additional one or two hours do not have to be allotted for each procedure.

11.0 EVs – THE NEXT HORIZON

Authors of this manuscript believe that EVs will prove to be the Penicillin of our age! We are only now starting to realize the first step of the innovative process. Generic neonatal MSC EVs are clearly immunomodulatory and pro-growth, and they are applicable to numerous indications (see appendix for author's protocols utilizing current products) but the next steps in the process will likely be the future of medicine.

As cells are known to modify their inherent cargos, growing cells in different conditions will induce differing outputs of products. Culturing cells in hypoxic or acidic environments modify the inherent secretions (32). Such "tuning" or "licensing" as it is called is a natural next step in the scientific process. Other types of cells (e.g., Leukocytes) and more differentiated cells (e.g., pre-cardiomyocytes) secrete their own valuable cargo which can also be "tuned" accordingly.

A natural progression in this process will be to utilize today's genetic engineering to internally modify the cargo. By upregulating miRNA and mRNA in different cells we will eventually be able to upcode those that are most responsible for regenerative effects. Key players like miRNA 133b, which is a known promoter of neurogenesis will change the playing field for severe neurodegenerative conditions and traumatic CNS lesions (33).

The last and most intriguing step in the process will be the utilization of the "Trojan Horse" phenomenon. Loading EVs with proteins, RNAs, and small-molecule drugs and making use of the receptor-ligands of their tiny membranes to deliver these products. Chemical processes such as electroporation, transient osmotic shock, and reversible chemical covalent modifications would allow post-isolation loading of numerous agents. Early studies utilizing Doxorubicin for breast cancer and curcumin for brain inflammation have yielded promising results (4). Such nanoparticle carriers may prove to be instrumental in the ongoing war against cancer and other degenerative

diseases. Of course, even the receptor-ligands of the tiny membranes can also be modified to optimize delivery. Table 4 shows multiple exosome protocols.

Table 4. *Exosome protocols*.

Condition	Protocol
OA - Large Joints (hips, shoulders, knees)	*Day 0 and Day 14:* 5mL PRP/PRFM plus 5mL Exosomes
Smaller Joints/Tendons/Ligaments	1mL Exosomes (consider ultra-concentrated) versus 2mL (normal concentrate) with or without PRP/PRFM
Lumbar Discs	*Day 0 and Day 14*: 1mL ultra-concentrated Exosomes plus 1mL PRP/PRFM
Thoracic Discs	*Day 0 and Day 14*: 1mL ultra-concentrated Exosomes plus 1mL PRP/PRFM, total volume less than 1mL per disc
Cervical Discs	*One procedure only:* Exosomes only up to 0.25mL per disc. Utilize ultra-concentrated Exosomes.
Erectile Dysfunction	5mL Exosomes plus 5mL PRP/PRFM. Injection in corpus cavernosum bilateral after penile block or Benzocaine/Lidocaine/Tetracaine 20%/8%/8% cream
Urinary Incontinence (Women)	5mL Exosomes plus 5mL PRP/PRFM. Injection roof of vagina subjacent to urethra.
Hair	Scalp Block* plus 10mL Exosomes after 10mL PRP/PRFM
Wounds, Ulcers, and Burns	Inject periphery and base of lesion liberally with 5mL Exosomes after debridement. Cover and keep dry with Telfa dressing for 7 days
Peripheral Neuropathy/Peripheral Arterial Disease (Lower Extremities)	5mL Exosomes pretibially and 5mL Exosomes in sole of foot. Severe Diabetic Neuropathy +/- consider intrathecal injection Exosomes. *Consider tibial nerve block under ultrasound versus Ethyl chloride for sole injections.*
Neurodegenerative Disease/Traumatic Brain Injury/Stroke/Spinal Cord Injury - cervical or high thoracic/transverse myelitis	• 0/C1 or C1/2 puncture • Remove 3mLs of CSF • Slowly inject 3 x 1mL of ultra-concentrated Exosomes intrathecally plus 15mL of standard Exosomes in 250mL NS via IV***
Lower Thoracic Spinal Cord Injury/Arachnoiditis	• Lumbar Cistern puncture • Remove 3 mL of CSF • Slowly injection 3 x 1mL of ultra-concentrated Exosomes intrathecally plus 15 mL of standard Exosomes in 250 mL NS via IV***
Type II Diabetes Mellitus	• 3 5mL Exosomes in 250mL NS via IV *** • Evaluate HgbA1C every 6 weeks • +/- consider SQ injection Exosomes • Consider retreatment Q12 weeks
Autoimmune Disease	3 x 5mL Exosomes in 250mL NS via IV***, re-evaluate at 6 weeks
Autism	• 3 x 5mL Exosomes in 250mL NS via IV*** plus intranasal** • 3 x 1mL ultra-concentrated Exosomes (0.25mL BID) versus intrathecal exosomes for severe, older patients
Concussion	3 x 1mL Exosomes in via intranasal** +/- 3 x 5mL in 250 NS via IV
COPD/Interstitial Fibrosis	Obtain PFTs, 2mL Exosomes QOD via HHN after bronchodilator treatment (12-15 treatments); Follow up in office after first 2 treatments to auscultate.
Aging	3 x 5mL Exosomes in 250mL NS via IV Q3 months prn

PRP/PRFM = Platelet Rich Plasma/Platelet Rich Fibrin Matrix

Additional Notes
No steroids 4 weeks prior to treatment. No NSAIDs 5 days prior to treatment. Suspend NSAIDs/steroids 12 weeks post op.
Exosome Concentrations 1mL vial = 8mg/mL (ultra-concentrate) 2mL vial = 3 mg/mL (standard) 5mL vial = 3 mg/mL (standard)

12.0 IS CLINICAL IMMORTALITY WITHIN OUR REACH?

I had recently participated in a roundtable discussing the possibilities of clinical immortality. Some key aspects of the conversations deserve memorialization here as they relate closely to what I believe is possible both today and within the near future.

It is the premise of most age management physicians and scientists that aging results in an abnormal imbalance of anabolism and catabolism. As we grow and in our youth, anabolism either outdistances catabolism or keeps pace with it. At some point, we can no longer keep pace with the degradative properties of aging and we begin to lose the battle. The Hayflick Hypothesis refers to "replicative senescence" but I believe most of us fall prey to "premature senescence." In this case, exogenous stressors bring physiological changes to bear which stress our systems beyond their abilities – ultimately yielding autoimmune diseases, cardiovascular disease, CNS disease, cancer, aging, and eventually death. The two biggest catabolic components are likely inflammation and poor redox homeostasis. For many years heart disease and stroke were linked primarily to lipid metabolism, but the last decade has seen a significant appreciation of the role of inflammation in these two entities. Similarly, we know all too well that aging compromises both the innate and adaptive immune systems. Memory B cells, T cells, neutrophils, and macrophages all decrease in effectiveness as father time marches by, further limiting our abilities to compensate with these inherent physiological changes (34). Finally, a new subset of medicine – age management medicine – has given rise to the understanding that we need energy to grow, repair, fend off infection/inflammation, and even fight cancer. At the heart of this therapy resides the main energy machines within our bodies – the mitochondria. Limited ability to produce energy (ATP/NADH), react to oxidative stress (reactive oxygen and nitrogen species) and dispose of damaged mitochondria (mitophagy) and other cellular debris (autophagy) are hallmarks of poor redox

homeostasis (35, 36, 37). Previously, and throughout this chapter, I have alluded to all of the numerous ways that generic MSC EVs combat these changes. They produce numerous anti-inflammatory substances, promote the production of energy through the sharing of key enzymes in the ATP glycolytic pathway, and even contribute to improved mitophagy and autophagy. In fact, it has been shown that MSC EVs cause microglia in the CNS to secrete neprilysin – combating ß-amyloid plaques via endogenous proteolytic pathways in mouse models of Alzheimer's disease. In mouse models of Parkinson's Disease, α-synuclein, another protein aggregate molecule, showed improved intracellular clearance through an increase in autophagy after MSC introduction (38).

Much time has been spent painting the picture of resident stem cells and their relative quiescent state without the requisite "young blood" bathing their niches. If in fact it is the limited milieus throughout our bodies that inhibit our regenerative potential – then might it be possible to turn back aging with simple injections at given times throughout the year? Maybe the next steps will include broths rich in GDF11, miRNA 133b (neuroregeneration), miRNA 133a (cardiac regeneration) (39) or similar substituents rich in the growth factors necessary to combat specific organ aging.

An interesting paper a few years ago by Joshua Schiffman, a pediatric oncologist at the University of Utah discussed the quandary known as Peto's Paradox. They addressed the mismatch of organism size and cancer rates in elephants. Obviously, bigger organisms have greater chances for cells going awry – yet elephants do not get cancer. Maybe the answer lies in the tumor-suppressing gene P53. We have one copy – elephants have 20 (40). Perhaps tomorrow's exosomes will also be enriched in P53. In this way, we may someday obviate the need for the Trojan horse.

13.0 REGULATORY LANDSCAPE

In the United States, exosome-based therapeutics will likely be regulated by the FDA's Office of Cellular, Tissue, and Gene Therapies (OCTGT) within the FDA Center for Biologics Evaluation and Research (CBER). CBER regulates "human cells, tissues, and cellular and tissue-based products" or "HCT/Ps" There are two different paths for these products defined as 361 products or 351 products according to what the FDA considers relative risk. 361 products do not require a license or approval by the FDA, whereas 351 products are "regulated as a drug, device, or biological product under the Federal Food, Drug, and Cosmetic Act (FDCA) and Section 351 of the PHS Act." 351 products require clinical trials. Stem cell exosomes could potentially be regulated under either pathway. They are currently unregulated. To date, only guidances have been published, which are not laws. Guidances are meant to provide cell therapy industry stakeholders with language and tools through which they can assess their compliance (5).

In December of 2016, President Barack Obama signed into law the 21st Century Cures Act. In this act, there are provisions for stem-cell based therapies. An important provision, which MSC Exosomes should qualify for is the regenerative Medicine Advanced Therapy designation (RMAT). It allows a fast track for accelerated approval of cell-based therapies that aim to treat serious medical conditions with high unmet needs and favorable preliminary clinical data (5).

14.0 TECHICAL ASPECTS

Prior to the administration of exosomes, multiple precautions have been described including necessity for appropriate preoperative lab recommendations as shown in Table 5 and relative contraindications as shown in Table 6.

*** Scalp Block**

1. Block above superior orbital fissure - above eyebrow - inject medial and lateral (trochlear nerve superior orbital nerves)
2. Auriculotemporal nerve (anterior to ear - posterior to temporal artery)
3. Greater and less occipital nerves

**** All Intranasal Injections**

- Utilize Tuberculin syringe with Luer Lock
- Pull up 0.25mL (remainder of 1cc kept in refrigerator)
- Attach 24G angiocath (remove internal needle), pull in excess air and tap syringe so fluid resides inferiorly.
- With patient supine, place along anterior wall of nose above middle turbinate and inject quickly towards cribriform plate.

***** Prior to IV Therapy**

Stop immune suppressant therapy for three half-lives. Pretreat with Benadryl PO 50mg and Tylenol 650mg 1 hour prior to procedure.

Table 5. *Pre-operative lab recommendations.*

Final work up is at the discretion of the treating physician.

- CBC
- CMP
- UA
- CA-125 for females
- PSA for males over 40
- CEA for males and females over 40
- PT/INR
- EKG

Table 6. Relative contraindications

- Cancer
- Myeloproliferative Disease
- Bone Marrow Dysplasia
- Sickle Cell
- Primary Pulmonary Hypertension
- Acute Bacterial Infection
- Recent Dental Work
- Macular Degeneration
- Any abnormal neovascularization
- Immuno-compromised

1) Mesenchymal Stem Cells are attractive targets for regenerative medicine because:
    A. They will predominantly engraft to tissues and become cartilage, bone, synovium, and other mesodermal tissues *in vivo*.
    B. They measure between 40 to 100 nm and easily bypass the blood brain barrier.
    C. They are devoid of DNA.
    D. They secrete paracrine factors which influence the body via immunomodulatory and trophic mechanisms.

    Key Concept: To recognize Mesenchymal Stem Cell's clinical benefits and cellular method of acting upon target tissues.

    Answer: D - The extracellular vesicles (exosomes and microvesicles) are responsible for the majority of effects of MSCs: **immunomodulation and growth**. Now termed "**medicinal signaling cells**," exert changes through **signaling rather than engraftment** as was previously thought.

2) Which of the following components is not found in extracellular vesicles:
    A. microRNA
    B. Mitochondia
    C. RNA
    D. Proteins

    Key Concept: To recognize the relative size of constituents of extracellular vesicles.

    Answer: B - Mitochondria - which are the powerhouse of the cells are too large to be contained within EVs. While exosomes are **less than 100 nm in size**, microvesicles may be hundreds of nanometers in size. Mitochondria however are on the scale of 1-2 microns and are too large to be contained within these tiny packets. In addition, it appears that **EVs improve oxidative stress via improving mitophagy and by supplying appropriate glycolytic pathway enzymes.**

3) The term "Resident Stem Cells" refer to cell precursors within many of the body's tissues. Which of the following cells are true resident stem cells?
    A. Autologous Chondrocyte Progenitor Cells
    B. Autologous Bone Marrow MSCs
    C. Autologous SVF derived MSCs
    D. Allogenic Wharton's Jelly MSCs

    Key Concept: To distinguish that resident stem cells are already "resident" to the organism.

Answer: A - Chondrocyte Progenitor Cells (CPCs) are one of the cells occupying the **functional niche** of the joint. CPCs are one of the many resident stem cells found within the joint. As discussed earlier, **MSCs exert their benefits through paracrine secretions**.

4) Heterochronic Parabiosis involves attaching two rodents, one old and one young together. What best explains the associated changes in each animal?
   A. Equilibrium or sharing of stem cells between the two organisms.
   B. An averaging of telomerase activity within both organisms thereby resetting the replicative senescence threshold of each animal.
   C. The procedure has been shown to be a scam.
   D. "Young blood" rejuvenating the older animal's stem cell niches and SASPs decreasing the functionality of the younger animal's niches.

   Key Concept: To hypothesize the cause of the reciprocal changes found in these experiments and how it would relate to aging.

   Answer: D - EVs of young cells are **progrowth and immunomodulatory** while older cells' EVs produce more autophagy and inflammation. The relative reciprocal young blood secretions and older blood secretions (**senescence-associated secretory phenotype - SASP**) upon **"premature" senescence** of the stem cell niches.

5) Distribution studies of EVs after intravenous infusion suggest rapid uptake and clearance with little traceable EVs seen after 6 hours. Yet a study utilizing EVs for patients with chronic renal failure demonstrated persistence of immunomodulation for greater than one year. This is suggestive evidence of:
   A. The long half-lives of anti-inflammatory proteins TGF-ß and IL-10.
   B. The relatively short half-life of pro-inflammatory protein TNF-α.
   C. The persistence and cascade of changes secondary to mRNA.
   D. The EVs pronounced and persistent upregulation of M1 macrophages.

   Key Concept: Differentiating between the different types of cell-to-cell signaling and the persistence of their effects.

   Answer: C - mRNA allow for significant upregulation of proteins on an ongoing basis while **proteins themselves have relatively ephemeral half-lives**. The cascade of benefits after EV administration is linked to the persistence of mRNA which can **persist for up to a month on their own and code for a thousand proteins each**.

6) It has been hypothesized that MSC exosomes could help fight or prevent Type II Diabetes Mellitus. All of the following are suggested possible routes except:
   A. MSC exosomes promote a systemic inflammatory milieu.

B. MSC exosomes promote the growth of new pancreatic B-cells.
C. MSC exosomes promote capillarization and activate satellite cells.
D. MSC exosomes bear mRNA which directly code for insulin

Key Concept: To recognize that MSC exosomes yield multiple positive benefits for patients suffering from Type II Diabetes Mellitus and impaired glucose tolerance.

Answer: C - MSC exosomes do not themselves produce insulin but do **prevent apoptosis** of pancreatic B cells, **promote an anti-inflammatory milieu**, and **yield capillarization** as well as **activation of satellite cells**.

7) Tomorrow's EVs may be "tuned" or "licensed" by:
   A. Upregulating nascent mRNA or miRNA.
   B. Removing the protein ligands from the plasma membranes.
   C. Removing the plasma membrane.
   D. Utilizing vesicles larger than 1000 nm

   Key Concept: Recognizing that "tuning" or "licensing" EVs will enable more potent and targeted therapies in the future.

   Answer: A - Selecting which mRNA and/or miRNA are present in EVs could allow for **more targeted therapies** for specific conditions in the future. Additional tuning can be achieved by using exosomes to deliver specific proteins directly to targets or inserting small-molecule drugs. Removing proteins from the membranes or the membranes themselves would decrease uptake by other cells. Similarly, using vesicles larger than 1000 nm would also limit uptake.

8) MSC EVs are notably immunomodulatory. Which of the following are related to their immunomodulatory effects:
   A. Production of TNFα
   B. Production of TGFß3
   C. Upregulation of TH_1 Cells
   D. Upregulation of M1 Cells

   Key Concept: To recognize that MSC EVs are immunomodulatory. They contain both proteins and mRNA, which code for immunomodulatory proteins and decrease circulating inflammatory cells.

   Answer: D - MSC **EVs cause a shift from M1 to M2 macrophages** and a **shift to T_{Reg} cells**.

9) MSC EVs utilize which of the following techniques to aid in preventing apoptosis by:
   A. Promoting appropriate mitophagy and autophagy.
   B. Donating mitochondria to stem cells in stress.
   C. Replicating nascent mitochondria.
   D. Increasing production of RNS and ROS.

Key Concept: Recognize that MSC EVs combat apoptosis through numerous techniques.

Answer: A - **Mitochondrial dysfunction with subsequent death is the leading cause of endothelial injury**. By removing the damaged mitochondria, oxidative cell damage can be limited.

10) Rapid uptake of intravenous EVs is seen in which of the following tissues:
    A. Brain
    B. Synovial joints
    C. Liver
    D. Retina

    Key Concept: Understanding that EVs show rapid uptake by macrophages of the mononuclear phagocyte system.

    Answer: C - Although MSC EVs can **cross the blood-brain barrier**, there is limited uptake in the brain. Following IV infusion, there is **significant uptake in the liver, spleen, and lungs**.

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